Journal of Hainan Medical University

http://www.hnykdxxb.com

Effect of carbamylated erythropoietin on retinopathy of diabetic rats

Lin Jiang✉

Ophthalmology Department, the Sixth People's Hospital of Chengdu Sichuan Province, Chengdu City, Sichuan Province, 610051

ARTICLE INFO

Article history:
Received 28 Aug 2017
Received in revised form 3 Sep 2017
Accepted 9 Sep 2017
Available online 14 Sep 2017

Keywords:
Diabetic retinopathy
Carbamylated erythropoietin
Apoptosis
Oxidative stress
Angiogenesis

ABSTRACT

Objective: To study the effect of carbamylated erythropoietin (CEPO) on retinopathy of diabetic rats. **Methods:** Male SD rats were selected as experimental animals and randomly divided into control group, DM group and CEPO group, and diabetic animal models were established and then given CEPO intervention. 2 weeks after intervention, the retina was collected to detect the expression of angiogenesis molecules, apoptosis molecules and oxidative stress pathway molecules. **Results:** HIF-1α, VEGF, Ang-1, Bax, Caspase-3, Nrf-2, ARE, HO-1 and NQO-1 mRNA expression in retina of DM group were significantly higher than those of control group while TKLK, PEDF, Bcl-2 and Survivin mRNA expression were significantly lower than those of control group; HIF-1α, VEGF, Ang-1, TKLK and PEDF mRNA expression in retina of CEPO group were not significantly different from those of DM group, Bcl-2, Survivin, Nrf-2, ARE, HO-1 and NQO-1 mRNA expression were significantly higher than those of DM group, and Bax and Caspase-3 mRNA expression were significantly lower than those of DM group. **Conclusion:** CEPO can reduce the apoptosis and oxidative stress injury of the retina tissue in diabetic rats without affecting the angiogenesis.

1. Introduction

Diabetic retinopathy (DR) is a common microvascular complication of diabetes that will affect patients' daily life and increase the risk of blindness. Long-term substandard blood glucose control will cause the retinal microvascular system lesions and affect the blood perfusion of the retina, and the continuous hypoxia stimulation can on the one hand, cause retinal nerve cell apoptosis and oxidative stress damage, and on the other hand, may stimulate the formation of new blood vessels and microaneurysm, further oppress the retina and affect the vision[1,2]. Erythropoietin (EPO) is an endocrine hormone that has anti-oxidative stress and anti-apoptotic effects, and it can reduce the damage of the retinal nerve cells when used for treatment of DR. However, EPO also promotes angiogenesis, and will increase the number of new blood vessels in the retinal tissue of DR patients and exacerbate the damage to the vision[3]. Carbamylated erythropoietin (CEPO) is the product of EPO carbamylation, which has cellular protective activity and does not have the pro-angiogenic activity[4]. In the following studies, CEPO was used to treat DR, and the effect of CEPO on retinopathy of diabetic rats was specifically analyzed.

✉Corresponding author: Lin Jiang, Ophthalmology Department, the Sixth People's Hospital of Chengdu Sichuan Province, Chengdu City, Sichuan Province, 610051.
Tel: 13388190962
Fund Project: Projects of Nanchong Science and Education Bureau No: 14A0026.

2. Experimental materials and methods

2.1 Experimental materials

The male SD rats with body mass of 180-220 g were selected as experimental animals and purchased in Shanghai Slac Laboratory Animal Co., Ltd., and the license number was SCXK (Shanghai) 2016-0002. All animal experiments passed the hospital ethics review and the procedures were followed for animal experiments and animal treatment after death. CEPO was synthesized by Pharmacy Department of the Sixth People's Hospital of Chengdu, streptozotocin was purchased in the Sigma Company, and RNA extraction kits, reverse transcription kits and fluorescence quantitative PCR kits were purchased from Beijing CWBIO Company.

2.2 Experimental methods

2.2.1 Diabetic animal model preparation

SD rats were randomly divided into control group, DM group and CEPO group, and the DM group and CEPO were made into diabetic animal models according to the following method: after fasting

for solids and liquids overnight, 60 mg/kg dose was referred for intraperitoneal injection of streptozotocin, caudal venous blood was collected 3 d after injection to test random blood glucose, and the random blood glucose > 16.7 mmol/L was the standard of successful preparation of diabetic animal model. Control group were fasting for solids and liquids overnight and then given intraperitoneal injection of same dose of sodium citrate buffer solution.

2.2.2 Drug intervention methods

The drug intervention was started 4 weeks after the establishments of animal models, CEPO rats were given intraperitoneal injection of 50 μg/kg CEPO, and the control group and the DM group was given intraperitoneal injection of same dose of saline. Injection was done once every day for 2 weeks in a row.

2.2.3 Retinodialysis and molecule expression test

2 weeks after drug intervention, the SD rats were beheaded, the eyeballs were anatomized, the anterior segment tissue was removed, the retinal tissue was separated under operating microscope and frozen rapidly by liquid nitrogen, RNA extraction kit was used to get the total RNA in retinal tissue, then reverse transcription kit was used for reverse transcription from total RNA to cNDA, finally fluorescence quantitative PCR kit was used to amplify HIF-1 α, VEGF, Ang-1, TKLK, PEDF, Bcl-2, Bax, Caspase-3, Survivin, Nrf-2, ARE, HO-1 and NQO-1, and the mRNA expression was calculated.

2.3 Statistical methods

SPSS 19.0 software was used to input data, the measurement data among three groups were by variance analysis and $P<0.05$ indicated statistical significance in differences in analysis results.

3. Results

3.1 Angiogenesis molecule expression in retina

Analysis of angiogenesis molecules HIF-1 α, VEGF, Ang-1, TKLK and PEDF expression in retina of three groups of rats was as follows: HIF-1 α, VEGF and Ang-1 mRNA expression in retina of DM group were significantly higher than those of control group while PEDF and TKLK mRNA expression were significantly lower than those of control group ($P<0.05$); HIF-1 α, VEGF, Ang-1, TKLK and PEDF mRNA expression in retina of CEPO group were not significantly different from those of DM group ($P>0.05$).

3.2 Apoptosis molecule expression in retina

Analysis of apoptosis molecules Bcl-2, Bax, Caspase-3 and Survivin expression in retina of three groups of rats was as follows: Bcl-2 and Survivin mRNA expression in retina of DM group were significantly lower than those of control group while Bax and Caspase-3 mRNA expression were significantly higher than those of control group ($P<0.05$); Bcl-2 and Survivin mRNA expression in retina of CEPO group were significantly higher than those of DM group while Bax and Caspase-3 mRNA expression were significantly lower than those of DM group ($P<0.05$).

3.3 Oxidative stress pathway molecule expression in retina

Analysis of oxidative stress pathway molecules Nrf-2, ARE, HO-1 and NQO-1 expression in retina of three groups of rats was as follows: Nrf-2, ARE, HO-1 and NQO-1 mRNA expression in retina of DM group were significantly higher than those of control group ($P<0.05$); Nrf-2, ARE, HO-1 and NQO-1 mRNA expression in retina of CEPO group were significantly higher than those of DM group ($P<0.05$).

4. Discussion

The occurrence of diabetic macrovascular complications and microvascular complications is related to long-term substandard blood glucose control and the increased generation of glycation end products. Diabetic retinopathy (DR) is an important microvascular complication in diabetic patients and is also a common clinical cause of blindness. The damage of the retinal microvascular

Table 1.
Comparison of angiogenesis molecule expression in retina among three groups of rats.

Groups	*n*	HIF-1 α	VEGF	Ang-1	TKLK	PEDF
Control group	8	1.03±0.14	1.01±0.12	0.98±0.11	1.05±0.14	0.95±0.13
DM group	8	2.42±0.35*	2.33±0.32*	1.83±0.20*	0.44±0.05*	0.36±0.05*
CEPO group	8	2.39±0.31	2.38±0.37	1.86±0.22	0.47±0.06	0.39±0.06

*: compared with molecule expression of control group, $P<0.05$.

Table 2.
Comparison of apoptosis molecule expression in retina among three groups of rats.

Groups	*n*	Bcl-2	Bax	Caspase-3	Survivin
Control group	8	1.05±0.12	0.97±0.13	0.96±0.11	1.03±0.15
DM group	8	0.39±0.06*	2.84±0.41*	2.47±0.35*	0.32±0.05*
CEPO group	8	0.75±0.09#	1.58±0.20#	1.71±0.22#	0.65±0.08#

*: compared with molecule expression of control group, $P<0.05$; #: compared with molecule expression of DM group, $P<0.05$.

Table 3.
Comparison of oxidative stress pathway molecule expression in retina among three groups of rats.

Groups	*n*	Nrf-2	ARE	HO-1	NQO-1
Control group	8	1.02±0.14	0.97±0.11	1.04±0.15	0.94±0.12
DM group	8	1.77±0.20*	1.84±0.22*	1.62±0.19*	1.96±0.23*
CEPO group	8	2.94±0.38#	3.19±0.47#	3.31±0.39#	2.89±0.35#

*: compared with molecule expression of control group, $P<0.05$; #: compared with molecule expression of DM group, $P<0.05$.

system can directly affect the blood perfusion and cause local tissue ischemia and hypoxia, and neuron damage will occur in the the hypoxic retina[5]. On the one hand, the hypoxia can cause the apoptosis of the retinal ganglion cells directly, thereby causing the corresponding nerve function injury[6]; on the other hand, hypoxia can cause activated oxidative stress reaction and increased oxygen free radical generation, which can cause nerve cell damage and destruction. Therefore, anti-oxidation and anti-apoptosis are the important targets for clinical treatment of DR[7,8]. EPO is an important endocrine hormone in the body, it can not only promote bone marrow hematopoiesis as well as red blood cell differentiation and maturation, but also has significant anti-oxidative stress and antiapoptotic activity as well as neurotrophic value, it can inhibit apoptosis, relieve oxidative stress reaction and promote the regeneration of neurons when used for the treatment of nerve damage, and it helps alleviate nerve function damage and promote the nerve function reconstruction.

In the pathological process of DR, persistent hypoxia state can stimulate angiogenesis, and the constantly formed new blood vessels can cause micro aneurysm formation, increase local exudation and cause retinal damage. Although EPO has a neuroprotective effect, it also promotes angiogenesis, which can increase the number of blood vessels and affect the function of the retina when used for DR treatment. CEPO is the product of EPO carbamylation reaction, which turns lysine groups of EPO into homocitrulline, and can prevent CEPO combination with EPOR and affect its activities on promoting angiogenesis, but retain the cell protection activity[9,10]. The retinal angiogenesis is associated with increased HIF-1 α expression caused by hypoxia stimuli, and high protein of HIF-1 α can increase the expression of angiogenesis molecules VEGF and Ang-1[11,12]; at the same time, hypoxia can lead to decreased expression of anti-angiogenesis molecules such as TKLK and PEDF, thereby enhancing the pro-angiogenesis effect of VEGF, Ang-1 and other molecules[13,14]. In the study, analysis of the expression of the angiogenesis molecules in the retina showed that HIF-1 α , VEGF and Ang-1 mRNA expression in retina of DM group were significantly higher than those of control group while TKLK and PEDF mRNA expression were significantly lower than those of control group. This indicates that the high expression of pro-angiogenesis molecules and the low expression of anti-angiogenesis molecules are associated with the retinopathy in the course of diabetes. Further analysis of the effects of CEPO on the expression of angiogenesis molecules showed that HIF-1 α , VEGF, Ang-1, TKLK and PEDF mRNA expression in retina were not significantly different between CEPO group and DM group. This shows that CEPO does not affect the angiogenesis of the retina in the course of diabetes.

The persistent retinal hypoxia condition in the course of diabetes can initiate mitochondrial pathway of apoptosis, and the excessive apoptosis in the retina can cause cell damage. Bax/Bcl-2 are the important molecules regulating mitochondrial pathway of apoptosis, Bax can increase the release of mitochondrial cytochrome C to the cytoplasm so as to promote apoptosis, and Bcl-2 can antagonize Bax function and reduce the release of mitochondrial cytochrome C to cytoplasm so as to inhibit apoptosis; Caspase-3 is a key molecule that executes apoptosis in the downstream of cytochrome C[15,16]. Survivin is the most powerful anti-apoptotic molecule known at present, which can antagonize the apoptosis cascade activation reaction mediated by multiple caspase molecules[17]. In the study, analysis of the expression of apoptosis molecules in retina showed that Bcl-2 and Survivin mRNA expression in retina of DM group were significantly lower than those of control group while Bax and Caspase-3 mRNA expression were significantly higher than those of control group. This indicates that the high expression of pro-apoptosis molecules and the low expression of anti-apoptosis molecules are related to the retinopathy in the course of diabetes. Further analysis of the CEPO effect on apoptosis molecule expression in the retina showed that Bcl-2 and Survivin mRNA expression in retina of CEPO group were significantly higher than those of DM group while Bax and Caspase-3 mRNA expression were significantly lower than those of DM group. This means that CEPO can decrease the expression of pro-apoptosis molecules and increase the expression of anti-apoptosis molecules in retina in the course of diabetes, thereby inhibiting the retinal nerve cell apoptosis and reduce the neurological damage.

The hypoxia condition in the course of DR will not only cause apoptosis, but also increase the oxygen free radical generation and cause oxidative stress damage in the retina[18,19]. Nrf-2/ARE is an important antioxidant pathway in the body, and the activated Nrf-2 transfers into the nucleus and is combined with ARE to start the expression of HO-1, NQO-1 and other antioxidant enzymes, then remove oxygen free radicals and reduce oxidative stress damage through the biological activities of HO-1 and NQO-1[20,21]. In the study, analysis of the expression of above oxidative stress pathway

molecules in retina showed that Nrf-2, ARE, HO-1 and NQO-1 mRNA expression in retina of DM group were significantly higher than those of control group. This indicates that the activation of oxidative stress response and the compensatory activation of the antioxidant pathway Nrf-2/ARE are related to the retinopathy in the course of diabetes. Further analysis of the CEPO effect on the oxidative stress pathway molecule expression in the retina showed that Nrf-2, ARE, HO-1 and NQO-1 mRNA expression in retina of CEPO group were significantly higher than those of DM group. This indicates that CEPO can promote the activation of antioxidant pathway Nrf-2/ARE in the retina in the course of diabetes, thus enhancing the antioxidant capacity of local tissues and reducing oxidative stress damage.

CEPO can regulate the expression of apoptosis molecules and oxidative stress pathway molecules in retina tissue of diabetic rats, inhibit apoptosis, enhance antioxidant capacity and relieve oxidative stress damage without affecting the process of angiogenesis in the retina.

References

[1] Coucha M, Elshaer SL, Eldahshan WS, Mysona BA, El-Remessy AB. Molecular mechanisms of diabetic retinopathy: potential therapeutic targets. *Middle East Afr J Ophthalmol* 2015; **22**(2): 135-144.

[2] Hampton BM, Schwartz SG, Brantley MA Jr, Flynn HW Jr. Update on genetics and diabetic retinopathy. *Clin Ophthalmol* 2015; **23**(9): 2175-2193.

[3] Jo HR, Kim YS, Son H. Erythropoietin and carbamylated erythropoietin promote histone deacetylase 5 phosphorylation and nuclear export in rat hippocampal neurons. *Biochem Biophys Res Commun* 2016; **470**(1): 220-225.

[4] Ding J, Wang J, Li QY, Yu JZ, Ma CG, Wang X, et al. Neuroprotection and CD131/GDNF/AKT pathway of carbamylated erythropoietin in hypoxic neurons. *Mol Neurobiol* 2017; **54**(7): 5051-5060.

[5] Petrovski G, Kaarniranta K, Petrovic D. Oxidative stress, epigenetics, environment, and epidemiology of diabetic retinopathy. *J Diabetes Res* 2017; *2017*: 6419357.

[6] Kowluru RA, Shan Y. Role of oxidative stress in epigenetic modification of MMP-9 promoter in the development of diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol* 2017; **255**(5): 955-962.

[7] Pelikanova T. Diabetic retinopathy: pathogenesis and therapeutic implications. *VnitrLek Fall* 2016; **62**(7-8): 620-628.

[8] deMoraes G, Layton CJ. Therapeutic targeting of diabetic retinal neuropathy as a strategy in preventing diabetic retinopathy. *Clin Exp Ophthalmol* 2016; **44**(9): 838-852.

[9] Chen J, Yang Z, Zhang X. Carbamylated erythropoietin: a prospective drug candidate for neuroprotection. *Biochem Insights* 2016; **8**(Suppl 1): 25-29.

[10]Liu X, Zhu B, Zou H, Hu D, Gu Q, Liu K, et al. Carbamylated erythropoietin mediates retinal neuroprotection in streptozotocin-induced early-stage diabetic rats. *Graefes Arch Clin Exp Ophthalmol* 2015; **253**(8): 1263-1272.

[11]Wei J, Jiang H, Gao H, Wang G. Blocking mammalian target of rapamycin (mTOR) attenuates HIF-1 α pathways engaged-vascular endothelial growth factor (vegf) in diabetic retinopathy. *Cell Physiol Biochem* 2016; **40**(6): 1570-1577.

[12]Cheng L, Yu H, Yan N, Lai K, Xiang M. Hypoxia-inducible factor-1 target genes contribute to retinal neuroprotection. *Front Cell Neurosci* 2017; **27**(11): 20.

[13]Iwona BS. Growth factors in the pathogenesis of retinal neurodegeneration in diabetes mellitus. *Curr Neuropharmacol* 2016; **14**(8): 792-804.

[14]Farnoodian M, Halbach C, Slinger C, Pattnaik BR, Sorenson CM, Sheibani N. High glucose promotes the migration of retinal pigment epithelial cells through increased oxidative stress and PEDF expression. *Am J Physiol Cell Physiol* 2016; **311**(3): 418-436.

[15]Wang XL, Yu T, Yan QC, Wang W, Meng N, Li XJ, et al. AGEs promote oxidative stress and induce apoptosis in retinal pigmented epithelium cells RAGE-dependently. *J Mol Neurosci* 2015; **56**(2): 449-460.

[16]Pei-Yan Han, Hong-Bin Lyu. Research progress of diabetic retinopathy and apoptosis-relating genes. *Int Eye Sci* 2016; **16**(5): 859-862.

[17]Liu N, Zhao N, Chen L, Cai N. Survivin contributes to the progression of diabetic retinopathy through HIF-1 α pathway. *Int J Clin Exp Pathol* 2015; **8**(8): 9161-9167.

[18]Guzman DC, Olguin HJ, Garcia EH, Peraza AV, de la Cruz DZ, Soto MP. Mechanisms involved in the development of diabetic retinopathy induced by oxidative stress. *Redox Rep* 2017; **22**(1): 10-16.

[19]Gehl Z, Bakondi E, Resch MD, Hegedus C, Kovacs K, Lakatos P, et al. Diabetes-induced oxidative stress in the vitreous humor. *Redox Biol* 2016; **9**: 100-103.

[20]Batliwala S, Xavier C, Liu Y, Wu H, Pang IH. Involvement of Nrf2 in ocular diseases. *Oxid Med Cell Longev* 2017; **2017**: 1703810.

[21]Ren X, Sun H, Zhang C, Li C, Wang J, Shen J, et al. Protective function of pyridoxamine on retinal photoreceptor cells via activation of the p Erk1/2/Nrf2/Trx/ASK1 signalling pathway in diabetic mice. *Mol Med Rep* 2016; **14**(1): 420-424.