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## Original article

# Effects of Puerarin on Experimental Model of Retinal Vein Occlusion in Rats

Chun-hua Hao, Wei-ting Wang, Ming-xiang Shao, Zhuan-you Zhao*, Li-da Tang*

*State Key Laboratory of Pharmacokinetics and Pharmacodynamics, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China*

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ABSTRACT

**Objective** To establish a model of retinal vein occlusion (RVO) in rats and to study the effect of puerarin on ischemic retinal disease and the corresponding mechanism. **Methods** RVO was induced in 10 adult male Sprague-Dawley (SD) rats by laser photothrombosis. Retinal blood flow was examined before and after 1 h of the operation, and model rats with the retinal vein blood flow decreasing by 50% compared to the basic value were chosen and then puerarins (20, 40, and 80 mg/kg) were given. The levels of vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), and nitric oxide (NO) were analyzed. In addition, the histopathology of RVO-eyes was performed. **Results** RVO-eyes displayed the signs of retinal damage and ischemia on Doppler Flowmeter and histopathology. Puerarin (20, 40, and 80 mg/kg) increased blood flow by 9.3% ($P < 0.05$), 33.1% ($P < 0.001$), and 41.5% ($P < 0.001$), respectively. On the other hand, the histological changes were less severe at different degrees, relieving the symptoms such as edematous and thick neuroretinal layers, lax, edematous, and disorganized optic fibers layers, swollen and confused inner and outer nuclear layer. Besides, dose-dependent decrease of VEGF and IL-1β and increase of NO in vitreous fluid were observed, with respect to the model group. **Conclusion** A rat model of laser photochemical-induced RVO is established and a decrease in the retinal blood flow and histological damage is detected. The puerarin has therapeutic benefit in the rat model of RVO, through the pathway of neovascularization, anti-inflammation, and increase of NO.

*Key words*

interleukin-1β; puerarin; retinal vein occlusion; vascular endothelial growth factor



## 1. Introduction

Retinal ischemia results from several causes such as retinal vein occlusion (RVO) mainly, which in turn can result in vitreous hemorrhage, tractional retinal detachment, or neovascular glaucoma (Shin et al, 1999). RVO is a common retinal vascular disease involving blindness due to the severe complications such as macular edema and neovascular glaucoma (Arevalo et al, 2008; Zhang et al, 2008). In animals, the earliest microvascular changes after RVO include venous dilation, decreased and/or reversed venous flow, arterial constriction, and increased venous pressure (Ben et al, 2001). In clinical practice, RVO has a wide diversity of pathologic manifestation, including neovascularization, hemorrhage,

* **Corresponding author: Zhao ZY Tang LD** Tel/Fax: +86-22-8484 5240 E-mail: haochunhua810207@163.com

exudation or leakage, filling defect of retinal vessels, capillary non-perfusion, dilation of small vessels, and lateral circulation (Yuan and Yuan, 2004). The pathogenesis of RVO is not very well understood and remains controversial. In order to elucidate thepathogenesis, model of RVO was used in our study.

In the current treatments for RVO such as anti-coagulation and thrombolysis, steroid is unsatisfactory. Puerarin, the active ingredient of Chinese herbal medicine, is a flavonoid glycoside derived from *Pueraria lobata* (Willd.) Ohwi or *P. lobata* (Willd.) Ohwi var. *thomsonii* (Benth.) Vaniot der Maesen in Leguminosae family. Recently, more and more researches have reported that puerarin had protective effect against cerebral ischemia / reperfusion injury (Zhao et al, 2013), cardiac hypertrophy (Yuan et al, 2013), chronic alcohol-induced liver injury (Li et al, 2013), and so on (Zhao et al, 2014). However, whether puerarin affects the course of RVO or cures the disorder by reversing the main pathology is still unclear. Here photochemically induced model in rats was applied to studying the effect of puerarin on RVO.

## 2. Materials and methods

### *2.1 Animals*

Adult male Sprague-Dawley (SD) rats weighing 220 to 260 g [SCXK(JING)2012-0001] were obtained from Vital River Laboratory Animal Technology Co., Ltd.

### *2.2 Drugs, reagents, and devices*

Puerarin (Batch No. 20120933) was from Taian Drug Manufactory, Lukang Medicine Co., Ltd., Shandong, China. Reagents including Rose Bengal (Batch No. 20121030) were purchased from The Third Reagent Factory (Shanghai, China). IL-1β and VEGF ELISA assay kit (Batch No. 201306) were from Bio-Swamp (Shanghai, China); 532 nm diode laser equipment was from aviation and spaceflight 8358 station (Tianjin, China); LDF100C Doppler Flowmeter was from Biopac System Inc (USA).

### *2.3 Experimental procedure*

SD rats were anesthetized with ip injection, and the left eye was chosen as the experimental eye. The pupil was dilated with 1% tropicamide (5 μL, Batch No. 20121030, The Third Reagent Factory, Shanghai, China). After 15 min, the rat was topically anesthetized in eye with 0.5% proparacaine. To occlude the retinal vein, a bolus injection of Rose Bengal (20 mg/kg) into the femoral vein was performed befor the laser, the laser spot (duration of 120 s, power of 50 mW, and spot size of 1 mm) was placed on a superior retinal vein, approximately two disc diameters away from the disc. Retinal blood flow was measured using Doppler Flowmeter. The RVO model was successfully established when the blood flow decreased by 50% compared with the basic value in major veins.

The rats that had successfully received laser photo thrombosis were randomly divided into four groups such as model (A, saline solution) and puerarin (B, 20 mg/kg; C, 40 mg/kg; and D, 80 mg/kg) groups. In addition, the rats in the control group were also given with the saline solution. Fifteen rats in each group was treated for 7 d and administered via vena caudalis.

### *2.4 Measurement of retinal venous blood flow*

To observe the evolution of venous occlusion, Doppler Flowmeter measurements of blood flow were performed before the occlusion and lasted for 1 h following the treatment with puerarin. The blood flow in the occluded retinal veins at the end of the experiment was compared with those taken at the start of therapeutic as the basic value.

### *2.5 Measurement of vascular endothelial growth factor (VEGF), interlukin-1β (IL-1β), and nitric oxide (NO) levels*

Samples of undiluted vitreous fluid (30–50 μL) were obtained from experimental eyes by puncturing the pars plana with sterile syringe at one week after RVO being induced. The samples were collected in sterile tubes and rapidly frozen at –80 °C.

The concentration of VEGF and IL-1β was measured by enzyme-linked immunosorbent assay (ELISA) using rat VEGF and IL-1β immunoassays (Bio-Swamp, China). The concentration of NO was measured by nitrate reduction according to agent instruction (Jiancheng Technology, China).

### *2.6 Histopathology*

The experimental eyes were enucleated, fixed in 10% formaldehyde for 24 h and then embedded in paraffin. Blocks were obtained by cutting through the whole globe oriented perpendicular to the medullary wings. Sections (5 μm-thick) obtained by using microtome were stained with Hematolxylin and Eosin (H&E) for light microscopic (LM) examination of retinal structure.

### *2.7 Statistical analysis*

All results were expressed as $\bar{x} \pm s$. Comparison on multiple groups was performed with One-way analysis of variance (ANOVA). Differences were considered statistically significant at a value of $P < 0.05$. Analyses were performed using software SAS9.1.

## 3. Results

### *3.1 Effect of puerarin on retinal vein blood flow in RVO rats*

The RVO was quantified by retinal vein blood flow to assess the severity of the disease. At the end of the experiment, the mean venous blood flow for the model group decreased by 73.5% relative to the basic value, with a statistically significant difference ($P < 0.001$). The mean blood flow for

puerarin (20, 40, and 80 mg/kg) groups was improved by 9.3% ($P < 0.05$), 33.1% ($P < 0.001$), and 41.5% ($P < 0.001$), respectively (Table 1).

### 3.2 Effect of puerarin on vitreous VEGF, IL-6, and NO levels of RVO rats

To evaluate the mechanism of puerarin on RVO rats, the levels of EGF, IL-1β, and NO in vitreous fluid were measured by assay kits. Vitreous fluid levels for VEGF and IL-1β increased while NO decreased in the model group compared with those of the control group ($P < 0.05$). Treatment with puerarin (20, 40, or 80 mg/kg) can decrease VEGF and IL-1β while increase NO in vitreous fluid dose-dependently, with respect to the model group (Table 2). So there might be a certain relationship between the protective role of puerarin on RVO rats and VEGF, IL-1β, and NO in vitreous fluid.

### 3.3 Effect of puerarin on pathohistological changes in RVO rats

The optical microscopic examination of the serial sections in impact areas demonstrated that there was no morphological change in the normal rats, such as the clear 10 layers, regular arrangement in inner and outer nuclear layer, aequalis cell of pigment epithelial, and density of capillary vessel. Compared with the retina of normal rats, neuroretinal layers were markedly edematous and thicker in the model group, with lax, edematous, and disorganized phenomenon in the optic fibers, inner plexiform, and bacillary layers. Cells in the inner and outer nuclear layers were swollen and confused, and numerous vacuoles could be observed. In each treatment group, these histological changes were less severe, differing in extent according to the dose of puerarin (Figure 1).

## 4. Discussion

The RVO model induced by photochemistry was used in this study. After the Rose Bengal was spired by laser for 532 nm, the photochemical oxidizing reaction was induced, which caused the damage of blood vessel endothelium and platelet aggregation, in addition, venous thrombogenesis in retinal vein was induced at last. This model in the current study was easy to reproduce, had nearly all the signs of ischemia without any complication, and offered the advantage of study for the neovascular retinopathy (Yuan et al, 2011). Thus, it could be an ideal tool to investigate ischemic retinal disease.

Until recently, the treatment options for RVO included anticoagulation, thrombolysis, steroid, and so on. However,

**Table 1 Effect of puerarin on retinal vein blood flow in RVO rats ($\bar{x} \pm s$, $n = 10$)**

| Groups | Doses / (mg·kg$^{-1}$) | Retinal vein blood flow / V | | |
|---|---|---|---|---|
| | | Basic value | Before treatment | After treatment |
| control | − | 1.58 ± 0.30 | 1.59 ± 0.22 | 1.61 ± 0.27 |
| model | − | 1.59 ± 0.30 | 0.44 ± 0.14$^{\triangle\triangle\triangle}$ | 0.43 ± 0.14$^{\triangle\triangle\triangle}$ |
| puerarin | 20 | 1.63 ± 0.26 | 0.49 ± 0.14 | 0.54 ± 0.11$^{*}$ |
| | 40 | 1.65 ± 0.48 | 0.44 ± 0.15 | 0.82 ± 0.19$^{***}$ |
| | 80 | 1.55 ± 0.27 | 0.41 ± 0.14 | 0.92 ± 0.2$^{***}$ |

$^{\triangle\triangle\triangle}P < 0.001$ *vs* control group; $^{*}P < 0.05$ $^{***}P < 0.001$ *vs* model group

**Table 2 Effect of puerarin on vitreous VEGF, IL-1β, and NO levels in RVO rats ($\bar{x} \pm s$, $n = 5$)**

| Groups | Doses / (mg·kg$^{-1}$) | VEGF / (pg·mL$^{-1}$) | IL-1β / (pg·mL$^{-1}$) | NO / (μmol·L$^{-1}$) |
|---|---|---|---|---|
| control | − | 853.2 ± 88.0 | 16.5 ± 3.0 | 37.6 ± 7.0 |
| model | − | 1243.9 ± 223.7$^{\triangle\triangle}$ | 29.7 ± 3.6$^{\triangle\triangle\triangle}$ | 18.8 ± 4.7$^{\triangle\triangle}$ |
| puerarin | 20 | 1065.2 ± 117.1 | 23.2 ± 4.6$^{*}$ | 20.4 ± 6.2 |
| | 40 | 978.8 ± 81.3$^{*}$ | 20.5 ± 3.0$^{**}$ | 28.4 ± 7.3$^{*}$ |
| | 80 | 931.9 ± 164.1$^{*}$ | 17.8 ± 4.7$^{**}$ | 30.0 ± 7.6$^{*}$ |

$^{\triangle\triangle}P < 0.01$ $^{\triangle\triangle\triangle}P < 0.001$ *vs* control group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model group

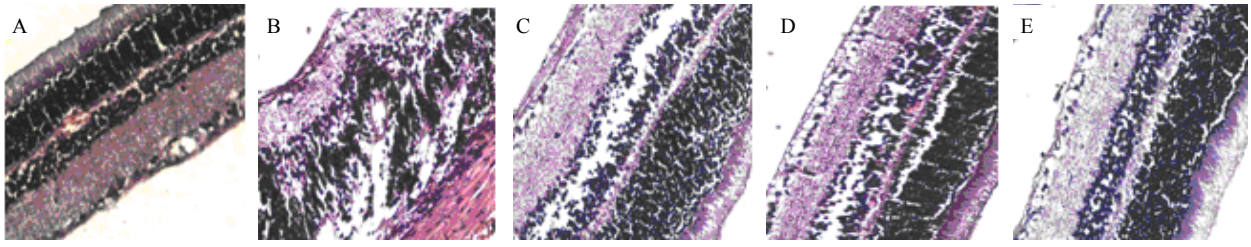


**Figure 1 Effect of puerarin at different concentration on pathohistological changes in RVO rats (HE staining)**

A: control group; B: model group; C: puerarin 20 mg/kg group; D: puerarin 40 mg/kg group; E: puerarin 80 mg/kg group

most visual outcomes were not satisfied (Zhou et al, 2010). The unsatisfactory results of such rapeutic efforts led to the development of new treatment strategies. Puerarin, the active ingredient of Chinese herbal medicine, is a flavonoid glycoside derived from *P. lobata* or *P. lobata* var. *thomsonii* in Leguminosae family. Previous studies have reported that puerarin played an important role in protecting retinal secondary lesion against ischemic insult, such as cerebral, cardiac, hepatic, and nephric ischemia injury. In our study, the protective effect of puerarin was observed to show a potential therapeutic effect as demonstrated by the significant reduction in RVO signs.

In our experiments, Doppler Flowmeter measurements were used to define the status of blood flow. Following laser application, it was observed that there was the decreased blood flow in the treated segments of the retinal veins. It was deemed that the RVO model of rats was successfully induced when the blood flow decreased by 50% of basic value. With the treatment of puerarin, the significant upgrade of blood flow was observed, which suggested there might be reopening or anastomosis of vessels and reperfusion of certain parts of the retina. Our pathohistological experiment also found that neuroretinal in the layers was markedly edematous and thicker in the model group, with lax, edematous, and disorganized phenomenon in the optic fibers, inner plexiform, and bacillary layers. However, in each treatment group, the pathohistological changes were less severe, differing in extent according to the dose of puerarin. In conclusion, our results verified that puerarin was belonged to the protective drugs against RVO of rats.

Nevertheless, the mechanisms underlying these effects remain incompletely defined. It is widely accepted that VEGF plays an important role in RVO development and is considered as one of the pivotal factors during ischemia and neovascularization (Yuan et al, 2007). Anti-VEGF therapy, including intravitreal bevacizumab, has proven to be effective in improving neovascularization (Tong et al, 2011; Gregori et al, 2008; Hou et al, 2009). Our results of ELISA indicated that VEGF was decreased after the injection of puerarin, which agreed with previous reports that intravitreal VEGF antagonist bevacizumab could lower the concentration of VEGF in aqueous fluid and vitreous (Park and Ahn, 2009; Lim et al, 2009; Miyake et al, 2010). Pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 are the principal mediators of the inflammatory reaction; They are produced mainly by immune cells. IL-1β is a major promoter of most acute-phase proteins, which could stimulate cell proliferation and antibody secretory. Noma et al (2011) demonstrated that the severity of macular edema was correlated with cytokine imbalance in central RVO. Our experiments found that IL-1β increased in vitreous fluid of RVO model. Puerarin could dose-dependently increase IL-1β in vitreous fluid with respect to the model group. NO is an important signaling molecule and a vessel dilator that mediates a variety of essential physiological processes. In our model groups, NO decreased compared with that in the control group, in accordance with the reports by Li et al (2010) and Zhou et al (2010). The vasoconstriction mediated by decrease of NO might be the cause of blood flow degression. Treatment with puerarin could dose-dependently increase NO level compared with the model group, and which might be the main reason of blood flow upgrade. These results indicate that puerarin may improve retinal ischemia after RVO by preventing neovascularization, anti-inflammation, and increase of NO. However, our results just only provide a basis for investigation into the role of puerarin on RVO in rats, further more studies should be done by increasing sample size and investigating the effects with other therapeutic mechanism.

## 5. Conclusion

In summary, a rat model of laser photochemical-induced ischemic retinal diseases was successfully established and a decrease of the retinal blood flow and histological changes were observed in association with the retinal lesions. The puerarin had therapeutic benefit in the RVO model of rats, including the decrease of the blood flow and less severe histological changes, might through a pathway mediated by preventing neovascularization, anti-inflammation, and increase of NO. That may provide a therapeutic drug against the RVO of rats.

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