

Molecular mechanism of the role of carbamyl erythropoietin in treating diabetic retinopathy rats

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Abstract. The aim of the present study was to investigate the therapeutic effects of carbamyl erythropoietin (CEPO) and safflor yellow (SY) in the treatment of rats with diabetic retinopathy (DR) as well as exploring the mechanism of action. Male SD rats were used to establish a diabetes model and streptozotocin-induced retinopathy was also performed in rats. A total of 126 rats with DR were obtained, and model rats were randomly divided into the model (n=42), experimental (n=42) and control (n=42) groups. The rats in the model group were injected with saline, the rats in the experimental group were treated with CEPO, and the rats in the control group were treated with SY. After treatment for 2 weeks, the retinas were harvested for quantitative analysis of the mRNA expression levels of angiogenesis-promoting and -inhibiting molecules, apoptosis-promoting and -inhibiting molecules, and oxidative stress pathway-related factors by Reverse transcription-quantitative PCR (RT-qPCR). No significant differences in expression levels of hypoxia-inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF), angiopoietin (Ang-1), tissue kallikrein (TKLK) and pigment epithelium-derived factor (PEDF) were observed between the experimental and model groups ($P>0.05$). The expression levels of apoptosis-promoting molecules Bcl-2 related X protein (Bax) and cysteine aspartate specific protease (caspase-3) mRNA in the retina of the experimental group was significantly lower than those in the control group ($P<0.05$). The expression levels of Bcl-2 and survivin mRNA were significantly higher in the experimental group than in the control group ($P<0.05$). The expression levels of the oxidative stress pathway nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) mRNA were significantly higher in the experimental group than in the control group. Therefore, the therapeutic

effects of CEPO in treating DR are better than those of SY. As a result, CEPO may inhibit apoptosis and oxidative stress damage of retinal tissue cells in DR rats without affecting angiogenesis.

Introduction

Diabetic retinopathy (DR) is an important manifestation of microangiopathy and is one of the most important causes of blindness in diabetic patients (1). The incidence of retinopathy is as high as 23% in diabetic patients (2). DR has become the main cause of adult blindness (3). Pathology of DR is mainly the damage of retinal capillary endothelial cells, and changes in expression patterns of angiogenesis, apoptotic and oxidative stress pathway molecules are usually involved (4,5).

Safflor yellow (SY) is the main component of the safflower in Compositae. SY has the function of inhibiting platelet aggregation, antioxidation and tumor growth (6). Recent studies suggest that SY can be used to effectively treat early retinopathy in patients with type II diabetes (7,8). Erythropoietin (EPO) can antagonize oxidation and inhibit apoptosis. On the one hand, EPO reduces damage of retinal nerve cells, and on the other hand, it can promote angiogenesis, leading to the development of the disease (9). Thus, carbamyl erythropoietin (CEPO) loses its ability to promote angiogenesis, but it still maintains other functions (10).

In this study, CEPO and SY were used to treat DR rats and their therapeutic effects were observed. Thus, the molecular mechanism of the action of CEPO in the treatment of DR rats was analyzed. Our study provided reference for further clinical studies.

Materials and methods

Experimental animals. Healthy adult male SD rats (200 \pm 20 g) were provided by China Medical University (Taichung, Taiwan, R.O.C.). The rats were kept in cage with controlled-temperature and light cycles (24°C and 12/12 light cycles) with free access to water and food. The humidity was 60 \pm 10%. The study was approved by the Ethics Committee of The Eye Hospital of Wenzhou Medical University (Wenzhou, China). The study was approved by the Ethics Committee of The Eye Hospital of Wenzhou Medical University (Wenzhou, China).

Drugs and main reagents. High-fat and high-sugar food (Beijing Botai Hongda Biotechnology Co., Ltd., Beijing,

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China), streptozotocin (Solarbio Science & Technology Co., Ltd., Beijing, China), CEPO (Jiangsu Tailida Co., Ltd., Rugao, China), SY (state approval no. Z20050594; Shanxi Huahui Kaide Pharmaceutical Co., Ltd., Jinzhong, China), TRIzol (Sangong Pharmaceutical Co., Ltd., Shanghai, China), cDNA first-strand synthesis kit (Sangong Pharmaceutical Co., Ltd.), RT-qPCR kit (Takara Bio, Inc., Otsu, Japan). The primer sequences were synthesized by GenScript (Nanjing, China).

Construction of the diabetes rat model. SD rats were adaptively fed for 1 week, and then fed with high-fat and high-sugar diet for 4 weeks. Intraperitoneal injection of streptozotocin (50 mg/kg) was performed every week after rats were fasted for 12 h. The last injection was performed after the rats were fasted for 8 h. Blood was extracted from the tail vein to detect random blood glucose and blood glucose levels, which were >17 mmol/l, indicating a successfully established model. A total of 126 DR model rats were obtained.

Animal grouping and drug intervention. One hundred twenty-six rat models were randomly divided into the model (n=42), experimental (n=42) and control (n=42) groups. The rats in the experimental group were treated with intraperitoneal injection of CEPO (50 µg/kg-day), the rats in the control group were treated with intraperitoneal injection of SY (40 mg/kg), and the rats in the control and experimental groups were treated for 2 weeks. Finally, the rats in the model group were injected with 1 ml normal saline for 2 weeks.

Measurement of expression levels of angiogenetic, apoptotic and oxidative stress pathway molecules at the mRNA level. After being treated for 2 weeks, the rats were sacrificed and the eyeballs were dissected to obtain retinas. TRIzol was used to extract total RNA from the retinal tissue according to the protocol, and then cDNA was synthesized according to the instructions of the cDNA first-strand synthesis kit. Reverse transcription-quantitative PCR (RT-qPCR) reactions were performed using β -actin as endogenous control. Expression of angiogenesis-promoting molecules [hypoxia-inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF), angiopoietin (Ang-1)], angiogenesis-inhibiting molecules [tissue kallikrein (TKLK), and pigment epithelium-derived factor (PEDF)], apoptosis-promoting molecules [Bcl-2 related X protein (Bax) and cysteine aspartate specific protease (caspase-3)], apoptosis-inhibiting molecules (Bcl-2 and survivin), oxidative stress pathway molecules [nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1)] was detected. RT-qPCR reaction system consisted of 10 µl of SYBR-Green Master Mix (Takara Bio, Inc., Otsu, Japan), 0.5 µl of upstream and downstream primers, 1 µl of cDNA, and 8 µl ddH₂O was added to make a total volume of 20 µl. RT-qPCR reaction conditions were: 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec and 55-61°C (depending on specific genes) for 1 min. The primer sequences used in PCR reactions are listed in Table I.

Statistical analysis. SPSS 17.0 (Beijing Xinmeijiahong Technology Co., Ltd., Beijing, China) was used for all statistical analyses. Measurement data were expressed as

Table I. Primer sequences used in PCR reactions.

Genes	Primer sequences (5'→3')	TM (°C)
<i>HIF-1α</i>	F: GAACCCATTTTCTACTCAGGACACAG R: CCACTTTCATCCATTGATTGCC	55
<i>VEGF</i>	F: CGAGACCCTGGTGGACAT R: ACAAATGCTTTCTCCGCT	58
<i>Ang-1</i>	F: AGACCAGTACAACACAAAC R: CGGTCTGAGAGAGGAGGCT	58
<i>TKLK</i>	F: TCATCAATGAAGACCTATGCG R: AGCATCAGGTCATTGCTGTA	56
<i>PEDF</i>	F: CTACAGGTGCGCGCCAAC R: CTAAGGCGCACACATTTG	61
<i>Bax</i>	F: GCGAATTGGAGATGAACTGG R: GTGAGCGAGGCGGTGAGGAC	56
<i>Caspase-3</i>	F: GAAGCGAATCAATGGACTCTG R: GCACAAAGCGACTGGATGAA	58
<i>Bcl-2</i>	F: CTGGTGGACAACATCGCTCTG R: GGTCTGCTGACCTCACTTGTG	58
<i>Survivin</i>	F: ATCCACTGCCCTACCGAGAAC R: TGCTCCTCTATCGGGTTGTCAT	58
<i>Nrf-2</i>	F: TCCTCTGCTGCCATTAGTCA R: GTGCCTTCAGTGTGCTTCT	58
<i>HO-1</i>	F: CAGGAGCTGCTGACCCATGA R: AGCAACTGTCGCCACCAGAA	58
<i>NQO-1</i>	F: GGATTGGACCGAGCTGGAA R: AATTGCAGTGAAGATGAAGGCAAC	58
<i>β-actin</i>	F: GACCCAGATCATGTTTGAGACCTT R: GACTCGTCATACTCCTGCTTGC	58

F, forward; R, reverse; HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; Ang-1, angiopoietin; TKLK, tissue kallikrein; PEDF, pigment epithelium-derived factor; Bax, Bcl-2 related X protein; caspase-3, cysteine aspartate specific protease; Nrf-2, nuclear factor erythroid 2 (NFE2)-related factor 2; HO-1, heme oxygenase-1; NQO-1, NAD(P)H quinone dehydrogenase 1.

mean \pm SD, and analysis of variance was used for comparisons among groups and the post hoc was Least Significant Difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analysis expression of retinal angiogenesis-related molecules at the mRNA level. After the SY treatment, expression levels of HIF-1 α , VEGF and Ang-1 mRNAs in the control group were significantly lower than those in the model and experimental groups ($P < 0.05$). However, there was no significant difference in the expression levels of the genes in the CEPO-treated experimental and model groups ($P > 0.05$; Table II). The expression levels of TKLK and PEDF in the control group were significantly higher than those in the model and experimental groups ($P < 0.05$; Table III), while no significant differences were observed between the model and experimental groups ($P < 0.05$; Table III).

Table II. Expression levels of angiogenesis-promoting molecules in the rat retina at the mRNA level (n=42, mean \pm SD).

Groups	HIF-1 α	VEGF	Ang-1
Model	2.42 \pm 0.33	2.35 \pm 0.30	1.80 \pm 0.22
Experimental	2.40 \pm 0.31	2.38 \pm 0.28	1.83 \pm 0.25
Control	1.85 \pm 0.13 ^a	1.68 \pm 0.15 ^a	1.32 \pm 0.15 ^a
F	7.748	8.761	9.331
P-value	0.049	0.042	0.038

^aP<0.05, compared with the control and the model groups. HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; Ang-1, angiopoietin.

Table III. Expression levels of angiogenesis-inhibiting molecules in the rat retina at the mRNA level (n=42, mean \pm SD).

Groups	TKLK	PEDF
Model	0.45 \pm 0.08	0.38 \pm 0.05
Experimental	0.48 \pm 0.06	0.35 \pm 0.07
Control	0.88 \pm 0.10 ^a	0.76 \pm 0.03 ^a
F	33.823	140.691
P-value	0.004	<0.001

^aP<0.05, compared with the control and model groups. TCLK, tissue kallikrein; PEDF, pigment epithelium-derived factor.

Table IV. Analysis of apoptosis-promoting molecules in the rat retina at the mRNA level (n=42, mean \pm SD).

Groups	Bax	Caspase-3
Model	2.88 \pm 0.32	2.51 \pm 0.35
Control	2.01 \pm 0.29 ^a	2.09 \pm 0.24 ^a
Experimental	1.58 \pm 0.18 ^{a,b}	1.70 \pm 0.22 ^{a,b}
F	23.632	6.463
P-value	0.001	0.032

^aP<0.05, compared with the model group; ^bP<0.05, compared with the control group. Bax, Bcl-2 related X protein; caspase-3, cysteine aspartate specific protease.

Analysis of expression of the apoptosis-related molecules in the retina. The expression levels of the apoptosis-promoting molecules Bax and caspase-3 in the retinal tissue of DR rats in the control and experimental groups were significantly lower than those in the model group (P<0.05), and were significantly lower in the experimental group than in the control group (P<0.05; Table IV). The expression levels of the apoptosis inhibitors Bcl-2 and survivin were significantly higher in the control and experimental groups than in the model group (P<0.05), and were significantly higher in the experimental group than in the control group (P<0.05; Table V).

Table V. Analysis of apoptosis-inhibiting molecules in the rat retina at the mRNA level (n=42, mean \pm SD).

Groups	Bcl-2	Survivin
Model	0.41 \pm 0.05	0.35 \pm 0.04
Control	0.61 \pm 0.08 ^a	0.58 \pm 0.03 ^a
Experimental	0.73 \pm 0.08 ^{a,b}	0.68 \pm 0.03 ^{a,b}
F	15.373	75.794
P-value	0.004	<0.001

^aP<0.05, compared with the model group; ^bP<0.05, compared with the control group.

Table VI. Analysis of the oxidative stress pathway-related molecules in the rat retina at the mRNA level (n=42, mean \pm SD).

Groups	Nrf-2	HO-1	NQO-1
Model	1.87 \pm 0.22	1.65 \pm 0.20	1.98 \pm 0.23
Control	2.54 \pm 0.19 ^a	2.88 \pm 0.29 ^a	2.49 \pm 0.22 ^a
Experimental	2.92 \pm 0.25 ^{a,b}	3.23 \pm 0.28 ^{a,b}	2.91 \pm 0.32 ^{a,b}
F	17.304	30.606	9.583
P-value	0.003	0.001	0.014

^aP<0.05, compared with the model group; ^bP<0.05, compared with the control group. Nrf-2, nuclear factor erythroid 2 (NFE2)-related factor 2; HO-1, heme oxygenase-1; NQO-1, NAD(P)H quinone dehydrogenase 1.

Analysis of the oxidative stress pathway-related molecules in the retina. After treatment, the expression levels of Nrf-2, HO-1, and NQO-1 in the retina were significantly higher in the control and experimental groups than in the model group (P<0.05), and were also significantly higher in the experimental group than in the control group (P<0.05; Table VI).

Discussion

The mechanism of DR is complicated, and it often causes apoptosis of the retinal ganglion cells and damages the nerve function during the disease progression. During the disease development, hypoxia triggers the activation of oxidative stress to further damage the nerve cells. In addition, hypoxia also triggers neovascularization to cause retinal damage, and activates the retinal mitochondrial pathway to induce cell apoptosis and further damages the retina.

Long-term high blood glucose leads to imbalance between pro-angiogenic factors (HIF-1 α , VEGF and Ang-1) and anti-angiogenic factors (TKLK and PEDF) (11,12). In particular, the increased level of VEGF promotes the proliferation of the retinal pigment epithelial cells, resulting in increased vascular permeability and retinal exudation, hemorrhage as well as edema (13,14). In this study, SY and CEPO were used to treat DR rats. Results showed that expression levels of HIF-1 α , VEGF, and Ang-1 were significantly lower, while the levels of TCLK and PEDF were significantly higher in the SY-treated control group than in the model group (P<0.05),

indicating that SY has a certain regulatory effect on the generation of blood vessels. Song *et al* (15) confirmed that SY may alter the expression of VEGF and PDGF, regulating the process of angiogenesis. There was no significant difference in the expression levels of HIF-1 α , VEGF, Ang-1, TKLK and PEDF between the experimental group treated with CEPO and the model group, indicating that CEPO did not affect the angiogenesis of the retina during treatment, thus, resolving the disadvantages of EPO (16).

Retinal hypoxia induces apoptosis by regulating the expression of apoptosis-promoting factors (Bax and caspase-3) and apoptosis-inhibiting factors Bcl-2 and survivin (17,18). Wang *et al* (17) showed that Bax may increase the release of cytochrome *c* from mitochondria into cytoplasm and exert a pro-apoptotic effect. Bcl-2 antagonizes Bax by reducing cytochrome *c* release. In addition, some studies have shown that caspase-3 is a key molecule at the downstream of cytochrome *c* (19). Survivin inhibits cell apoptosis by antagonizing the apoptosis cascade mediated by multiple caspase molecules (18). Results of this study showed that compared with the model group, the expression levels of the apoptosis-promoting factors Bax and caspase-3 were significantly decreased in the experimental and control groups than in the model group, and were also significantly lower in the experimental group than in the control group. The expression levels of Bcl-2 and survivin were significantly higher in the experimental and control groups than in the model group, and were also significantly higher in the experimental group than in the control group, indicating that both SY and CEPO may improve DR by regulating the expression of apoptosis-related factors, and the effects of CEPO are stronger than those of SY.

In addition, retinal hypoxia also triggers the activation of the oxidative stress pathways to increase the levels of oxygen-free radicals, which in turn, damages the nerve cells (19). Nrf acts as a receptor for oxidative stress and plays a major role in the defense against oxidative stress. Nrf2 has 6 highly conserved domains that can specifically recognize and bind to ARE to form Nrf-2/ARE antioxidant pathway (20), and activation of Nrf2 forms a positive feedback to enhance the regulation of antioxidative stress (21). The binding of Nrf-2 to ARE can activate HO-1 and NQO-1 to remove excess oxygen-free radicals, and reduce stress injury (22). The results of this study showed that the expression levels of oxidative stress molecules were significantly higher in the experimental and control groups than in the model group, indicating that both SY and CEPO may improve the disease conditions by regulating the oxidative stress pathway. The expression level of each oxidative stress molecule in the experimental group was significantly higher than that in the control group, indicating that CEPO is more effective in scavenging oxygen-free radicals than SY.

In conclusion, the therapeutic effect of CEPO in treating DR is better than that of SY. CEPO may inhibit cell apoptosis and oxidative stress damage in the retinal tissues of diabetic rats without affecting angiogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XX drafted this manuscript and helped construction of diabetes rat model. YC and YY performed RT-qPCR. All authors read and approved the final study.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Eye Hospital of Wenzhou Medical University (Wenzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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