Anti-Angiogenic Effect of Luteolin on Retinal Neovascularization via Blockade of Reactive Oxygen Species Production

Sung Wook Park,[1,2,3] *Chang Sik Cho,*[1,3] *Hyoung Oh Jun,*[1] *Nam Hee Ryu,*[4] *Jin Hyoung Kim,*[1] *Young Suk Yu,*[1,2] *Jin Sook Kim,*[4] *and Jeong Hun Kim*[1,2]

Purpose. Oxidative stress-induced vascular endothelial growth factor (VEGF) is thought to play a critical role in the pathogenesis of retinopathy of prematurity (ROP). This study was performed to investigate the anti-angiogenic effect of luteolin against reactive oxygen species (ROS)-induced retinal neovascularization.

Methods. The toxicity of luteolin was evaluated through modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in human retinal microvascular endothelial cells (HRMECs) as well as TUNEL staining in the retina of C57BL/6J mice. After intravitreal injection of luteolin in the mouse model of ROP, retinal neovascularization was examined by fluorescence angiography and vessel counting. Anti-angiogenic activity of luteolin was evaluated by VEGF-induced migration and tube formation assay. The effect of luteolin on tertiary-butylhydroperoxide (t-BH)–induced ROS production was measured with 2′7′-dichlorofluorescein diacetate. The effect of luteolin on t-BH–induced and hypoxia-induced VEGF transcription and expression were evaluated by RT-PCR and Western blot, respectively.

Results. Luteolin never affected the viability of HRMECs up to 10 μM, where luteolin never induced any structural change in all retinal layers. Luteolin inhibited retinal neovascularization in the mouse model of ROP. Moreover, VEGF-induced migration and tube formation were significantly decreased by cotreatment of luteolin. Luteolin attenuated VEGF transcription via blockade of t-BH–induced ROS production. Luteolin suppressed hypoxia-induced VEGF expression via attenuating hypoxia inducible factor 1 α expression.

Conclusions. Our results suggest that luteolin could be a potent anti-angiogenic agent for retinal neovascularization, which is related to anti-oxidative activity to block ROS production and to subsequently suppress VEGF expression and the pro-angiogenic effect of VEGF. (*Invest Ophthalmol Vis Sci.* 2012; 53:7718–7726) DOI:10.1167/iovs.11-8790

Angiogenesis-related blindness, which is composed of retinopathy of prematurity (ROP), diabetic retinopathy (DR), and AMD is most common cause of blindness in each age group and characterized by pathologic angiogenesis.[1,2] Among angiogenesis-related blindness, ROP occurs through the partial regression of pre-existing vessels by vaso-obliteration followed by the pathologic angiogenesis in developing retinal vasculature, where excessive oxygen supply in the premature retina leads to the generation of free radical and reactive oxygen species (ROS).[3] In this oxidative stress condition, production of ROS increased with correlation of vascular endothelial growth factor (VEGF) expression and angiogenesis.[4] In addition, the anti-oxidant systems in preterm infants is highly stressed and incompletely developed.[5]

In oxygen-induced retinopathy (OIR) as a model of ROP, ROS were increased in the retina and that nicotinamide adenine dinucleotide phosphate-oxidase was activated to trigger the apoptosis of endothelial cells, which contributed to avascular retina and exacerbated with supplemental oxygen supply contributing to angiogenic blood vessel growth into the vitreous.[6] Recently, it was reported that the Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling pathway can lead to angiogenesis through ROS by triggering VEGF.[7] Therefore, ROP could be developed by the action of ROS.

ROS regulates VEGF transcriptional activation through hypoxia inducible factor 1 α expression (HIF-1α) expression in ovarian cancer cells.[8] ROS, the production of which is increased by VEGF stimulation, activate Vascular endothelial growth factor receptor 2 (VEGFR2) downstream signaling linked to endothelial cell proliferation and migration[9,10] Thus, we hypothesized that blocking ROS production during oxidative stress could attenuate neovascularization via regulating both VEGF expression and VEGFR2 downstream signaling pathway in OIR.

During our efforts on developing anti-angiogenic drugs,[11–15] we recently extracted luteolin as a potent candidate from *Platycodon grandiflorum*, which is widely used in Asian traditional herbal medicine.[16] Luteolin is a natural flavonoid, which can provide both short and long term anti-oxidative effect by directly neutralize toxic ROS by donating hydrogen ions and also modulate cell-signaling pathways.[17] Based on the anti-oxidative activity, luteolin has variable biological activities

From the [1]Fight against Angiogenesis-Related Blindness Laboratory and the [2]Department of Ophthalmology, Clinical Research Institute, Seoul National University Hospital, Seoul, Korea; and the [4]Diabetic Complications Research Center, Division of Traditional Korean Medicine Integrated Research, Korea Institute of Oriental Medicine, Daejeon, Korea.

[3]These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported by grants from the National Research Foundation (NRF) funded by the Korea government (MEST; 2012-0006019), the Bio-Signal Analysis Technology Innovation Program of MEST/NRF, Republic of Korea (2012-0006058), the Mid-Career Researcher Program of MEST/NRF, Republic of Korea (2012-0004931), and the Korea Institute of Oriental Medicine (KIOM; K11040) (NHR, JSK).

Submitted for publication October 13, 2011; revised March 26, August 10, and September 27, 2012; accepted October 21, 2012.

Disclosure: **S.W. Park**, None; **C.S. Cho**, None; **H.O. Jun**, None; **N.H. Ryu**, None; **J.H. Kim**, None; **Y.S. Yu**, None; **J.S. Kim**, None; **J.H. Kim**, None

Corresponding author: Jeong Hun Kim, Fight against Angiogenesis-Related Blindness (FARB) Laboratory, Clinical Research Institute, Seoul National University Hospital and Department of Ophthalmology, Seoul National University College of Medicine, Seoul National University, Seoul, Korea, 110-744; steph25@snu.ac.kr.

Investigative Ophthalmology & Visual Science, November 2012, Vol. 53, No. 12

of antiproliferative, antimetastatic, and anti-angiogenic.[18] Recently, it was also reported that luteolin protects retinal ganglion cells and retinal pigment epithelial cells from oxidative injury.[19,20] Luteolin inhibits hypoxia-induced VEGF expression by a HIF-1 independent mechanism, suppression of STAT3 tyrosine phosphorylation.[21]

In the present study, we demonstrated that luteolin effectively inhibited in vitro angiogenesis in HRMECs and retinal neovascularization in OIR without toxicity, which would be related to that luteolin effectively inhibited VEGF expression via HIF-1α dependent mechanism by blockade of ROS production, and VEGF-induced angiogenesis via regulating possibly VEGFR2 signaling pathway. Taken together, our results suggest that this anti-angiogenic activity of luteolin as an antioxidant could be applied to variable vasoproliferative retinopathies induced by ischemia as well as ROP.

Materials and Methods

Luteolin Extraction and Isolation

Extraction of luteolin from *Platycodon grandiflorum* was performed as previously described.[16] Briefly, the fresh plant materials were extracted with ethanol (EtOH) by maceration at room temperature for 3 days. The extracts were combined and concentrated in vacuo at 40°C. The concentrated extract was suspended with n-hexane, ethyl acetate (EtOAc) and n-butanol (n-BuOH), respectively. The EtOAC-soluble fraction was silica gel chromatographed (230–400 mesh) using a chloroform ($CHCl_3$)/methanol (MeOH) gradient (from 9:1 to 1:1 vol/vol, finally 100% MeOH) to yield eight fractions. After recrystallization (MeOH) of the fraction eluted with $CHCl_3$-MeOH (9:1 vol/vol), the rest of fraction was purified further over a silica gel column (230–400 mesh) eluted with dichloromethane (CH_2Cl_2)/MeOH gradient (from 19:1 to 9:1), yielding luteolin. We identified as luteolin by spectroscopic methods and comparisons with literature values.[22] The purity of luteolin was 100% as assessed by analytical high performance liquid chromatography (HPLC).

Animals

C57BL/6J mice were purchased from Samtako (Seoul, Korea). Care, use, and treatment of all animals in this study were in strict agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6J mice were kept in standard 12 hour dark-light cycle and at approximately 23°C room temperature.

Cell Cultures

HRMECs were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and were grown in a gelatin coated 75-cm^2 flask in an M199 medium (Gibco BRL, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS; Gibco BRL), 3-ng/mL basic fibroblast growth factor (Millipore, Bedford, MA), and 10-U/mL heparin (Sigma, St. Louis, MO) at 37°C in an incubator with a humidified atmosphere of 95% O_2 and 5% CO_2. The experiments were performed using cells between passages four and nine. Human brain astrocytes were purchased from the Applied Cell Biology Research Institute and were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific Hyclone, Logan, UT) supplemented with 20% FBS (Gibco BRL), and N-2 supplement (Gibco BRL). The experiments were performed using cells between passages nine and 18.

Cell Viability Assay

Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay with modifying.[20] Conventional MTT tetrazolium reduction assay was not suitable for cell viability assay since some flavonoids directly reduce MTT in the absence of living cells.[23] HRMECs were seeded into each well of 96-well plates at a concentration of 1×10^4 cells/well. After incubation for 24 hours, cells were treated with either dimethyl sulfoxide (DMSO) as a control or various concentration of luteolin (0.1–100 μM) for 2 days. The medium was exchanged with fresh media with MTT solution (2 mg/mL). After incubation at 37°C for 2 hours, the medium was carefully removed from the plate, and DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorption at 540 nm was measured using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Three independent experiments were performed for each experimental condition.

TUNEL Assay

10-μM luteolin 1 μL was injected intravitreously to 8-week-old female C57BL/6J mice. The mice were sacrificed at 3 days after injection and enucleated. For the positive control of TUNEL assay in the retina, the mice were sacrificed 3 days after carotid artery occlusion and enucleated as our previous description.[24] Enucleated globes were fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin. TUNEL staining was performed with a kit (ApopTag Fluorescein Green; Intergen, Purchase, NY), according to the manufacturer's instructions. TUNEL-positive cells were evaluated in randomly selected fields (×400) under fluorescein microscopy (BX50; OLYMPUS, Tokyo, Japan).

Oxygen-Induced Retinopathy

With some modifications,[12] OIR was induced as described by Smith et al.[25] Briefly, newborn C57BL/6J mice were randomly assigned to two experimental ($n = 6$, respectively) and control ($n = 6$) groups. At postnatal day (P) 7, mice pups were subjected to hyperoxia (75 ± 0.5% oxygen) for 5 days (from P7 to P12) and return to room air (21% oxygen) for 5 days. Neovascularization occurs upon return to room air and peaks at P17. Oxygen was checked twice daily with an oxygen analyzer (Miniox I; Bertocchi srl Elettromedicali, Cremona, Italy). To assess the anti-angiogenic activity of luteolin, pups were injected 1 μL intravitreously with 0.1-μM luteolin or 1-μM luteolin on P14, when retinal neovascularization began. These experiments were repeated at least three times.

Qualitative Assessment of Retinal Neovascularization by Fluorescein Angiography

As our previous description,[13] at P17, deeply anesthetized mice were perfused through the tail vein with a fluorescein conjugated dextran (molecular weight = 500,000; Sigma-Aldrich Ltd.) dissolved in PBS. After 1 hour perfusion, the eyes were enucleated and fixed in 4% paraformaldehyde for 2 hours. The retina were dissected, flat-mounted in Dako mounting medium (DakoCytomation, Glostrup, Denmark), and viewed by a fluorescein microscopy (BX50; OLYMPUS) at a magnification of 4×.

Quantitative Assessment of Retinal Neovascularization

As our previous description,[13] at P17, the eyes were removed from animals with OIR, fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin. Sagittal sections of 4 μm, each 30 μm apart, were cut through the cornea parallel to the optic nerve. The sections were stained with hematoxylin and eosin (H&E) to assess the vascular lumens of new vessels growing into the vitreous via light microscopy (Carl Zeiss, Chester, VA). Vascular lumens between posterior lens capsule and the inner limiting membrane were counted in at least 10 sections from each eye (at least five on each side of the optic nerve) by two independent observers blinded to treatment. The average number of intravitreal vessels per section was calculated for each group.

VEGF-Induced Wound Migration Assay on HRMECs

Cell migration was evaluated with modified wound migration assay as our previous description.[24] HRMECs (1×10^6 cells) were plated onto

gelatin-coated culture dishes at 90% confluence, and wounded with a razor blade. After wounding, plates were rinsed with serum-free medium to remove cellular debris. The wounded monolayer was incubated with treatment of 1-μM luteolin or 10-ng/mL VEGF (Cell Signaling Technology, Danvers, MA) for 24 hours. The cells were fixed with absolute methanol and stained with Giemsa's solution (BDH Laboratory Supplies, London, UK). Migration was quantified by counting the number of cells that moved beyond the reference line.

VEGF-Induced Tube Formation Assay on HRMECs

Tube formation was assayed as our previous description.[26] Matrigel (BD Biosciences, Franklin Lakes, NJ) was coated on 48-well culture plates and polymerized for 30 minutes at 37°C. HRMECs (5×10^4 cells) were seeded on the surface of matrigel and treated with 1-μM luteolin or 10-ng/mL VEGF for 8 hours. The morphologic changes of the cells and formed tubes were observed and photographed (×200). Tube formation was quantified by counting the number of connected cells in randomly selected fields and dividing that number by the total number of cells in the same field.

Intracellular ROS Measurement

As our previous description,[24] Intracellular ROS was measured using the fluorescence dye 2′7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Ltd.), which is converted to dichlorofluorescein (DCF) by the presence of peroxidase. HRMECs were treated with 1-μM luteolin or 100-μM tertiary-butylhydroperoxide (t-BH). After 30 minutes, cells were labeled with 20-μM of DCFH-DA for 30 minutes at 37°C in the dark. The cells were washed with Hank's buffered salt solution, and then incubated for 10 minutes at 37°C. Intracellular ROS production was measured by microplate fluorometer at excitation and emission wavelengths of 495 and 530 nm.

RT-PCR Analysis

HRMECs were treated with 1-μM luteolin or 100-μM t-BH for 2 hours. Human brain astrocytes were incubated with 1-μM luteolin for 8 hours under hypoxia (1% O_2). Total RNA was obtained from cells using the TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. 5 μg of total RNA was converted to first-stranded cDNA using moloney murine leukemia virus reverse transcriptase and oligo-$(dT)^{16}$ (Invitrogen, Rockville, MD). Equal amounts of cDNA were subsequently amplified by PCR in a 20-μL reaction volume containing 1 × PCR buffer, 300-μM deoxyribonucleotide triphosphates (dNTPs) 10-μM specific primer for VEGF (5′-GAGAATTCGGCCTCCGAAACCATGAACTTTCTGT-3′ and 5′-GAG CATGCCCTCCTGCCCGGCTCACCGC-3′), HIF-1α (5′-AGTCGGACAG CCTCAC-3′ and 5′-TGCTGCCTGTATAGGA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′), and 1.25 U Taq DNA polymerase (Kapa Biosystems, Woburn, MA). PCR products were separated on 1% agarose gels and visualized using SYBR Safe DNA gel stain (Invitrogen) under UV transillumination.

Western Blotting

Western blotting was performed using standard Western blotting methods. Human brain astrocytes were incubated with 1-μM luteolin for 8 hours under hypoxia (1% O_2). Cell proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (Tris 50 mM pH 7.4; NaCl 150 mM; SDS 0.1%; NaDeoxycholate 0.5%; Triton X-100 1%; Cell Signaling Technology) with a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), and incubated on ice for 1 hour and centrifuged at 20,000×g for 30 minutes at 4°C. The protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of protein from the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either 6% to approximately 10% Tris-Tricine gel (Bio-Rad Laboratories, Inc., Hercules, CA) and transferred to nitrocellulose membranes (Amersham Hybond ECL, GE healthcare, Piscataway, NJ). The membranes were blocked in PBS with 0.05% Tween 20 (PBST; Bio-Rad Laboratories, Inc.) containing 5 % dry skim milk and incubated in PBST with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at room temperature. Rabbit monoclonal antibodies directed against VEGF (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and actin (1:5000, Sigma-Aldrich Ltd.), and mouse monoclonal antibody directed against HIF-1α (1:1000, BD Biosciences) were obtained from Invitrogen, Cell Signaling. The blots were scanned the band intensity analyzed using ImageJ 1.40 software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, IL). *P* values less than 0.05 were considered to be statistically significant. Figures are depicted as mean ± SD.

Results

Effect of Luteolin on Viability of Retinal Endothelial Cells and Retina

To evaluate the cytotoxicity of luteolin on HRMECs, we performed modified MTT assay with different concentration of luteolin (0.01–100 μM). As shown in Figure 1A, up to 10 μM, luteolin did not affect to the cell viability of HRMECs. However, significant reduction of cell viability was observed in 100-μM of luteolin. Retinal toxicity of luteolin was also evaluated by histologic examination with H&E stain and TUNEL assay after 1 μL intravitreal injection of 10-μM luteolin. As shown in Figure 1B, the retina was normal thickness, and all retinal layers were clear without any inflammatory cells in the vitreous, retina, or choroid. Moreover, 1 μL intravitreal injection of 10-μM luteolin did not increase TUNEL-positive cells in all retinal layers. Thus, luteolin less than 10 μM did not show cytotoxicity on HRMECs or retinal toxicity in mice.

Anti-Angiogenic Effect of Luteolin on Retinal Neovascularization in OIR

To determine whether luteolin could inhibit retinal neovascularization in OIR, luteolin 1 μL (0.1 μM and 1 μM) was intravitreously injected on P14 of OIR. Retinal neovascularization was qualitatively analyzed with fluorescein angiography and quantitatively analyzed with counting vascular lumen. As shown in Figure 2A, neovascular tufts of intravitreous neovascularization were easily observed at the border of vascular and avascular retina. However, the neovascular tufts were significantly decreased in luteolin-treated mice (Figs. 2B, 2C). In the vascular lumen analysis, many neovascular lumens were observed in control mice (Fig. 2D), whereas those were markedly decreased in luteolin-treated mice (Figs. 2E, 2F). Number of vascular lumens in luteolin-treated mice (0.1 μM: 14.7 ± 1.2, 1 μM: 13.5 ± 1.5) were significantly decreased than those in control mice (20 ± 0.8) ($P < 0.05$, respectively) (Fig. 2G).

Inhibitory Effect of Luteolin on Migration and Tube Formation of Retinal Endothelial Cells

To determine the effect of luteolin on in vitro angiogenesis, wound migration assay and tube formation assay were performed on HRMECs. The VEGF-induced migration of HRMECs was significantly decreased by cotreatment of 1-μM luteolin. (Fig. 3A) The VEGF-induced tube formation of HRMECs was also significantly inhibited by cotreatment of 1-

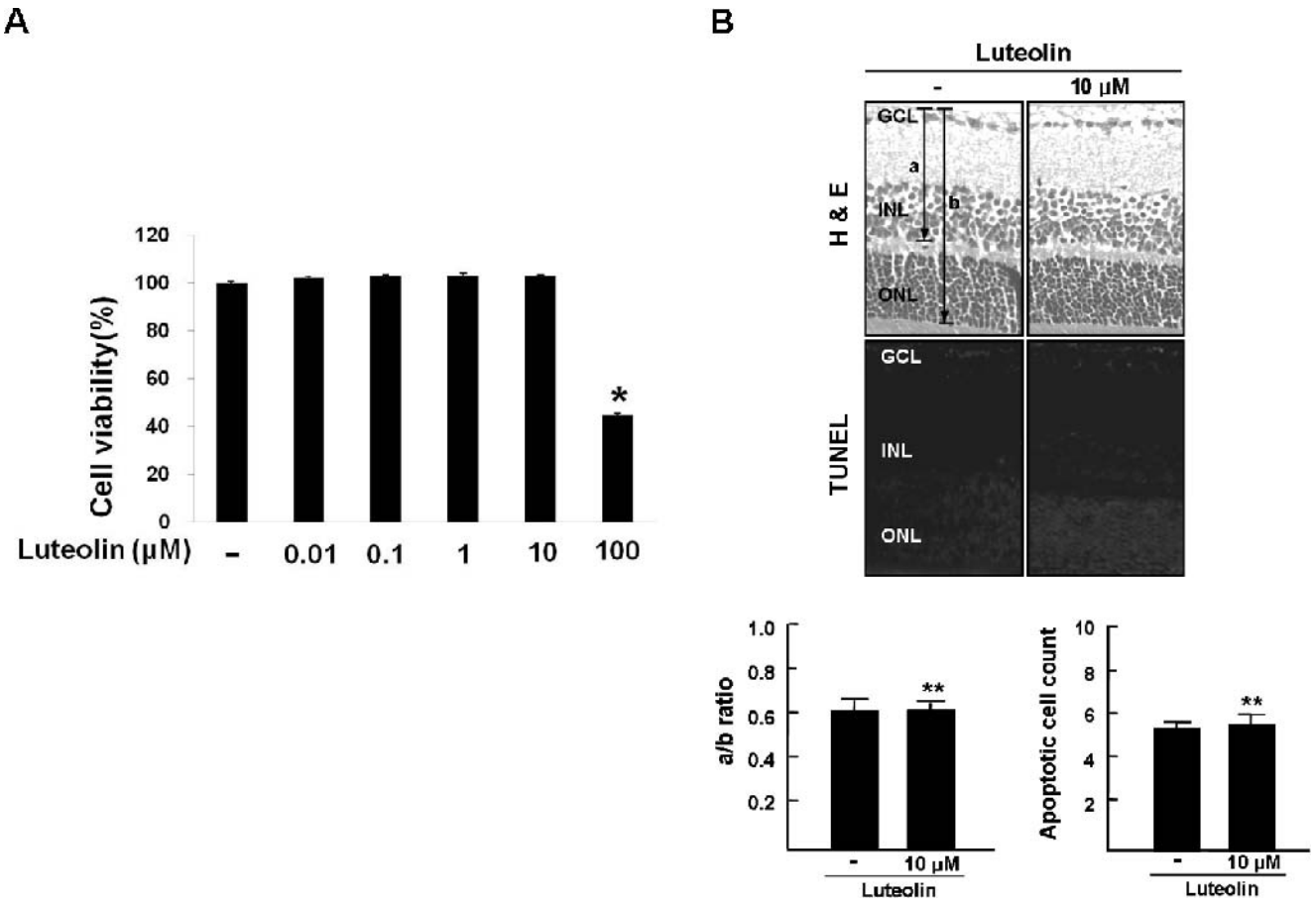

Figure 1. Luteolin does not affect the viability of retinal endothelial cells, nor induces retinal toxicity. (**A**) HRMECs were treated with luteolin (0.01–100 μM) and incubated for 48 hours. Cell viability was measured by modified MTT assay. (**B**) Retina was evaluated 3 days after intravitreous injection of 10-μM luteolin 1 μl. The retina was normal without structural change or any inflammatory cells in all retinal layers. TUNEL-positive cells were not increased after treatment. Each value represents the mean (±SD) of three independent experiments (*$P < 0.05$, **$P > 0.05$).

μM luteolin. (Fig. 3B) Thus, VEGF-induced in vitro angiogenesis was effectively reduced by luteolin on HRMECs.

Blockade of t-BH–induced ROS Production and Down-Regulation of VEGF on Retinal Endothelial Cells by Luteolin

To determine whether anti-angiogenic effect of luteolin on HRMECs was mediated by anti-oxidant activity of luteolin, we measured the inhibitory activity of luteolin to ROS production and VEGF mRNA expression after treatment of t-BH on HRMECs. As shown in Figure 4A, 100-μM t-BH increased intracellular ROS production about 2-fold compared with control ($P < 0.05$). However, t-BH–induced intracellular ROS production was significantly decreased to 1.4-fold compared with control with cotreatment of 1-μM luteolin ($P < 0.05$). In addition, t-BH–induced VEGF transcription (1.8-fold, $P < 0.05$) was significantly reduced to 1.2-fold compared with control with cotreatment of 1-μM luteolin ($P < 0.05$, Fig. 4B).

Effect of Luteoiln on Hypoxia-Induced HIF-1α and VEGF Transcription and Expression

Because astrocyte is one of the major sources of VEGF expression in the retina under hypoxia, we investigated whether luteolin had an anti-angiogenic effect on astrocyte under hypoxia. We measured the inhibitory activity of luteolin to hypoxia-induced HIF-1α and VEGF transcription and expression.

Human brain astrocytes were incubated under hypoxia with treatment of 1-μM luteolin for 8 hours. As shown in Figure 5A, hypoxia-induced VEGF transcription was significantly decreased with treatment of 1-μM luteolin. Interestingly, 1-μM luteolin never affected the transcriptional activity of HIF-1α under hypoxia. However, 1-μM luteolin reduced HIF-1α expression under hypoxic condition, which led to the decrease of VEGF transcription and expression. (Figs. 5A, 5B). In addition, hypoxia-induced VEGF transcriptions (VEGF 165: 4.7-fold, VEGF 121: 3.5-fold, $P < 0.05$) were significantly reduced to 3.0-fold and 2.4-fold compared with control, respectively ($P < 0.05$, Fig. 5C).

Discussion

In the present study, we, for the first time, demonstrated that luteolin inhibited retinal neovascularization via blockade of ROS production. Under therapeutic concentration less than 10-μM of luteolin where it never affect the viability of HRMECs, nor show retinal toxicity, luteolin significantly inhibited in vitro angiogenesis processes and retinal neovascularization. Luteolin suppressed t-BH–induced mitochondrial ROS production, and hypoxia-induced mitochondrial ROS production,[27] leading to inhibition of HIF-1α and VEGF expression in vitro, and retinal neovascularization in vivo. In addition, luteolin inhibits VEGF

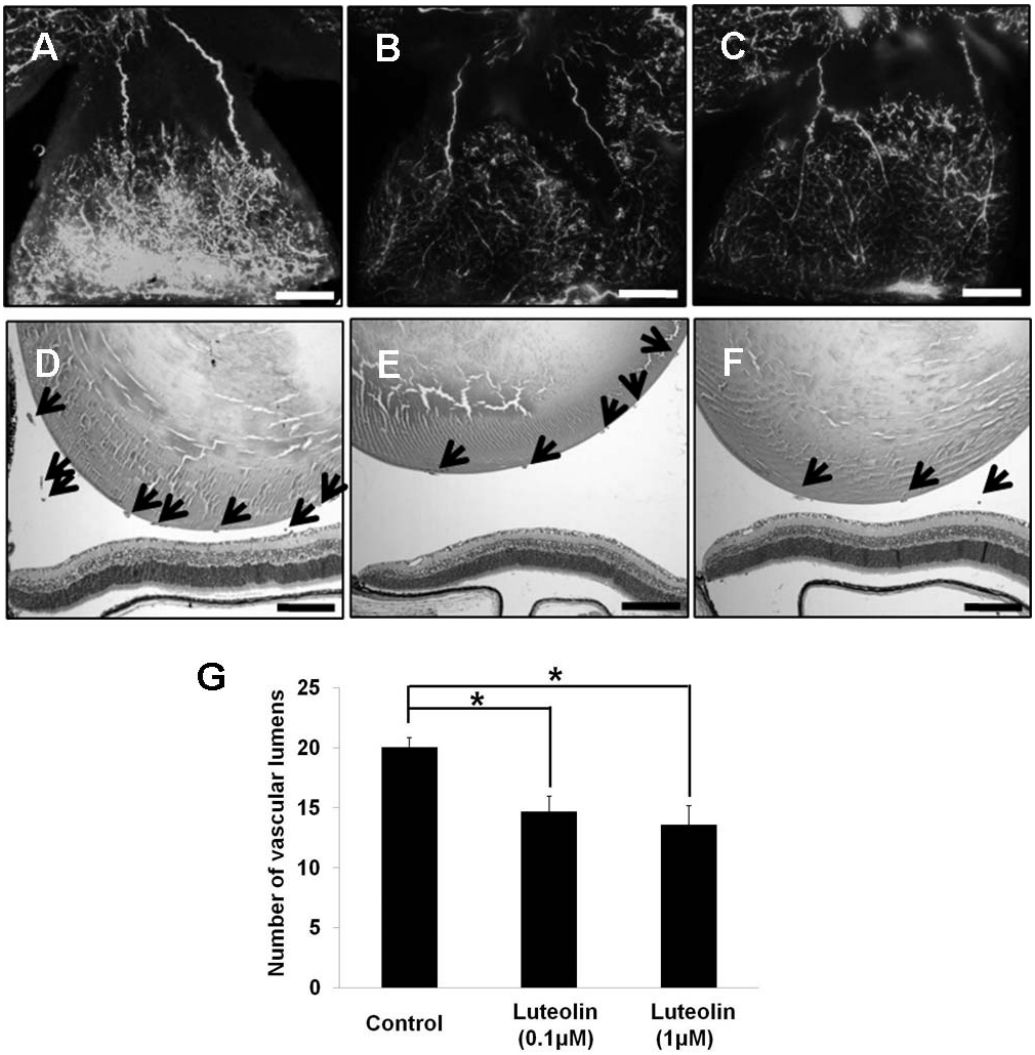

Figure 2. Luteolin inhibits retinal neovascularization in OIR. (**A**, **B**, **C**) Retinal vasculatures of control and luteolin-treated mice with OIR were evaluated with fluorescein angiography. Whole mount retinal preparation from P17 control (**A**) and 0.1-μM luteolin (**B**), 1-μM luteolin-intravitreously injected mice (**C**) of OIR was performed after 1 hour perfusion of fluorescein conjugated dextran. Neovascular tufts of intravitreous neovascularization were observed at the border of vascular and avascular retina. (**D**, **E**, **F**) Hematoxylin-stained cross-sections were prepared from P17 control (**D**), 0.1-μM luteolin (**E**), and 1-μM luteolin-treated mice (**F**) of OIR. *Arrows* indicate the vascular lumens of new vessels growing into the vitreous. (**G**) Number of vascular lumens was counted from randomly selected ×40 magnification view. *Scale bars*: 50 μm. Each value represents the mean (±SD) of three independent experiments (*$P < 0.05$).

signaling by suppressing the generation of ROS derived from mitochondria in ECs,[9,10] resulting in the inhibition of VEGF-induced angiogenesis in vitro. The anti-angiogenic effect of luteolin on retinal neovascularization might be related to its anti-oxidant activity.

Conventional MTT tetrazolium reduction assay was not suitable for luteolin, since luteolin directly reduces MTT in the absence of living cells.[23] It leads to underestimation of the toxicity of an anti-oxidant. Thus, we used modified MTT assay for cell viability assay. The results of modified MTT were not underestimated than trypan blue exclusion assay (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8790/-/DCSupplemental). That is, because with modified MTT methods, changing the condition media with luteolin to fresh media before adding MTT lead to no direct contact with MTT and luteolin. Further, luteolin toxicity was not significantly different between HRMECs and human brain astrocytes (data not shown).

ROP, a leading cause of blindness in newborns, is a proliferative disease of the retinal vascular in premature infants,[1,2] whose antioxidant system is highly stressed and incompletely developed.[5] Considering the pathogenesis of ROP that the pathologic angiogenesis in developing retinal vasculature occurs after the vaso-obliteration, ROP could be thought to be related to the action of ROS.[3,28] In addition, oxidative stress plays an important pathogenic role in retinal inflammation in DR and oxidation also plays an important role in angiogenesis.[29,30] The elevation of intracellular ROS generation in the retina is a characteristic of the oxidative stress found in DR.[31]

In general, high levels of ROS are toxic and cause cell damage and cell death, whereas low and adequate amounts of ROS can serve as signaling molecules to induce proliferation and migration of endothelial cells.[32] The importance of ROS production in angiogenesis has been documented in diabetic eyes, in balloon-injured arteries,[33] and in pulmonary artery obstruction.[34] ROS increased VEGF expression and enhanced neovascularization of atherosclerotic and restenotic arteries.[33] Therefore, early and transient increase of ROS production played an initiating and promoting role in subsequent

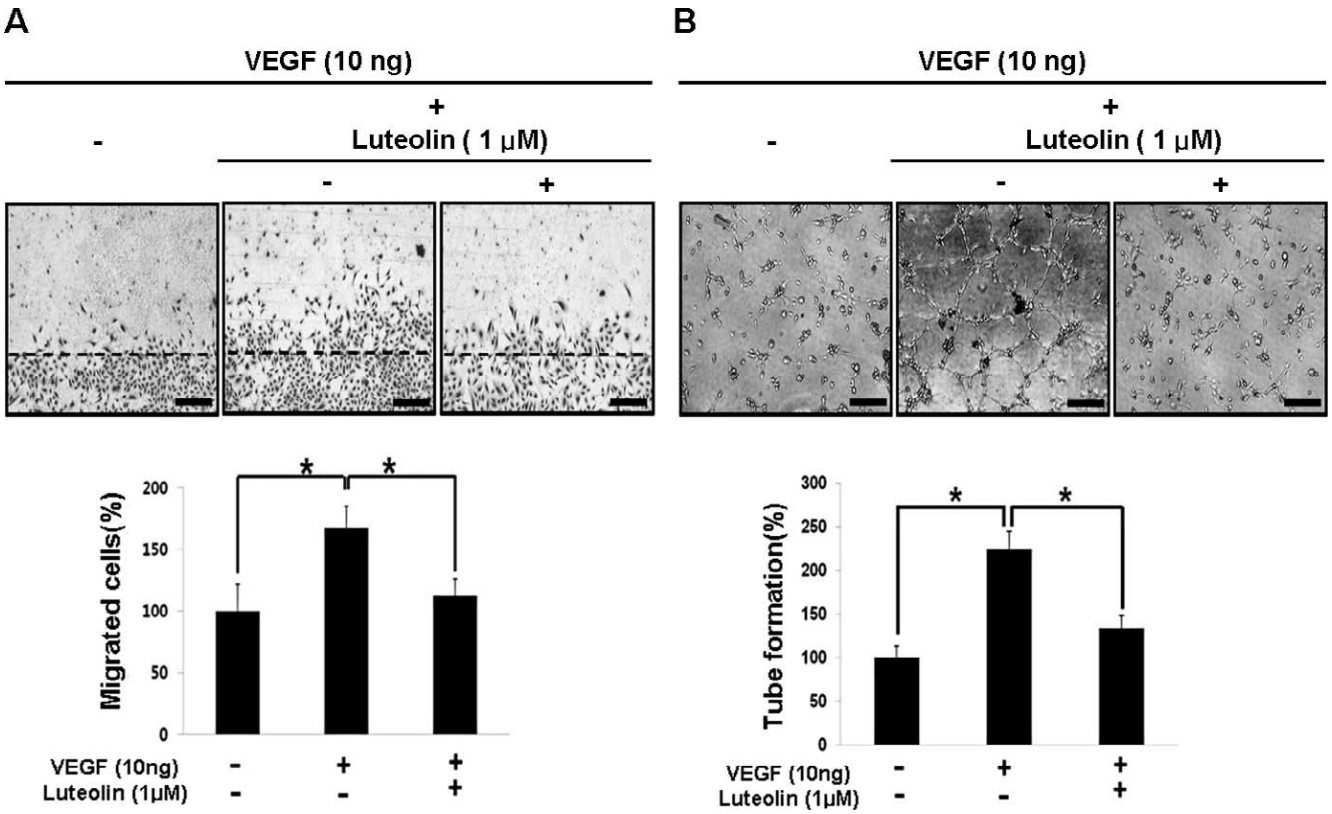

Figure 3. Luteolin inhibits VEGF-induced migration and tube formation of HRMECs. (**A**) HRMECs (1×10^6 cells) were plated onto gelatin-coated culture dishes at 90% confluence, and wounded with a razor blade. The wounded monolayer was incubated with treatment of 1-μM luteolin or 10-ng/mL VEGF for 24 hours. Migration was quantified by counting the number of cells beyond the reference line. The basal migration of HRMECs without VEGF and luteolin was normalized to 100%. (**B**) HRMECs (5×10^4 cells) were placed on Matrigel-coated plates and incubated with treatment of 1-μM luteolin or 10-ng/mL VEGF for 8 hours. Tube formation was quantified by counting the number of connected cells in randomly selected fields and dividing that number by the total number of cells in the same field. The basal tube formation of HRMECs without VEGF and luteolin was normalized to 100%. *Scale bars*: 100 μm. Each value represents the mean (±SD) of three independent experiments (*$P < 0.05$).

ischemia-induced angiogenesis, when IL-2 could activate angiogenesis via ROS production.[34] In addition, ischemia-mediated hypoxia/reoxygenation caused a progressive increase of ROS production to lead the activation of the nuclear transcription factor kappa B, which resulted in active angiogenesis to ischemic injury and were inhibited by ROS-scavengers.[4] Interestingly, it was reported that ROS is directly involved in VEGF-mediated signaling pathways linked to angiogenic responses, whereas VEGF itself could stimulate ROS production through activation of an endothelial nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase.[35] Furthermore, antioxidants such as N-acetylcysteine (NAC) have been shown to inhibit angiogenesis both in vitro and in vivo.[36] Therefore, antioxidants could be applied to inhibit angiogenesis based on blockade of ROS-mediated angiogenic processes.[4]

Platycodon grandiflorum is widely used in Asian traditional herbal medicine, which has variable biological activities as an anti-oxidant.[17] Recently we isolated and identified luteolin from an EtOAc-soluble fraction of the flowers of *Platycodon grandiflorum*.[16,22] In addition, we found out that luteolin extracted from *Platycodon grandiflorum* protected retinal pigment epithelial cells from oxidative stress induced apoptosis as other researchers' reports that luteolin blocked ROS accumulation in retinal cells.[19,20]

We showed that luteolin reduced t-BH–induced VEGF transcription via attenuating ROS production. Though luteolin could inhibit hypoxia-induced VEGF expression by a HIF-1α independent mechanism, suppression of STAT3 tyrosine phosphorylation,[21] we also confirmed that luteoiln reduced hypoxia-induced VEGF transcription and expression via attenuating HIF-1α expression, not HIF-1α transcription. The anti-angiogenic mechanism of luteolin as anti-oxidant could be explained in that it blocks VEGF expression via regulating ROS.[27] The stability and expression levels of HIF-1α can be regulated by ROS under nonhypoxic and hypoxic conditions. Under normoxia, HIF-1α is stabilized by oxidative stress induced by hydrogen peroxide, and antioxidants markedly attenuate HIF-1α protein accumulation, perhaps by the inactivation of the proline hydroxylase (PHD).[37] Hypoxia inhibits prolyl hydroxylation, resulting in stabilization of HIF-1α, and through increased ROS, which oxidize PHD-bound iron. Under hypoxic conditions, the regulation mechanisms of HIF-1α by ROS are complex in that ROS levels are elevated in hypoxia[38] and hypoxia-induced HIF-1α stability through inactivation of PHD by binding to iron.[39] Thus, anti-angiogenic effect of luteolin on both t-BH–induced VEGF and hypoxia-induced VEGF expression could be explained by the anti-oxidant activity of luteolin regulating ROS.

We demonstrated that luteolin inhibited VEGF-induced migration and tube formation of HRMECs. The mechanism of this anti-angiogenic activity of luteolin could be also explained by the anti-oxidant activity of luteolin regulating ROS.

Accumulating evidence indicates that ROS are associated with VEGF-stimulated VEGFR2 autophosphorylation in ECs.[9,10] Recent evidence has revealed that ROS derived from mitochondria regulate VEGF-induced angiogenesis.[40] We could not determine the exact mechanism how luteolin inhibit VEGF-induced angiogenesis in this study. However, we suggested that

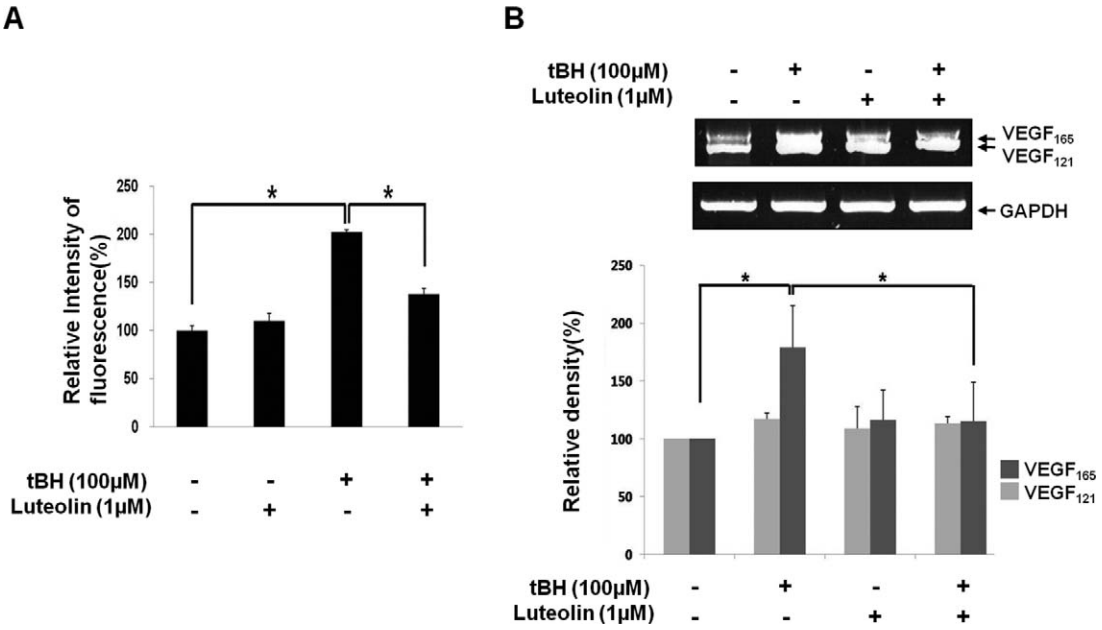

FIGURE 4. Luteolin inhibits t-BH–induced ROS production and VEGF transcription in HRMECs. (**A**) HRMECs were exposed to 100-μM t-BH with treatment of 1-μM luteolin for 30 minutes and were incubated with DCFH-DA for 30 minutes. Intracellular ROS production was measured on a spectrofluorometer, excitation and emission settings of 495 and 525 nm, respectively. Quantitative analysis was performed by measuring the fluorescence intensity relative to the control. (**B**) HRMECs were treated with 100-μM t-BH or 1-μM luteolin for 2 hours. VEGF mRNA transcription was analyzed by RT-PCR. Quantitative analysis was performed by measuring the density relative to the control. Each value represents the mean (±SD) of three independent experiments (*$P < 0.05$).

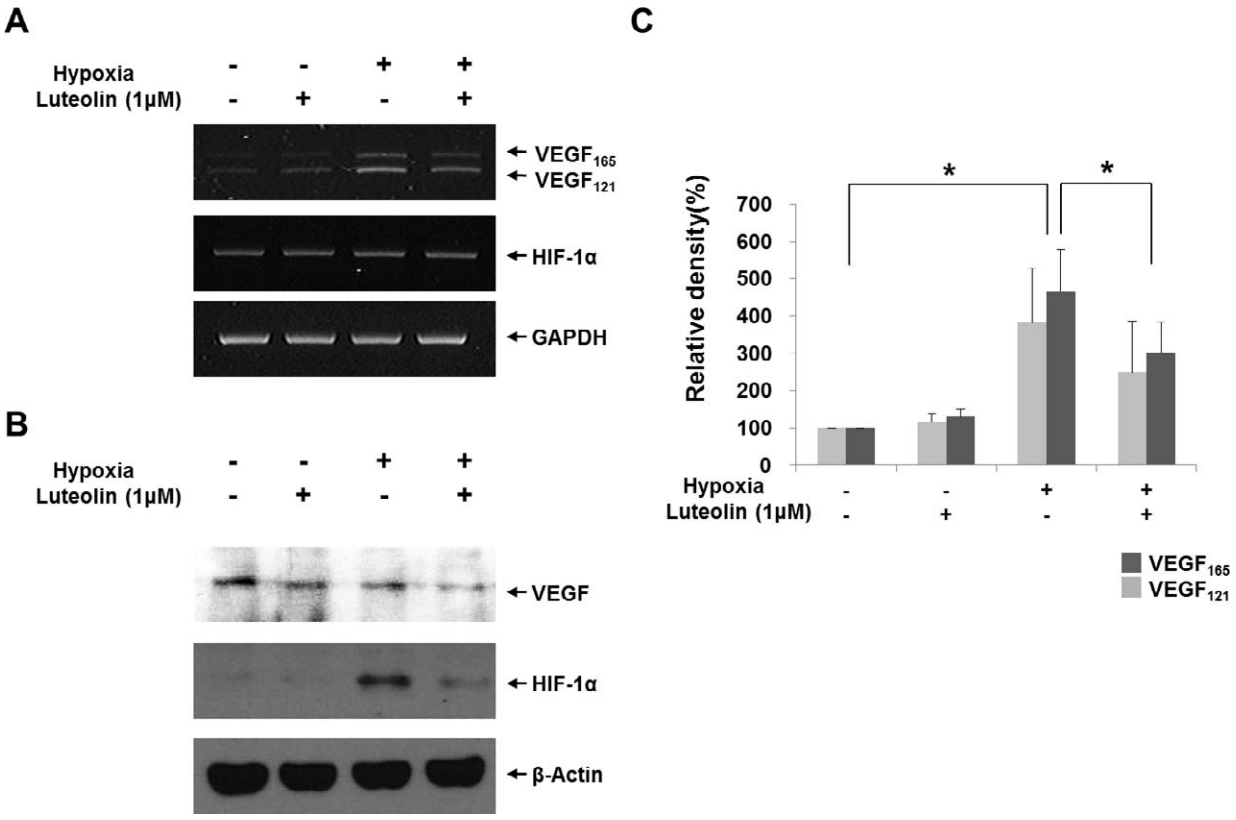

FIGURE 5. Luteolin inhibits hypoxia-induced VEGF transcription and expression in human brain astrocytes. Human brain astrocytes were incubated under hypoxia with treatment of 1-μM luteolin for 8 hours. (**A**) HIF-1α and VEGF mRNA transcription were analyzed by RT-PCR. (**B**) HIF-1α and VEGF protein expression were analyzed by Western blot. (**C**) Quantitative analysis of VEGF mRNA transcription was performed by measuring the density relative to the control. Each value represents the mean (±SD) of six independent experiments (*$P < 0.05$).

luteolin could inhibit VEGF-induced angiogenesis via blockade of ROS production[40] or by blocking PI3K/Akt pathway or partially blocking phosphorylation and kinase activation of VEGFR2.[41] Indeed, luteolin acted through an intracellular route to block the accumulation of reactive oxygen species in retinal ganglion cell (RGC)-5 cells.[19] It also protected ARPE-19 cells even 2 hours after exposure to t-BH–induced oxidative stress.[20] It implied that luteolin could not only inhibit the intracellular ROS production, but also reduce accumulated intracellular ROS.

Thus, we suggested that the mechanism of luteolin's inhibition of neovascularization in the ROP model could be both because it blocked VEGF expression and because it blocked VEGF signaling pathway after it is expressed by regulating ROS as anti-oxidant.

In our study, anti-angiogenic effect of luteolin was observed to reduce retinal neovascularization in OIR mice, as a ROP model. Interestingly, luteolin inhibited both t-BH–induced VEGF transcription via blockade of ROS production and hypoxia-induced VEGF expression via attenuating HIF-1α expression. This effect was confirmed in vitro angiogenesis, by inhibiting VEGF-induced migration and tube formation in HRMECs. It implied that luteolin could inhibit retinal neovascularization not only reducing VEGF transcription itself, but also inhibiting the pro-angiogenic effect of VEGF signaling pathway via blockade of ROS.

In conclusion, the anti-angiogenic activity of luteolin in HRMECs and OIR might be related to the anti-oxidant activity of luteolin mediated by ROS-induced VEGF expression and ROS-regulating VEGF effect. We suggest that luteolin could be safely applied to inhibit retinal neovascularization in ROP. Furthermore, it may be applicable to other retinopathy that was mediated by ROS-induced angiogenesis, such as AMD or DR.

References

1. Jo DH, Kim JH. How to overcome retinal neuropathy: the fight against angiogenesis-related blindness. *Arch Pharm Res*. 2010; 33:1557–1565.
2. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*. 1994; 331:1480–1487.
3. Sapieha P, Joyal JS, Rivera JC, et al. Retinopathy of prematurity: understanding ischemic retinal vasculopathies at an extreme of life. *J Clin Invest*. 2010;120:3022–3032.
4. Lelkes PI, Hahn KL, Sukovich DA, Karmiol S, Schmidt DH. On the possible role of reactive oxygen species in angiogenesis. *Adv Exp Med Biol*. 1998;454:295–310.
5. Buhimschi IA, Buhimschi CS, Pupkin M, Weiner CP. Beneficial impact of term labor: nonenzymatic antioxidant reserve in the human fetus. *Am J Obstet Gynecol*. 2003;189:181–188.
6. Saito Y, Uppal A, Byfield G, Budd S, Hartnett ME. Activated NAD(P)H oxidase from supplemental oxygen induces neovascularization independent of VEGF in retinopathy of prematurity model. *Invest Ophthalmol Vis Sci*. 2008;49:1591–1598.
7. Liu T, Castro S, Brasier AR, Jamaluddin M, Garofalo RP, Casola A. Reactive oxygen species mediate virus-induced STAT activation: role of tyrosine phosphatases. *J Biol Chem*. 2004; 279:2461–2469.
8. Xia C, Meng Q, Liu LZ, Rojanasakul Y, Wang XR, Jiang BH. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Res*. 2007;67:10823–10830.
9. Ushio-Fukai M, Nakamura Y. Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Lett*. 2008;266:37–52.
10. Ushio-Fukai M. Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res*. 2006;71:226–235.
11. Kim J, Ahn JH, Kim JH, et al. Fenofibrate regulates retinal endothelial cell survival through the AMPK signal transduction pathway. *Exp Eye Res*. 2007;84:886–893.
12. Kim JH, Kim KH, Yu YS, Kim YM, Kim KW, Kwon HJ. Homoisoflavanone inhibits retinal neovascularization through cell cycle arrest with decrease of cdc2 expression. *Biochem Biophys Res Commun*. 2007;362:848–852.
13. Kim JH, Yu YS, Shin JY, Lee HY, Kim KW. Deguelin inhibits retinal neovascularization by down-regulation of HIF-1alpha in oxygen-induced retinopathy. *J Cell Mol Med*. 2008;12:2407–2415.
14. Kim JH, Oh M, Yu YS, Kim KW, Kwon HJ. N-hydroxy-7-(2-naphthylthio) heptanomide inhibits retinal and choroidal angiogenesis. *Mol Pharm*. 2009;6:513–519.
15. Kim JH, Lee YM, Ahn EM, Kim KW, Yu YS. Decursin inhibits retinal neovascularization via suppression of VEGFR-2 activation. *Mol Vis*. 2009;15:1868–1875.
16. Jang DS, Lee YM, Jeong IH, Kim JS. Constituents of the flowers of *Platycodon grandiflorum* with inhibitory activity on advanced glycation end products and rat lens aldose reductase in vitro. *Arch Pharm Res*. 2010;33:875–880.
17. Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: antioxidants or signalling molecules? *Free Radic Biol Med*. 2004;36:838–849.
18. Lee WJ, Wu LF, Chen WK, Wang CJ, Tseng TH. Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K-Akt pathways. *Chem Biol Interact*. 2006;160:123–133.
19. Maher P, Hanneken A. Flavonoids protect retinal ganglion cells from oxidative stress-induced death. *Invest Ophthalmol Vis Sci*. 2005;46:4796–4803.
20. Hanneken A, Lin FF, Johnson J, Maher P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Invest Ophthalmol Vis Sci*. 2006;47:3164–3177.
21. Anso E, Zuazo A, Irigoyen M, Urdaci MC, Rouzaut A, Martinez-Irujo JJ. Flavonoids inhibit hypoxia-induced vascular endothelial growth factor expression by a HIF-1 independent mechanism. *Biochem Pharmacol*. 2010;79:1600–1609.
22. Han XH, Hong SS, Hwang JS, Lee MK, Hwang BY, Ro JS. Monoamine oxidase inhibitory components from Cayratia japonica. *Arch Pharm Res*. 2007;30:13–17.
23. Talorete TP, Bouaziz M, Sayadi S, Isoda H. Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells. *Cytotechnology*. 2006;52:189–198.
24. Kim JH, Lee BJ, Yu YS, Kim KW. Anti-angiogenic effect of caffeic acid on retinal neovascularization. *Vascul Pharmacol*. 2009;51:262–267.
25. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35: 101–111.
26. Kim JH, Lee BJ, Yu YS, Kim MY, Kim KW. Rosmarinic acid suppresses retinal neovascularization via cell cycle arrest with increase of p21(WAF1) expression. *Eur J Pharmacol*. 2009; 615:150–154.
27. Kuo MT. Redox regulation of multidrug resistance in cancer chemotherapy: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal*. 2009;11:99–133.
28. Chen J, Smith LE. Retinopathy of prematurity. *Angiogenesis*. 2007;10:133–140.
29. Altomare E, Grattagliano I, Vendemaile G, Micelli-Ferrari T, Signorile A, Cardia L. Oxidative protein damage in human diabetic eye: evidence of a retinal participation. *Eur J Clin Invest*. 1997;27:141–147.

30. Connor KM, Subbaram S, Regan KJ, et al. Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation. *J Biol Chem*. 2005;280:16916–16924.
31. Kanwar M, Chan PS, Kern TS, Kowluru RA. Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. *Invest Ophthalmol Vis Sci*. 2007;48:3805–3811.
32. Zhang DX, Gutterman DD. Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. *Am J Physiol Heart Circ Physiol*. 2007;292:H2023–2031.
33. Ruef J, Hu ZY, Yin LY, et al. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ Res*. 1997; 81:24–33.
34. Nijmeh J, Moldobaeva A, Wagner EM. Role of ROS in ischemia-induced lung angiogenesis. *Am J Physiol Lung Cell Mol Physiol*. 2010;299:L535–541.
35. Ushio-Fukai M, Tang Y, Fukai T, et al. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res*. 2002;91: 1160–1167.
36. Cao Y, Cao R. Angiogenesis inhibited by drinking tea. *Nature*. 1999;398:381.
37. Pouyssegur J, Mechta-Grigoriou F. Redox regulation of the hypoxia-inducible factor. *Biol Chem*. 2006;387:1337–1346.
38. Taylor CT. Mitochondria and cellular oxygen sensing in the HIF pathway. *Biochem J*. 2008;409:19–26.
39. Guzy RD, Hoyos B, Robin E, et al. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metab*. 2005;1:401–408.
40. Wang Y, Zang QS, Liu Z, et al. Regulation of VEGF-induced endothelial cell migration by mitochondrial reactive oxygen species. *Am J Physiol Cell Physiol*. 2011;301:C695–704.
41. Bagli E, Stefaniotou M, Morbidelli L, et al. Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3′-kinase activity. *Cancer Res*. 2004;64:7936–7946.