Inhibition of Obtusifolin on retinal pigment epithelial cell growth under hypoxia

Li-Fei Wang¹, Zhong-Yang Yan¹, Ya-Lin Li¹, Yan-Hui Wang¹, Sheng-Juan Zhang¹, Xin Jia¹, Lu Lu², Yan-Xia Shang², Xin Wang³, Yun-Huan Li¹, Shan-Yu Li¹

¹Fundus Surgery Ward, Hebei Provincial Eye Hospital, Xingtai 054001, Hebei Province, China
²Diabetic Eye Disease Ward, Hebei Provincial Eye Hospital, Xingtai 054001, Hebei Province, China
³Corneal Disease Ward, Hebei Provincial Eye Hospital, Xingtai 054001, Hebei Province, China

Co-first authors: Li-Fei Wang and Zhong-Yang Yan

Correspondence to: Li-Fei Wang. Fundus Surgery Ward, Hebei Provincial Eye Hospital, No.399 East Quanbei Street, Qiaodong District, Xingtai 054001, Hebei Province, China.

Tel: 8629-82245172     8629-82210956      Email: ijopress@163.com

Received: 2018-08-15        Accepted: 2019-06-11

DOI:10.18240/ijo.2019.10.04


INTRODUCTION

As a degenerative cause, choroidal neovascularization (CNV) is the pathological basis of various eye diseases such as age-related macular degeneration (AMD), myopic macular degeneration (PM) and central exudative chorioretinopathy (CEC)⁴⁵. The mechanism of occurrence and development of CNV is complex, the principle is not yet clear, and treatment is difficult. Current treatments for CNV include surgery to remove or block CNV, intravitreal injections of anti-angiogenic drugs such as anti-vascular endothelial growth factor (VEGF), and using of glucocorticoids and inflammatory reactions⁶⁷⁸⁹. However, the efficacies of the above methods are not satisfactory, because of poor long-term efficacy, high recurrence rate, high price, and many adverse reactions⁶⁷⁸⁹. Although modern medicine has developed rapidly in CNV studies, the clinical efficacy of current CNV is not effective. Therefore, it is of great significance to explore new treatments.

Semen Cassiae is dry, mature seed of the leguminous plant Cassia obtusifolia L. or Cassia tora L. It is an ancient Chinese medicine that can be used as a food and medicine⁹⁰. The main active ingredient of cassia is Obtusifolin, which has antioxidant and nominal effects⁹⁰. Study has reported that the activity of ciliary lactate dehydrogenase (LDH) in obtusifolin-fed dogs and rabbits was significantly elevated⁹⁰. Therefore, we speculate that Obtusifolin has effects on the treatment of CNV.

The generation of blood vessels refers to the process of forming a new capillary network by sprouting or intussusception after the body or tissue receiving the stimulus⁹¹. Current research suggests that hypoxia is one of the most important causes of the occurrence and development of CNV and studies have confirmed that VEGF plays a key role in the formation of CNV⁹²⁹³. The hypoxia inducible factor-1(HIF-1)/VEGF/eNOS pathway is mainly induced by hypoxic environment,
activates eNOS release of NO and other factors through
signal transduction, regulates cell proliferation, apoptosis, and
migration\(^{14-15}\). It is considered that VEGF-related pathways
and proteins are overexpressed in ocular diseases where CNV
is the pathological basis\(^{16-17}\).

This study explored the effects of Obtusifolin on cell viability
and VEGF in human retinal epithelial cells under hypoxic
conditions, and explored its effects on CNV.

**MATERIALS AND METHODS**

**Cells Culture and Observation** The human retinal epithelial
cells line (ARPE-19) was purchased from ATCC (USA). The
cells were cultured in RPMI 1640 medium containing 10% fetal
bovine serum and 100 U/mL of penicillin-streptomycin
mixture in an incubator at 37°C in 5% CO\(_2\). According to
different groups, the corresponding concentration (100, 200,
400 μg/mL) of Obtusifolin was added to the culture medium
and incubated at 37°C in 5% CO\(_2\) for 24h. Obtusifolin was
dissolved in DMSO and the amount of DMSO did not exceed
0.1% of the total volume of the medium. An *in vitro* chemical
hypoxia model was established by adding cobalt chloride
(CoCl\(_2\); Sigma, USA) to the culture medium. Cell culture-
related reagents were purchased from Gibco (USA). All cells
in this experiment were within 5 passages. ARPE-19 cells
morphology was observed through a light microscope (Nikon,
Japan).

**Cell Viability Analysis** Cell counting kit-8 (CCK-8) assay
was used to detect cell viability at 12, 24, and 48h after added
0, 50, 100, 150, 200 μmol/L CoCl\(_2\). The kit was purchased
from Tongren (Japan). Diluted CCK-8 reagent were added and
cultured at 37°C in 5% CO\(_2\) atmosphere for 4h. The absorbance
of each well at 450 nm was measured using a microplate reader
(ELX 800, Bio-Teck, USA), and cell viability was calculated
according to the standard curve.

**Real-time Quantitative Polymerase Chain Reaction**

**Analysis** Real-time quantitative polymerase chain reaction
analysis (RT-qPCR) was used to detect the mRNA expression
levels of HIF-1α, Cyclin D1, proliferating cell nuclear antigen
(PCNA), p53, p21, VEGF, VEGFR2 and eNOS. The cells were
triturated and lysed using Trizol (TaKaRa, Japan) at 0°C for
5min. The RNAs were extracted by CCl\(_3\) (Aladdin, China) and
dissolved in DEPC water (Sigma aliquots). RNA concentration
was measured by using a UV spectrophotometer (NanoDrop
One Microvolume UV-Vis spectrophotometer, Thermo, USA).
Reverse transcription assays were performed on RNA
samples using a reverse transcription kit (TaKaRa, Japan) to
synthesize cDNA. Reverse transcription reaction conditions
was 37°C for 15min and reverse transcriptase inactivation
condition was 85°C for 15s. RT-qPCR experiments were
performed with the SYBR Premix Ex TaqTM Real-Time PCR
Kit (TaKaRa, Japan). PCR was performed by activating the
DNA polymerase at 95°C for 5min, followed by 40 cycles
of two-step PCR (95°C for 10s and 60°C for 30s) and a final
extension at 75°C for 10min and held at 4°C. DnaSe and
RNase-free water were used as the templates of negative
control experiences. All primers were obtained from Genewiz
(Suzhou, Jiangsu China) and listed in Table 1. GAPDH was
considered as an internal control. The formula 2\(^{-\Delta\Delta CT}\) was
implemented to analyze the gene expression.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α-forward</td>
<td>ACCTATGACCTGCTTCCTGC</td>
<td>98</td>
</tr>
<tr>
<td>HIF-1α-reverse</td>
<td>TTTAACTCAAGCTGCTCGC</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1-forward</td>
<td>CTGGCCATGAACTCCTCGA</td>
<td>245</td>
</tr>
<tr>
<td>Cyclin D1-reverse</td>
<td>GTCACACTTGATGCTTCTCG</td>
<td></td>
</tr>
<tr>
<td>PCNA-forward</td>
<td>CACCTTACGCTTGATGCTTCTCG</td>
<td>137</td>
</tr>
<tr>
<td>PCNA-reverse</td>
<td>CACCCGACGGCATCTGTTACATAC</td>
<td></td>
</tr>
<tr>
<td>p53-forward</td>
<td>CTGAGGTCGCTGCTGGATGCTGTCG</td>
<td>360</td>
</tr>
<tr>
<td>p53-reverse</td>
<td>CTGATTCGCTGCTGGATGCTGTCG</td>
<td></td>
</tr>
<tr>
<td>P21-forward</td>
<td>AGTATGCGCTGCGTCTGCTGCTG</td>
<td>229</td>
</tr>
<tr>
<td>P21-reverse</td>
<td>CTGATCCTGCTGCTGCTGCTGCTG</td>
<td></td>
</tr>
<tr>
<td>VEGF-forward</td>
<td>CTGGAGCGGCTGCTGGATGCTGTCG</td>
<td>177</td>
</tr>
<tr>
<td>VEGF-reverse</td>
<td>TTTAACTCAAGCTGCTTCCTCG</td>
<td></td>
</tr>
<tr>
<td>VEGFR2-forward</td>
<td>CCAGGCAAGCTAGTGTGCTGAGAGAG</td>
<td>243</td>
</tr>
<tr>
<td>VEGFR2-reverse</td>
<td>GGGACCCACGTCCTAACAACAGAGAGAG</td>
<td></td>
</tr>
<tr>
<td>eNOS-forward</td>
<td>ACCCTACGCGCTACACATC</td>
<td>217</td>
</tr>
<tr>
<td>eNOS-reverse</td>
<td>GCTCATCCTCAGGTCGCTC</td>
<td></td>
</tr>
<tr>
<td>GAPDH-forward</td>
<td>CCATCTTCAAGGAGCGGAGATAGGAG</td>
<td>222</td>
</tr>
<tr>
<td>GAPDH-reverse</td>
<td>TGCTGTAGATCTTCTGAGGCTG</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 The sequences of primers
Western Blot Western blot was applied to detect protein expression. Cells were lysed with liquid nitrogen and blocked with RIPA (Abmole, USA), followed by 1% cleavage in PMSF and phosphatase inhibitors (Abmole, USA) and lysis for 30 min at 4°C. The supernatant was collected by centrifugation at 12,000 rpm at 4°C for 15 min. A standard curve was drawn using the BCA method to determine the protein concentration. A 10% SDS-PAGE gel was prepared without RNase dH2O and used to electrophoresis. The PVDF membrane (Bio-Rad, USA) was transferred using a Trans-Blot Transfer Slot (Bio-Rad, USA) and blocked with 5% fat-free milk for 2 h at room temperature. The primary antibody (anti-HIF-1α, Abcam, ab51608, dilution: 1:800; anti-Cyclin D1, Abcam, ab134175, dilution: 1:800; anti-PCNA, ab29, Abcam, dilution: 1:700; anti-p53, Abcam, ab26, dilution: 1:600; anti-p21, Abcam, ab109520, dilution: 1:600; anti-VEGF, Merck Millipore, ABS82, dilution: 1:800; anti-VEGFR2, Abcam, ab2349, dilution: 1:600; anti-eNOS, Abcam, ab76198, dilution: 1:900) was added according to the kit instructions, shaking at room temperature for 2 h, then incubated at 4°C for 12 h. The secondary antibody (goat anti-mouse IgG, Abcam, ab6785, 1:8000; rabbit anti-mouse IgG, Abcam, ab99697, dilution: 1:9000; mouse anti-rabbit IgG, Invitrogen, BA1034, 1:7000; donkey anti-rabbit IgG, R&D, NL004, 1:5000; rabbit anti-human IgG, Abcam, ab6759, dilution: 1:10000) was added and incubated at room temperature for 1.5 h. Chemiluminescence detection was carried out using ECL reagents (Huiying, Shanghai, China).

Evaluation of Cell Cycle Cell cycle was tested by flow cytometry. The cells were collected and washed with PBS at 0°C, and then fixed with 75% ethanol at -20°C for 12 h. After fixation, the cells were treated with 10 μL of RNase A (10 mg/mL, TaKaRa, Japan) for 30 min at 37°C. And then detected by flow cytometer (Becton Dickinson, SanJose, CA, USA). The flow cytometry results were processed by FlowJo V10 software (Becton, Dickinson & Company, USA).

Enzyme Linked Immunosorbtent Assay The VEGF concentration of culture fluid was tested using enzyme linked immunosorbent assay (ELISA). The kits were purchased from Nanjing Kajii Biotechnology Co., Ltd. (China). The primary antibody was added at 4°C overnight, after washing blocking solution was added at 4°C for 2 h. And then the secondary antibody was added and incubated for 1 h at room temperature. Horseradish peroxidase (HRP) was dropped for 0.5 h at room temperature, and tetramethylbenzidine (TMB) was added for 10 min. The absorbance value was measured at 450 nm by a microplate reader (ELX 800, Bio-Teck, USA) and the concentration was calculated according to the standard curve.

Statistical Analysis All the experimental data were presented as mean±standard deviation (SD). Statistical analysis used
Inhibition of Obtusifolin on RPE cells

could promote cell proliferation and division by regulating cell cycle-associated proteins, while Obtusifolin could reduce cell proliferation by affecting cell cycle-associated proteins and promote cells retention in the G1 phase.
Effects of Obtusifolin on HIF-1, VEGF, and eNOS

To study the effects of Obtusifolin on the HIF-1, VEGF, and eNOS in the hypoxic cell model, the expression levels of the relevant mRNA and protein in the pathway were detected by RT-qPCR and Western blot respectively. When ARPE-19 cells were exposed to hypoxia, the levels of HIF-1α, VEGF, VEGFR2 and eNOS proteins and mRNA were significantly increased (Figure 4). Obtusifolin could dose-dependently down-regulate the expression of the pathway to make it close to the control group (Figure 4). The level of VEGF secreted by ARPE-19 cells was significantly elevated under the induction of hypoxia. Obtusifolin dose-dependently down-regulated VEGF secretion (Figure 4F).

Figure 3 Effect of Obtusifolin on cell cycle associated proteins

Figure 4 Effect of Obtusifolin on HIF-1, VEGF, and eNOS

A-D: RT-qPCR was applied to detect HIF-1α, VEGF, VEGFR2 and eNOS mRNA expressions under 100, 200, 400 μg/mL Obtusifolin; E: Western blot was used to test HIF-1α, VEGF, VEGFR2 and eNOS protein expressions under 100, 200, and 400 μg/mL concentrations respectively. *P<0.05, **P<0.01 versus control group; *P<0.05, **P<0.01 versus CoCl2 group.
Effects of Obtusifolin on ARPE-19 Cells  The effects of different concentrations of Obtusifolin on cells was observed under a microscope (Figure 5). The possible mechanism of Obtusifolin was shown in Figure 6.

DISCUSSION

The main pathological basis of angiogenesis caused by hypoxia or inflammatory cytokines is overexpression of VEGF\(^{[18]}\). Angiogenesis is a complex process that involves the proliferation, migration and tube formation of vascular endothelial cells\(^{[19-20]}\). CNV blood vessels mainly come from the retinal pigment epithelial cell\(^{[18]}\), so this study uses human retinal pigment epithelial cells line ARPE-19 cells as the research object. CoCl\(_2\) has a low affinity with O\(_2\) and does not have the effect of regulating O\(_2\) concentration. However, Co\(^{2+}\) can replace the chelation of Fe\(^{2+}\) and hemoglobin, which destroys the ability of cells to sense hypoxia and thus mimic the hypoxic microenvironment\(^{[21]}\). The study also finds that CoCl\(_2\) can protect cells through anti-apoptosis pathways, and the method is simple, stable and easy to control\(^{[22]}\). Therefore, CoCl\(_2\) was used to simulate an in vitro chemical hypoxia microenvironment in this study. The results showed that the HIF-1\(\alpha\) and cell viability were increased in a dose-dependent manner in the ARPE-19 cells treated with CoCl\(_2\), demonstrating the successful establishment of an in vitro chemical hypoxia model.

Obtusifolin, including Emodin, Chrysophanol, Rhein, and Aloe-emodin, has a variety of biological activities, of which the eyesight is one of its most importance\(^{[10]}\). The results of this study showed that Obtusifolin had the effect of reducing the cell viability of ARPE-19 cells under hypoxic conditions. Further studies have also found that Obtusifolin could promote the retention in the G1 phase and inhibit the proliferation of ARPE-19 cells. Hou et al\(^{[23]}\) found that Obtusifolin has the effect of promoting apoptosis of retinal capillary cells in diabetic retinopathy rats. And other studies suggest that for hyperlipidemic rats, Obtusifolin shows anti-oxidation and NO regulation\(^{[24]}\). This showed that Obtusifolin inhibits the proliferation and differentiation of ARPE-19 cells, suggesting that it has a certain anti-angiogenic ability.

To further explore the mechanism of the effect of Obtusifolin on cell viability, we studied cell cycle-related proteins by Western blot and RT-qPCR. The results showed that Obtusifolin could dose-dependently down-regulate Cyclin D1 and PCNA in ARPE-19 cells under hypoxia and up-regulate p53 and p21 levels. Cyclin D1 is one of the most...
important proteins that regulate cell cycle, it can bind and activate the unique cyclin-dependent kinase CDK4 during G1, promoting cell cycle progression from G1 to S, thereby promoting cell proliferation⁴¹. PCNA is involved in cellular DNA synthesis. PCNA was not expressed in G0-G1 phase cells, but it was significantly increased in the late G1 phase, and PCNA was a sensitive indicator of cell cycle response³⁹. As a tumor suppressor gene, p53 has the effect of inhibiting cell proliferation by tissue cycle²⁷. The p21 gene is a member of the Cip family and it is a cyclin-dependent kinase inhibitor downstream of the p53 gene⁴⁰. P21 can together with p53 constitute the cell cycle G1 checkpoint²⁹. The results of this study suggest that Obtusifolin could inhibit cell proliferation by up-regulating tumor suppressor genes and down-regulating cyclin proteins.

The proliferation of cells is affected by a variety of cellular pathways. For the proliferation of retinal pigment epithelial cells and the formation of blood vessels, the largest influencing factor is the hypoxic microenvironment, and overexpression of VEGF is the main cause of vascular proliferation³⁰⁻³¹. HIF-1 is a key upstream transcription factor in angiogenesis signaling pathway, HIF-1 can be divided into HIF-1α and HIF-1β³². When the body is under hypoxia, it will induce high expression of HIF-1α, and it will up-regulate the expression of VEGF after binding with VEGF gene through hypoxia response element (HER)³³. As a key protein in the pathway, VEGF mainly promotes the release of eNOS and NO through the activation of PI3K/Akt, MAPK and JAK/STAT pathway³⁴⁻³⁶. NO is an essential angiogenesis profile factor³⁷⁻³⁸. On the other hand, PI3K/Akt and other pathways also have the effect of promoting cell proliferation and anti-apoptosis³⁹⁻⁴⁰. The results of this study indicated that the hypoxic microenvironment could promote the expression and secretion of VEGF by increasing the expression of HIF-1α, and promote the expression of VEGFR2 and eNOS. Obtusifolin could down-regulate the HIF-1α, decrease the expression and secretion of VEGF. Previous studies have shown that improving the hypoxic state can play an anti-angiogenic role by inhibiting VEGF⁴¹⁻⁴². Studies have also found that inhibiting the expression and secretion of VEGF can exert an anti-angiogenic effect by inhibiting the expression of NO⁴³. Tang and Zhong’s study⁴⁴ shows that Obtusifolin can regulate oxidative stress levels associated with obesity and diabetes. Obtusifolin regulates the levels of SOD and MDA to down-regulate oxidative stress levels. In addition, the study also finds that Obtusifolin regulates the level of NO⁴⁴. Study has also shown that Obtusifolin could reduce the level of inflammatory factors by inhibiting nuclear factor-kappa B, which might also be related to angiogenesis⁴⁵. This study first discovered that Obtusifolin could inhibit angiogenesis by inhibiting signal transduction by downregulating HIF-1α and reducing VEGF expression. In addition, Obtusifolin may also inhibit VEGF expression and may also inhibit cell proliferation by inhibiting VEGF related pathways and further studies are needed. Hypoxia could promote angiogenesis possibly by inducing the expression of VEGF, while Obtusifolin could inhibit the expression and secretion of VEGF by down-regulating HIF-1α, thereby reducing the inhibition of angiogenesis by eNOS.

ACKNOWLEDGEMENTS

Authors’ contributions: Substantial contributions to conception and design: Wang LF, Yan ZY; Data acquisition: Li YL, Wang YH; Data analysis and interpretation: Zhang SJ, Jia X; Drafting the article or critically revising it for important intellectual content: Lu L, Shang YX; Final approval of the version to be published: Wang X, Li YH; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: Li SY; All authors read and approved the final manuscript.

Conflicts of Interest: Wang LF, None; Yan ZY, None; Li YL, None; Wang YH, None; Zhang SJ, None; Jia X, None; Lu L, None; Shang YX, None; Wang X, None; Li YH, None; Li SY, None.

REFERENCES

Inhibition of Obtusifolin on RPE cells


31 Liu NN, Zhao N, Cai N. Suppression of the proliferation of hypoxia-Induced retinal pigment epithelial cell by rapamycin through the mTOR/ HIF-1α/VEGF signaling. J Sep Sci 2015;38(6):446-452.


down-regulating the ERK1/2-HIF-1α and STAT3 signaling pathways. *Eur J Pharmacol* 2016;771:93-98.


