

# The Decoction of Tonifying Kidney and Activating Blood Circulation Directly Inhibits VEGF Pathway and Slows Down the Progression of Diabetic Retinopathy

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**Abstract:** To illustrate the effect of Bushen-Huoxue Decoction to the expression of advanced glycation end products (AGEs) and vascular endothelial growth factor (VEGF) in diabetic retinopathy (DR) rats and its effect on the process of DR. The blank group and STZ insulin resistance model group, positive group and administration group were established. The expression of VEGF and HIF-1  $\alpha$  and the concentration of AGEs in plasma were measured. The results of retina digestion showed that although there were different degrees of retinal vascular damage in the administration group, the damage was lighter than that in the model group, and the difference of E/P value was significant ( $P < 0.01$ ). The expression of VEGF and HIF-1  $\alpha$  in the administration group was between the blank group and the model group. The concentration of AGEs in the plasma of the positive group and the administration group was significantly lower, with a very significant difference ( $P < 0.01$ ). Bushen-Huoxue decoction has a marked therapeutic effect on DR, and its mechanism is closely related to the inhibition of VEGF expression.

## 1. Introduction

With the rapid increase of the number of diabetes patients in the world, diabetes related complications are rapidly becoming a global health problem. At the same time, diabetic retinopathy (DR), as one of the main blinding diseases of vision disability and blindness in the 20-65-year-old working population, threatens the vision health of the majority of patients [1]. The pharmacological effect of Traditional Chinese Medicine on DR is a comprehensive treatment with multi links, multi directions and multi targets from the whole, and each link is connected, interacted and influenced with each other. In recent years, many doctors have revealed the pathogenesis of the disease from Yin and Yang Qi and blood, and their understanding has been basically the same, that is, Qi and yin deficiency, blood stasis blocking collaterals are the basic pathogenesis of the disease. Based on the deficiency of both qi and Yin and blood stasis, the treatment should be based on the combination of the whole syndrome differentiation based on Benefiting Qi and nourishing Yin, nourishing liver and clearing eyes, and the local syndrome differentiation based on promoting blood circulation and removing blood stasis. With the increase of clinical reports on TCM treatment of this disease, it has initially shown its unique advantages [2-3]. However, most of the related researches were single or single Chinese medicine, and most of them focus on pharmacodynamics. There are few reports on the expression of VEGF and AGEs [4-6]. Therefore, under the guidance of traditional Chinese medicine theory, we used modern pharmacological experiments and detection methods to explore the therapeutic effect of Bushen-Huoxue Decoction (rehmannia, Danshen, ginseng and Gegen mixture Decoction) on DR.

## **2. Materials and Instruments**

### **2.1 Medicinal materials**

Rehmannia, Danshen, Ginseng and Gegen were purchased from the Hehuachi medicinal material market in Chengdu, and were identified by Professor Yan Zhu-yun of the school of medicine of Chengdu University of traditional Chinese medicine as: The dry roots of *Rehmannia glutinosa* Libosch, *Salvia miltiorrhiza* Bge, *Panax ginseng* C.A.Mey, *Pueraria lobata* (wild.) Ohwi. The four medicines were extracted twice with water in the proportion of 2:2:2:1 respectively. The first extraction was 8 times of the amount and the second extraction was 6 times of the amount. The extract of each part was combined and the solvent was removed to obtain the extract (32.93%). The extract was made into gastric juice of rats with a concentration of 1 g / ml of crude drug by adding water, and stored at 4 °C for future use. Qi-ming granules (Zhejiang Wan-sheng Pharmaceutical Co., Ltd., batch No.: 181088), a positive control drug, was added with water to prepare a gastric juice with a concentration of 1 g/ml.

### **2.2 Models**

SPF male SD rats with weighing 140 to 170 g (from experimental animal center of Sichuan University, certificate: SCXK (Sichuan) 2015-030). High fat and high sugar feed (47% conventional feed, 45% lard, 10% sucrose, 2% cholesterol, 1% bile salt) is provided by Jiangsu Nantong Trophy feed Technology Co., Ltd. The experimental animals were fed adaptively for one week.

### **2.3 Reagents and instruments**

HIF-1  $\alpha$  (Abcam, batch No. ab216842), VEGF (Proteintech, batch No. 19003-1-ap). Goat anti rabbit IgG H & L (batch No. sp-9001), normal goat serum (batch No. zli-9021), concentrated DAB Kit (batch No. k135925c), all purchased from Beijing zhong-shan-jin-qiao biological Co., Ltd. Rat ages ELISA Kit (Thermo, batch No. zc-37286). Rotary microtome (CUT4050, German gante tools and Machinery Manufacturing Co., Ltd), Embedding machine (KH-BL, Hubei Xiaogan kuohai Medical Technology Co., Ltd), PHY-III pathological tissue bleaching and drying instrument (Changzhou Zhong-wei Electronic Instrument Co., Ltd.), EVOS M7000 Imaging System (AMF 7000, Thermo Fisher Scientific Co., Ltd), etc.

## **3. Methods**

### **3.1 Grouping and treatment of experimental animals**

Forty-eight mice were randomly divided into four equal groups with 12 rats in each group. The blank group was fed with normal diet, while the other groups were fed with high fat and high sugar diet. After four weeks, the other groups were fasted for 12 hours, injected with 4.5 ml/kg dose volume and 1% Streptozocin(STZ) intraperitoneally, resulting in insulin resistance. The blank group was injected with the same amount of citric acid sodium citrate buffer. 72 hours later, blood was taken from the tail tip and blood glucose was measured. Blood glucose over 16.67 mmol·L<sup>-1</sup> was included in the model group, positive group or administration group. After that, the rats in each group were fed with normal diet for 12 weeks and then began to be administered. The rats in the administration group were given gastric juice (11.67 ml/kg) once a day for 4 weeks. The positive group was given Qi-ming granules (1.215 ml/kg). The blank group and model group were given the same dose of distilled water to monitor blood glucose. After the last administration, blood obtained from the abdominal aorta was put into heparin sodium anticoagulant tube, centrifuged at 3000 rpm for 10 min, and the plasma was detached to detect AGEs level. The corresponding index was taken from the eyeball tissue.

### **3.2 Detection of retina digestive patch**

The eyeball was fixed with POM, washed and soaked with water, the anterior segment and vitreous were removed under the operating microscope, the retina was peeled off, digested and prepared, the preparation was dried naturally at room temperature, and stored at 4 °C for 12 hours. The number of endothelial cells and the number of weekly cells were recorded by routine

hematoxylin eosin staining and microscopic examination respectively, and the ratio of the two (E/P) was calculated.

### 3.3 Detection of VEGF and HIF-1 $\alpha$ in retina

The retina was examined by routine operation according to pathological examination SOP. The concentrations of VEGF and HIF-1  $\alpha$  were 1:100. The standard of staining was blue as negative cell, white as substrate, yellow or brown as positive cell. The light density and area of the tissue in the sections were calculated by three different visual fields at 400 times of immunohistochemistry (IHC) staining, and then the average light density (OD) of each image was calculated.

### 3.4 Determination of AGEs in rat plasma

The content of AGEs in rat plasma was determined by ELISA, and the test procedure was in strict accordance with the instructions of rat ages ELISA Kit. At 450 nm wavelength, the Absorbance of the sample was measured by the enzyme reader to calculate the concentration of the sample.

## 4. Results

### 4.1 General state observation and blood glucose of rats

The rats in the blank group were in good growth state, with glossy hair color, normal two stools and little drinking water; the rats in the other groups increased their food and drinking water to 2-3 times of that in the blank group over time, resulting in fecal prevarication, more and turbid urine, yellow and coarse hair color and broken tail. The blood glucose in the blank group remained relatively unchanged all the time; the blood glucose in the other groups increased rapidly and maintained hyperglycemia after STZ injection, which indicated that DR model was established successfully. During the administration period, there was no significant difference in blood glucose between the administration group and the model group, indicating that it had no marked effect on the improvement of blood glucose. The blood glucose monitoring results are shown in Table 1.

Table 1. Effect of different polar parts of Bu-shen Huo-xue Recipe on fasting blood glucose in rats

Groups	$(\bar{x} \pm S, N = 12)$						
	First week	Fifth weeks	Seventh	11	15	17	20
Blank group	3.5 $\pm$ 0.7**	5.7 $\pm$ 0.4**	6.5 $\pm$ 1.2**	4.5 $\pm$ 0.4**	6.5 $\pm$ 2.5*	5.4 $\pm$ 0.6**	5.9 $\pm$ 1.0**
Model group	4.1 $\pm$ 1.2	5.6 $\pm$ 0.9	22.6 $\pm$ 11.5	30.3 $\pm$ 6.2	27.7 $\pm$ 8.2	25.4 $\pm$ 6.4	24.5 $\pm$ 4.6
Positive group	3.8 $\pm$ 0.5	5.9 $\pm$ 0.7	22.7 $\pm$ 4.2	22.7 $\pm$ 8.3	19.1 $\pm$ 8.1	26.0 $\pm$ 7.2	24.6 $\pm$ 7.4
Decoction group	4.0 $\pm$ 0.4	5.6 $\pm$ 0.2	24.7 $\pm$ 82	28.9 $\pm$ 6.7	27.0 $\pm$ 6.8	25.6 $\pm$ 7.4	33.3 $\pm$ 0.9

Compare with model group \*  $P < 0.05$  \*\*  $P < 0.001$

### 4.2 Test results of retinal patch

The results revealed that the distribution of retinal blood vessels in the blank group was even and in the model group the distribution of retinal blood vessels was disordered and distorted, which indicated that the retinal blood vessels in the model group were seriously damaged, while the retinal blood vessels in the drug administration group were slightly damaged compared with the model group. To compare with the blank group, the E / P value of the model group was significantly higher ( $P < 0.01$ ), as shown in Table 2. Compared with the model group, the positive group was significantly lower ( $P < 0.01$ ). The E / P value of the decoction had no significant change, without statistical value.

Table 2. Effect of Bushen Huoxue Recipe on the ratio of retinal microvascular skin cells to peripheral cells in rats

$(\bar{X} \pm S, N = 12)$		
Groups	Dose (ml/kg)	E/P
Blank control	—	$6.82 \pm 5.25^{**}$
Model comparison	—	$15.52 \pm 4.91$
Positive control	1.215	$7.95 \pm 3.51^{**}$
Water location	11.67	$12.63 \pm 4.58$

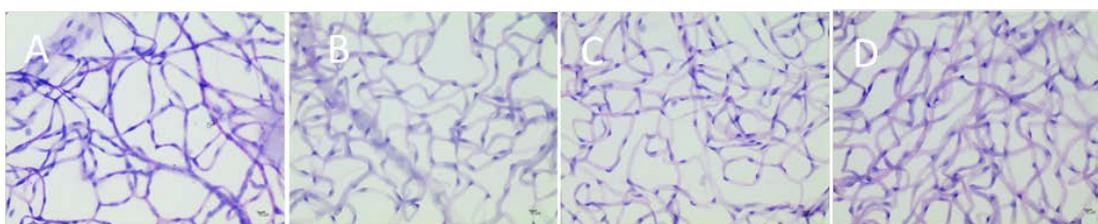


Figure 1. Effect of water extract of tonifying kidney and promoting blood circulation on retinal vascular patch ( $\times 400$ ). A: Blank control. B: Model control. C: Positive control. D: Water part.

### 4.3 Detection results of VEGF and HIF-1 $\alpha$ in retina

There was a small amount of VEGF and HIF-1  $\alpha$  expression in the retina of the blank group rats, while they were the most in the model group rats, and the expression of VEGF and HIF-1  $\alpha$  in the administration group was between the blank and the model group. The statistical analysis exhibited that compared with the blank group, the expression of VEGF and HIF-1  $\alpha$  in the eyeball tissue of the model group was relatively higher ( $P < 0.01$ ). Compared with the model group, the expression of VEGF in the administration group was considerably lower ( $P < 0.01, 0.05$ ). The content of HIF-1  $\alpha$  protein in each administration group was significantly lower ( $P < 0.01, 0.05$ ), except for the water part group. The results are shown in Table 3.

Table 3. Effect of different polar parts of Bu-shen Huo-xue Prescription on the expression of EGF and HIF-1 in retina of rats.

$(\bar{x} \pm S, N = 12)$			
Groups	Dose (ml/kg)	VEGF OD Value	HIF-1 $\alpha$ OD Value
Blank control	-	$0.235 \pm 0.009^{**}$	$0.286 \pm 0.015^*$
Model comparison	-	$0.259 \pm 0.005$	$0.318 \pm 0.016^*$
Positive control	1.215	$0.244 \pm 0.006^{**}$	$0.293 \pm 0.012^*$
Decoction	11.67	$0.241 \pm 0.008^{**}$	$0.301 \pm 0.010^*$

### 4.4 AGEs test results

See Table 4 for the content of AGEs in rat plasma. The statistical results showed that the plasma levels of AGEs in the model group were higher ( $P < 0.01$ ). Compared with the model group, the plasma levels of AGEs in the positive group and the administration group were significantly low ( $P < 0.01$ ).

Table 4. Effect of different polar parts of Bushen Huoxue Formula on ages concentration in plasma of rats.

( $\bar{x} \pm S, N = 12$ )		
Groups	Dose(ml/kg)	AGEs concentration(ng/ml)
Blank control	-	268 ± 25
Model comparision	-	443 ± 34
Positive control	1.215	286 ± 37
Aqueous extract	11.67	294 ± 74

## 5. Discussion

The occurrence and development of DR is a pathological process with multiple causes and stages. Based on the disorder of blood glucose metabolism and microcirculation, the typical pathogenesis is associated with abnormal cell proliferation, abnormal polyol inositol metabolism, nonenzymatic glycosylation of protein, protein kinase C pathway and so on [7]. However, at present, more interest has focused on the effects of VEGF and AGEs on the pathogenesis of DR.

In the early stage of hyperglycemia, abnormal tissue oxygenation results in the change of microvascular function and retinal vasodilation. Long term vasodilation results in microaneurysms and changes in vascular structure, peripheral cell degeneration, basement membrane thickening and endothelial cell proliferation [8,9]. The results showed that the distribution of retinal vascular network was disordered, local distortion and aggregation, and the ratio of endothelial cells to peripheral cells (E/P) increased. Nevertheless, retinal neovascularization and fibrosis will aggravate the disease. The expression of HIF-1  $\alpha$  and VEGF in the retina of DR rats is enhanced, which is closely related to the occurrence and development of DR [10]. Among them, VEGF overexpression in the whole process will lead to vascular leakage and neovascularization. HIF-1  $\alpha$  can activate and promote the transcription of downstream genes such as VEGF, which can be activated by AGEs, thus indirectly affecting the expression of VEGF. The degree of retinopathy is positively correlated with AGEs content [11-13].

These results indicated that the expression of VEGF, HIF-1  $\alpha$  and AGEs in the model group was noticeably higher than that in the blank group, suggesting that DR was related to the expression of VEGF, HIF-1  $\alpha$  and AGEs. The indexes of rats in the administration group decreased, which showed that Bu-shen Huo-xue Prescription had inhibited the development of DR to some extent. The treatment of DR might be related to the direct inhibition of VEGF expression or the indirect reduction of VEGF level, the protection of blood retinal barrier, the reduction of vascular permeability, the inhibition of neovascularization, and the protection of vascular endothelial function through the inhibition of HIF-1  $\alpha$  and AGEs expression. This prescription has a certain therapeutic effect on DR, and our study also verifies the effectiveness of targeted VEGF in the treatment of DR, which provides a reference for the research of traditional Chinese medicine prescriptions.

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## Reference

- [1] Zhang Z-Y, Miao L-F, Qian L-L, et al. Molecular Mechanisms of Glucose Fluctuations on Diabetic Complications. *Frontiers in endocrinology*, 2019, 10: 640-640.
- [2] Yang Min, Luo xiangxia, Kang Li, et al. Research progress on Mechanism of prevention and

treatment of diabetic retinopathy by traditional Chinese medicine. Chinese Journal of traditional Chinese medicine, 2018, 33 (11): 267-270.

[3]Li Rong, Wang Guoqin. 60 cases of diabetic retinopathy treated by Yiqi Yangyin Huoxue Method. Chinese medicine research, 2008,21 (5): 36-37.

[4]Song Wang, Yuan Yuan, Ge Zhengyan, et al. Research progress on the prevention and treatment of experimental diabetic retinopathy with single Chinese medicine and compound prescription. Chinese Journal of pharmacology and toxicology, 2019,33 (3): 224-231.

[5]Wu J, Ke X, Wang W, et al. Aloe-emodin suppresses hypoxia-induced retinal angiogenesis via inhibition of HIF-1 $\alpha$ /VEGF pathway. Int J Biol Sci,2016,12(11):1363-1371.

[6]Tan SQ, Geng X, Liu JH, et al. Xue-fu-Zhu-Yu decoction protects rats against retinal ischemia by downregulation of HIF-1 $\alpha$  and VEGF via inhibition of RBP2 and PKM2. BMC Complement Altern Med. 2017,17(1):365.

[7] Stitt A W, Curtis T M, Chen M, et al. The progress in understanding and treatment of diabetic retinopathy. Progress in Retinal and Eye Research, 2016, 51: 156-186.

[8] Jin Yamei, Wu Qiaomin, Qin zhengran. Correlation between retinopathy and vitamin D in type II diabetes. Zhejiang Journal of integrated traditional Chinese and Western medicine, 2019, 29 (02): 58-60.

[9]Wei J, Jiang H, Gao H, et al. Blocking Mammalian Target of Rapamycin (mTOR) Attenuates HIF-1 $\alpha$  Pathways Engaged-Vascular Endothelial Growth Factor (VEGF) in Diabetic Retinopathy. Cellular Physiology and Biochemistry, 2016, 40(6): 1570-1577.

[10]Simo R, Sundstrom J M, Antonetti D A. Ocular Anti-VEGF Therapy for Diabetic Retinopathy: The Role of VEGF in the Pathogenesis of Diabetic Retinopathy. Diabetes Care, 2014, 37(4):893-899.

[11] Wang Shan, Cheng Xian Kun, pan Wen Qiang, et al. Effect of gliclazide combined with calcium dobesilate on PKC, VEGF, HIF-1  $\alpha$  expression in diabetic retinopathy rats. Journal of Mudanjiang Medical College, 2019,40 (03): 11-14

[12]Vadlapatla R K, Vadlapudi A D, Pal D, et al. Ritonavir inhibits HIF-1 $\alpha$ -mediated VEGF expression in retinal pigment epithelial cells in vitro. Eye, 2014, 28(1): 93-101.

[13]Melincovici C S, Bosca A B, Susman S, et al. Vascular endothelial growth factor (VEGF) - key factor in normal and pathological angiogenesis. Rom J Morphol Embryol, 2018, 59(2): 455-467.