ORIGINAL ARTICLES

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Berberine prevents diabetic retinopathy through inhibiting HIF-1α/VEGF/NF-κB pathway in db/db mice

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Our paper investigated the effect of berberine on the diabetic retinopathy (DR) in *db/db* transgenic mice and explored its possible mechanisms. During chronic intragastric administration for ten weeks, berberine could decrease the levels of fasting blood glucose, TC and TG without hepatotoxicity. Moreover, berberine could protect the retinal morphology against the hyperglycemic insults and decrease glycogen accumulation, the contents of TNF-α and IL-1β in the retinas, as demonstrated by HE staining, PAS staining and ELISA kits, respectively. Immunofluorescence assay revealed that the protein expression of vascular endothelial growth factor (VEGF), VEGF receptor 2, hypoxia-inducible factor-1α (HIF-1α), and nuclear factor-κB (NF-κB) p65 was upregulated in *db/db* retinas compared with wild type ones, whereas berberine treatment could suppress their expression. Berberine prevent DR development through modulating the glucolipid metabolism and inhibiting the HIF-1α/VEGF/NF-κB pathway, suggesting that berberine maybe a potential agent for the treatment of DR.

1. Introduction

Diabetic retinopathy (DR) is a retinal microvascular dysfunction caused by chronic hyperglycemia, leading to microvascular leakage and blockage in retina. Diabetes dramatically increases the risk of eye diseases, including diabetic macular edema (DME), cataract, glaucoma, refractive error and diplopia, which ultimately lead to vision loss and even blindness (Association 2014). It was estimated that about one third of diabetic patients would suffer from diabetic retinopathy (Solomon et al. 2017). Currently, more than 93 million people are suffering from diabetic eye disease. The patients should be referred to the ophthalmology in time, once they have been diagnosed as diabetic retinopathy or DME, for laser photocoagulation, anti-vascular endothelial growth factor (VEGF) treatment (intraocular administration of VEGF inhibitors), glucocorticoids or even vitrectomy surgery to prevent further decline in vision or vitreous hemorrhage. However, long-term side effects, high economic cost, discomfort of the patients have limited the application of these approaches (Wong and Sabanayagam 2019). Therefore, new anti-DR therapies with safe, efficient and low-cost property are attracting the worldwide attention.

Long-term hyperglycemia in retina and surrounding tissue could increase intracellular osmotic pressure, oxidative stress, capillary permeability and thickness of retina by the overactive polyol metabolism pathway, advanced glycation end products (AGEs) generation, protein kinase C (PKC) and inflammation activation (Wong and Sabanayagam 2019). Without timely intervention, retinal hypoxia will cause proliferative diabetic retinopathy (PDR), which leads to retinal neovascularization, vitreous bleeding, retinal leakage. Hypoxia inducible factor-1α (HIF-1α), the active subunit of HIF-1 protein, is regulated by hypoxia signaling to affect the activity of HIF. Under hypoxia, stabilized HIF-1α combined with HIF-β to regulate a large number of genes involved in hypoxia adaptation, inflammation progression and tumor growth, including VEGF, erythropoietin (EPO), nitric oxide syntheses(NOSs), and glucose transporters (GLUTs) (Kurihara et al. 2014). VEGF is the key downstream effector of HIF-1α signaling and is related to the retinal angiogenesis. By binding to its receptors, VEGF regulates the vascular permeability, extracellular matrix degeneration, the migration and proliferation of vascular endothelial cells, and angiogenesis (Wang et al. 2015). A large number of studies revealed that retinal inflammation is triggered by heterogeneous factors, such as hyperglycemia, reactive oxygen species (ROS), increased circulating cytokines and chemokines, and AGEs (Al-Kharashi 2018; Santiago et al. 2018). It is worth noting that the persistent and low-degree inflammation induced by diabetes might promote the angiogenesis (Kim et al. 2013), thereby affecting the progression of DR. Therefore, tuning of HIF-1α/VEGF/NF-κB signaling as regulated by oxygen tension and long-term hyperglycemic might be a potential strategy for the prevention and treatment of DR.

Berberine, an isoquinoline alkaloid isolated from *Coptis chinensis* (Huanglian in Chinese), has been used to treat a variety of chronic disease, such as cardiovascular diseases, tumors, depression, diabetes and diabetic complications (Che et al. 2019; Gong et al. 2019; Kong et al. 2020; Zhang et al. 2019). Although the anti-diabetic capacity of berberine has been testified, the effect of berberine on DR has been rarely discussed. Recently, berberine was found to protect retinal Müller cells *in vitro* against the insults of modified low-density lipoproteins (oxidized-glycated LDL) and high glucose by regulating autophagy, apoptosis, oxidative stress, the NF-κB and AMPK/mTOR signaling (Fu et al. 2016; Zhong et al. 2020; Zhou et al. 2020). This suggested us to investigate the potential protective role of berberine on experimental diabetic retinopathy.

In this study, we applied *db/db* mice to establish an experiment diabetic retinopathy model. Hyperglycemia and retinal lesion, such as distorted ganglion cell layer and hemangiectasis, were found in *db/db* mice. However, hyperglycemia, hyperlipidemia and retinal lesion were restored by the treatment of berberine, without any hepatotoxicity. Furthermore, berberine could upregulate the protein expression of HIF-1α,VEGF, VEGFR2 and NF-κB. Our data might give rise to new strategy to control diabetic retinopathy and further understanding of HIF-1α/VEGF/NF-κB signaling regulation under the anti-diabetic effect of berberine.

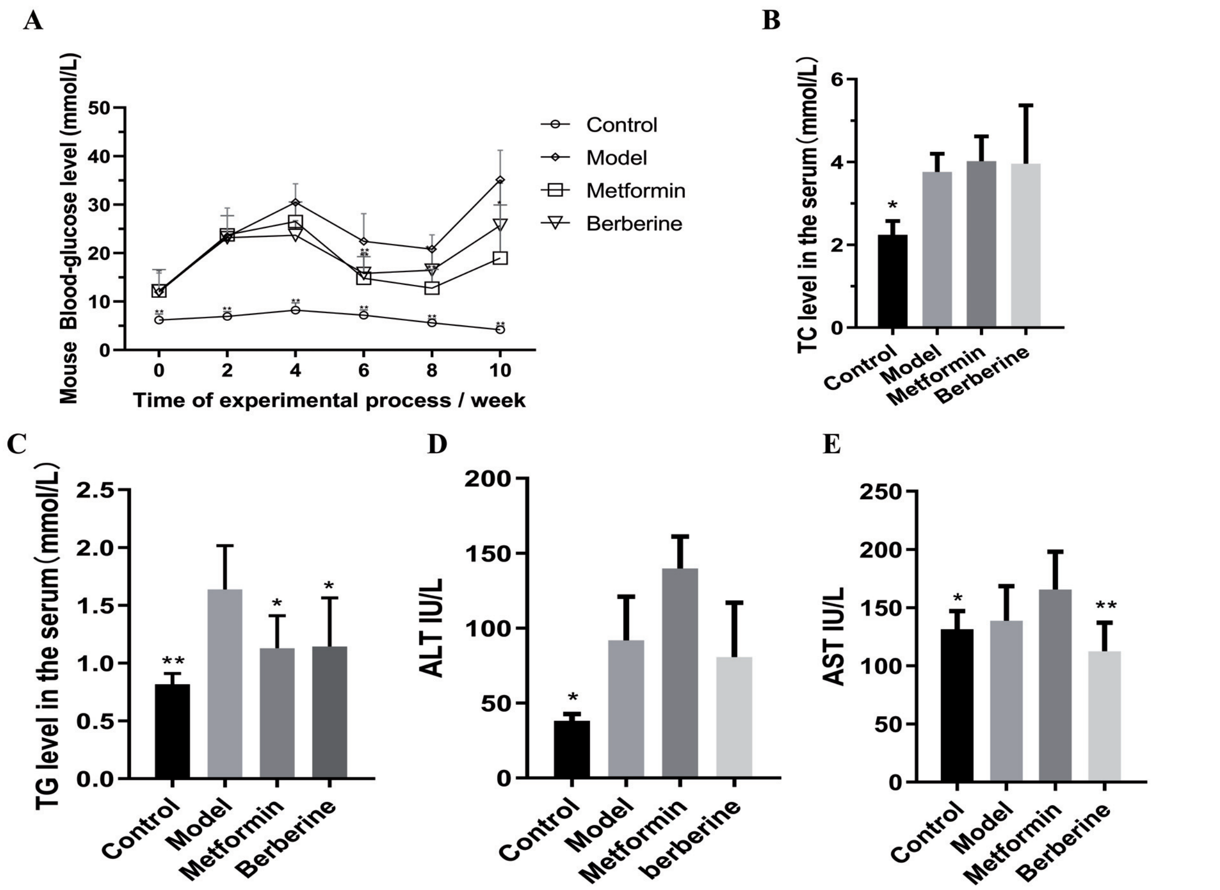

Fig. 1: Berberine alleviated hyperglycemia in diabetic *db/db* mice. (A) The levels of fasting blood glucose (FBG) was detected every 2 weeks from the beginning, when berberine was given, to the 10th week. Control, wild type mice treated with saline; Model, *db/db* mice treated with saline; metformin and berberine, *db/db* mice treated with metformin and berberine, respectively. After 10-week experiment, serum (B) total cholesterol (TC), (C) triglyceride (TG), (D) alanine aminotransferase (ALT), and (E) aspartate aminotransferase levels were detected. Data are represented as mean +/- SD. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. model.

2. Investigations and results

2.1. Berberine alleviates hyperglycemia in db/db mice

To investigate the anti-diabetic effect of berberine, the *db/db* mice were treated with berberine or not for 10 weeks. As shown in Fig. 1, the 18-week old *db/db* mice exhibited typical diabetic features, including increased FBG, the serum lipids including total cholesterol (TC) and triglyceride (TG), and the biomarkers of hepatotoxicity including alanine aminotransferase (ALT) and aspartate aminotransferase (AST). These data indicated that long-term hyperglycemia could induce dyslipidemia and the hepatic injury. Administration of berberine or metformin, a classical oral anti-diabetic medicine, prevented the increase of FBG, TG, and AST in *db/db* mice significantly (Fig. 1B). Note that the remarkable glucose-lowering capacity of the berberine was displayed earlier than metformin. Unfortunately, metformin elevated the levels of serum ALT without statistical significance. These results suggested that berberine might exert anti-diabetic and TG-lowering effect while did not aggravate hepatic injury.

2.2 Berberine restores the impaired morphology of retina in db/db mice

HE staining was performed to assess the retinal morphology. In the wild-type C57BL/6 mice, the structure of each layer in the retina was completed, including ganglion cells, inner granular and outer granular layers, and the cells were abundant and organized (Fig. 2A). In *db/db* mice, the retina was partly corrugated and the retinal ganglion cell layers exhibited disordered narrowing or widening arrangement. Some regions of the layers displayed a loss of cells (red arrow in Fig. 2Ab) and expanded capillaries. Treatment of berberine or metformin for ten weeks ameliorated the abnormal morphology in retina. The disordered and abnormal phenotype of ganglion cells, inner granular and outer granular layers were recovered (Fig. 2A). These results suggested that berberine could protect the retinal morphology and homeostasis in *db/db* mice.

2.3. Berberine inhibits glycogen accumulation in retina of db/db mice

To determine the glycogen deposition in the retina, PAS staining was performed. Compared with wild-type mice, *db/db* diabetic mice showed a higher ratio of regions with positive glycogen deposition (purple area in Fig. 2B) in retina to the whole area. Berberine or metformin group exhibited lower glycogen accumulation in the retina (Fig.2C). This further demonstrated that berberine could improve the diabetic retinal abnormalities.

2.4. Berberine inhibits inflammation in the retina of db/db mice

To investigate if berberine could regulate the inflammatory response in the retina, pro-inflammatory cytokines were detected in the retina homogenate, and the NF-κB p65 protein expression

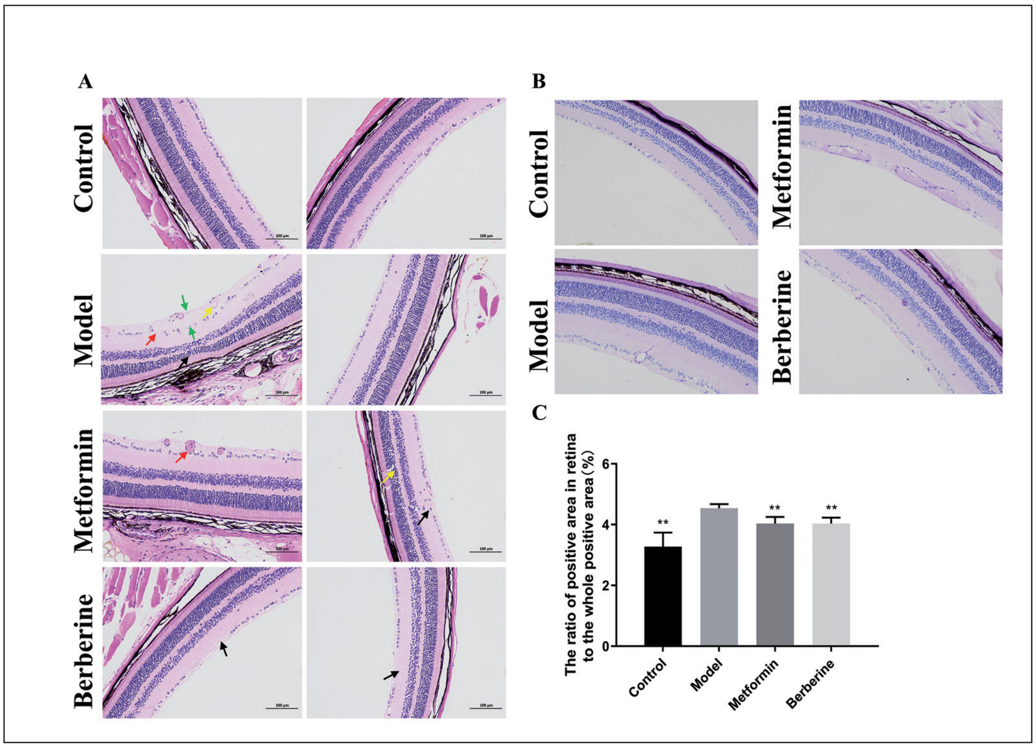

Fig. 2: Berberine restored the retinal morphopathology and decreased the intracellular accumulation of glycogen in retina of *db/db* mice. (A) Representative HE staining of retina in control, model, metformin and berberine groups. Black arrows indicate narrowing in the ganglion cell, inner granular and outer granular layers. Green arrows indicate widening in the ganglion cell layer. Red arrows indicate the lack of cells. Yellow arrows indicate hemangiectasis. (B) Representative PAS staining images of retina. (C) The ratio of PAS positive area in retina to the whole positive area. Data are represented as mean +/- SD. $^{**}P < 0.01$, vs. model.

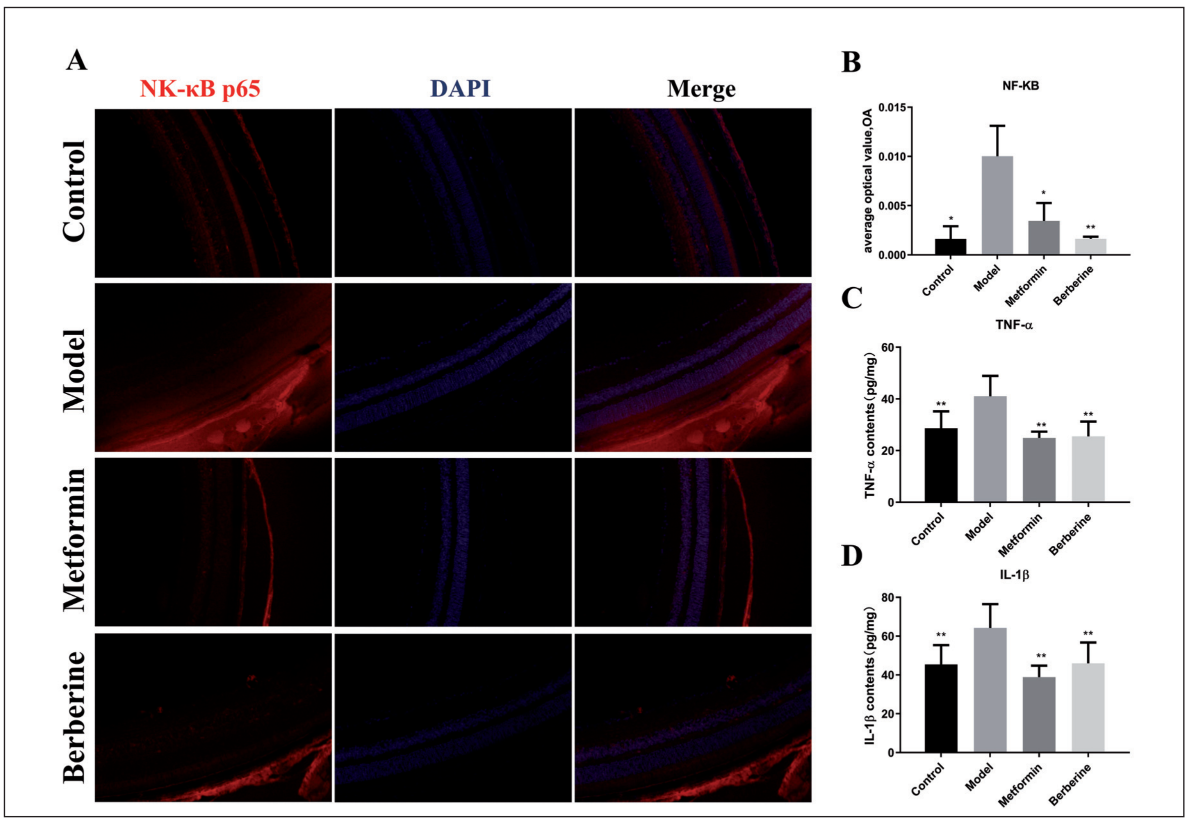

Fig. 3: Berberine suppressed the inflammation in the diabetic retina. (A) NF-κB p65 expression detected by immunofluorescence on sections of mice retinas. (B) Average optical density of NF-κB p65 quantified by image analysis system (NIKON DS-U3). (C) TNF-α levels in the retinal homogenate. (D) IL-1β levels in the retinal homogenate. Data are represented as mean +/- SD. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. model.

in the sections of mice retinas was detected by immunofluorescence. As depicted in Fig. 3C and Fig. 3D, the levels of IL-1β and TNF-α in the retina homogenate of diabetic *db/db* mice were dramatically increased, compared to the wild-type mice. However, administration of berberine or metformin significantly inhibited the production of IL-1flammatory cytokines were detected in *db/db* mice. Moreover, berberine could restore the elevation of NF-κB p65 protein expression in the retinas induced by long-term hyperglycemia. Thus, berberine might exert its protective effect through inhibiting inflammatory response in the diabetic retina.

2.5. Berberine prevents the DR development via regulating HIF-1α/VEGF/ VEGFR2 signaling

HIF-1α/VEGF/NF-κB is one of the key pathways contributed to diabetic angiogenesis. To further confirm the effect of berberine on HIF-1α/VEGF/NF-κB signaling in the retina, the immunofluorescence assay was performed. Compared with wild-type mice, the *db/db* mice showed markedly higher protein expression of HIF-1α, VEGF, VEGFR2 in retina (Fig. 4). Treatment of berberine inhibited the protein expression of HIF-1α, VEGF, VEGFR2 in *db/db* retina, suggesting that berberine could prevent DR development by down-regulating HIF-1α/VEGF/VEGFR2 signaling.

3. Discussion

Controlling of blood pressure, glucose and lipid levels are principal strategies for the prevention of DR. Good metabolic controlling could slow the progression of eye diseases, and great efforts should be made to achieve that goal after diabetes has been diagnosed. Thus, medications with hypoglycemic and lipid-lowering effects are essential for diabetes treatment. The distinctive advantages of Chinese medicine have been proven in the prevention and treatment of diabetes and its complications. For example, the benefits of berberine in diabetes have been demonstrated in some animal and clinical tests (Chen et al. 2018; Jin et al. 2017; Zhang et al. 2018; Zhang et al. 2010; Zhong et al. 2020).We further demonstrated the role of berberine on complications of diabetes, the diabetic retinopathy. Treatment with berberine for ten weeks significantly suppressed FBG, TC and TG in the serum of diabetic *db/db* mice. Previous studies suggested that berberine might exert a protecting effect on the retinal Müller cells and podocytes by anti-apoptosis, anti-oxidative, anti-inflammation, enhancing autophagy and activating the AMPK/mTOR signaling roles (Chen et al. 2018; Fu et al. 2016; Jin et al. 2017). Our study firstly showed that orally administration of berberine could prevent the onset of retinopathy induced by diabetes in *db/db* mice, and the underlying mechanism might be correlated with the inhibition of HIF-1α/VEGF/NF-κB signaling pathway.

DR patients and animal models commonly manifest prominent inflammatory characters, including increased levels of IL-1β, TNF-α and various inflammatory mediators in the vitreous humor and serum, increased vascular permeability, and enhanced macrophage infiltration and leukocyte adhesion (Antonetti et al. 2006; Demircan et al. 2006; Joussen et al. 2004; Miyamoto et al. 1999). Moreover, inflammation and angiogenesis act synergistically in the pathomechanism of DR as they share a variety of signaling pathway. Diacyl lipopeptide or triacyl ipopeptide induced by dyslipidemia could combine with toll-like receptor 2 (TLR2) and thereby activate NF-κB signaling and boost the expression of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-12. Simultaneously, inflammatory cytokines combining with cytokine receptors further enhance the NF-κB-mediated inflammatory cascade. On the other hand, the activation of NF-κB pathway

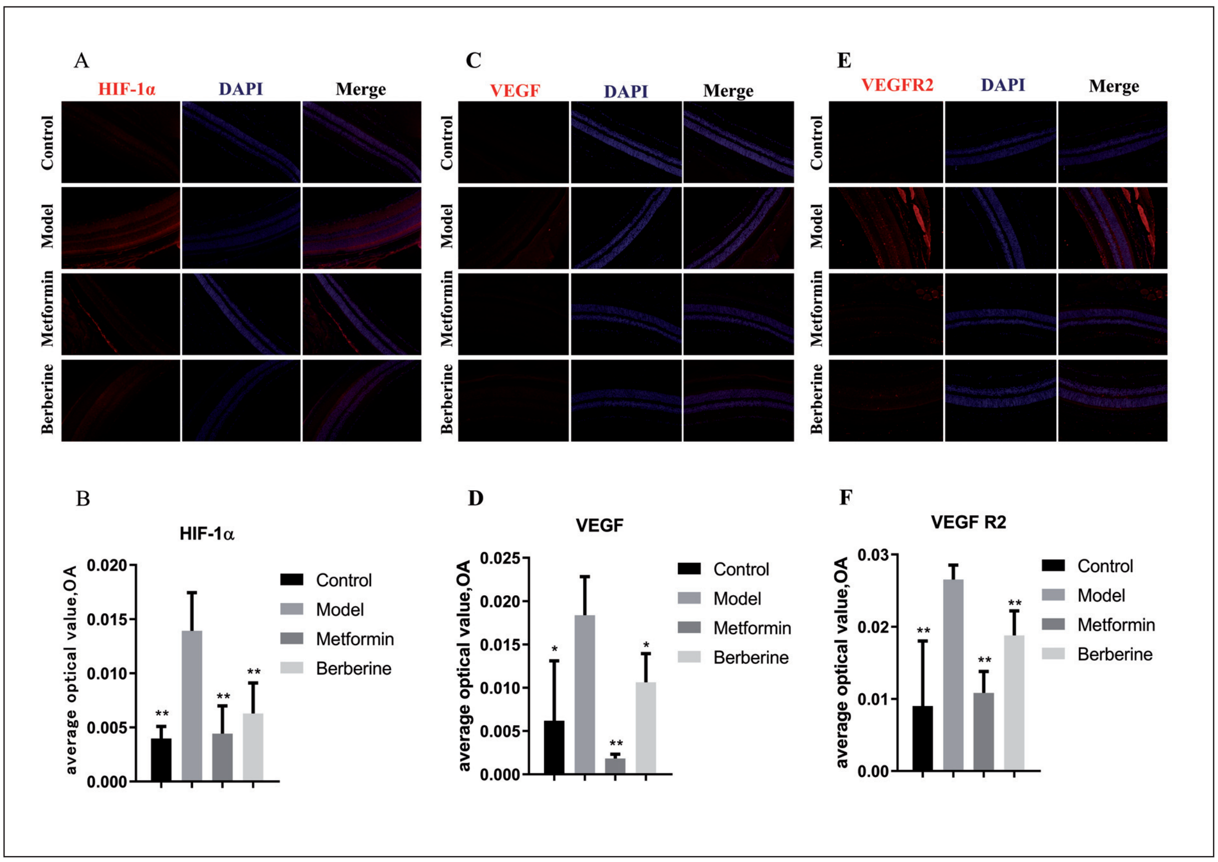

Fig. 4: Berberine inhibited HIF-1α/VEGF signaling in the diabetic retina. (A) HIF-1α expression detected by immunofluorescence on sections of mice retinas. (B) Average optical density of HIF-1α. (C) VEGF protein expression detected by immunofluorescence. (D) Average optical density of VEGF. (E) VEGFR2 protein expression detected by immunofluorescence. (F) Average optical density of VEGFR2. The optical density was quantified by image analysis system (NIKON DS-U3). Data are represented as mean +/- SD. *$P < 0.05$, **$P < 0.01$, vs. model.

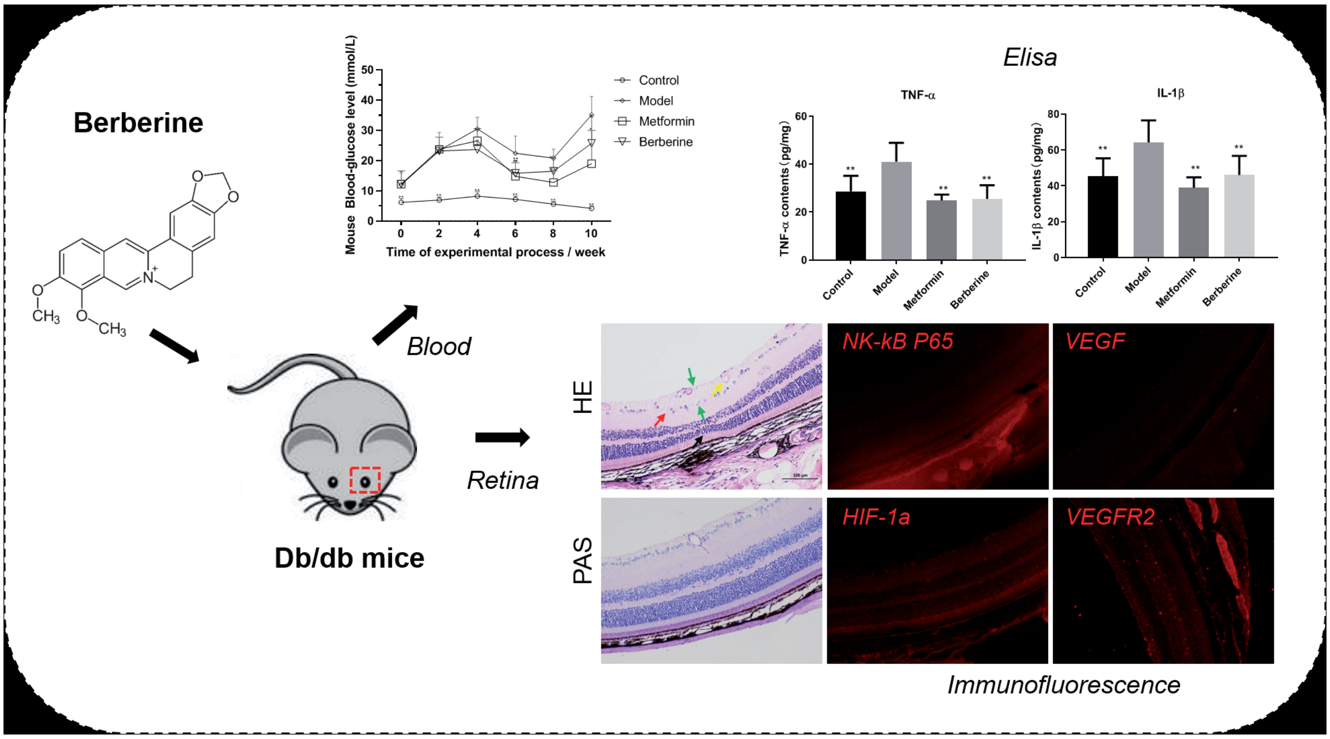

Fig. 5: Graphical overview about study design and results.

promotes the angiogenesis by upregulating the expression of VEGF and endothelial cells proliferation in the retina (Ho et al. 2006). Therefore, IL-1β, TNF-α and NF-κB could be potential therapeutic targets for the DR treatment. Recently, it has been confirmed that specific inhibition of IL-1β or gene knockout of the IL-1 receptor could protect retinal capillaries against hyperglycemia, and that TNF-α genetic or medicinal deficiency could significantly suppress leukocyte adhesion and blood retinal barrier (BRB) breakdown in the diabetes (Behl et al. 2008; Joussen et al. 2002; Vincent and Mohr 2007). In our study, there was a prominent increase of IL-1β and TNF-α levels in the retina of 18-week db/db diabetic mice. In addition, administration of berberine remarkably reduced the levels of IL-1β and TNF-α, and inhibited the expression of NF-κB p65 protein in the retina of db/db mice. These results agree with a previous study reporting that berberine mitigates HOG-LDL-induced TNF-α, IL-6 and IL-8 expression in the retinal Müller cells (Fu et al. 2016). A large number of studies has documented that berberine could inhibit the phosphorylation of IKK and activate PI3K/Akt signaling, thus restraining the transcription of NF-κB (Dinesh and Rasool 2018; Lee et al. 2007).

Hyperglycemia and hypoxia are responsible for aberrant proliferation of vessel cells. Persistent hyperglycemia disrupts the homeostasis of human endothelial cells and causes hyperosmolarity, angiogenesis and diabetic retinopathy (Madonna et al. 2016; Wang et al. 2019). Our research found excessive glycogen accumulation in the retina of *db/db* mice. It has been demonstrated that over-production of ROS was the consequence of overactive polyol pathway and AGEs accumulation respond to high-glucose-insultion in the retina (Yamagishi and Matsui 2011; Yamagishi et al. 2008). This process was accompanied by a rush consumption of oxygen, thus leading to retinal hypoxia. Under hypoxic conditions, the expression of endothelial tight junction proteins was downregulated significantly in the retina, which might lead to vascular endothelial dysfunction and the impairment of the BRB (Rathnasamy et al. 2015). On the one hand, HIFs were activated by the hypoxia and transported to the nucleus to regulate the transcription of downstream target genes, including VEGF, the essential factor for endothelial cell survival under hypoxia (Kurihara et al. 2014). On the other hand, the balance of pro-angiogenic and anti-angiogenic mediators was regulated by hypoxia, which could finally result in aberrant proliferation vessel cells (Simo et al. 2006). Considering these investigations, we evaluated the glycogen accumulation and the protein expression of HIF-1α in the berberine group and proved that poor control of diabetes could induce a prominent increment of glycogen, HIF-1α expression in the retina. Treatment of berberine could alleviate the excessive glycogen accumulation, as well as the HIF-1α expression, in the retina of db/db mice strikingly, along with the improved morphology of retina, further confirming the role of glycogen accumulation and HIF-1α dysregulation in the pathology of diabetic retinopathy.

VEGF is a key causative factor for retinal angiogenesis in diabetic macular oedema and proliferative DR (Ahuja et al. 2019). Dysregulation of glucolipids metabolism increases the production of ROS, exacerbates oxidative stress, mitochondrial dysfunction, inflammation, and hypoxia. As a consequence, VEGF was upregulated to respond to the hypoxia situation. However, at early stage of DR, VEGF overproduction or long-term duration in the serum was harmful to the eyes, activating the ERK1/2, JNK, Src, and FAK signaling to trigger vascular permeability, cell migration, proliferation and angiogenesis (Parra et al. 2005; Takahashi et al. 2001). In fact, anti-VEGF approaches were more effective targets for DR, compared to conventional therapeutic ways (Osaadon et al. 2014). Anti-VEGF drugs are applied when the patients suffer evident vision lesions. Therefore, it is an urgent requirement to develop new treatments to prevent the occurrence of DR or to counterwork its progression heading of impaired vascular phenotype. In agreement with these researches, we demonstrated a significant upregulation of VEGF and VEGFR2 proteins in the diabetic retina. Overall, it could be speculated that the antiangiogenic capacity of berberine is mainly mediated through the inhibition of HIF-1α, thus downregulating the expression of VEGF and VEGFR2 proteins in the retina induced by diabetes. This finding was consistent with the results of some anti-cancer studies with berberine (Jie et al. 2011). Since berberine has been proven to possess neuroprotective capacity (Chen et al. 2018; Fu et al. 2016), it is conceivable that berberine could rescue retinal neurons from high glucose/hypoxia damage, which was common in the early stage of DR (Hernández et al. 2016). Surprisingly, berberine attenuated retinal damage by regulating HIF-1α/VEGF and NF-κB pathway. However, more efforts need to be made to explain the exact target of berberine against DR and to address the related questions such as how berberine affects the downstream factors of VEGF signaling and what is the efficacy of berberine on the different cells comprising the retina.

In conclusion, berberine prevents DR development by inhibiting HIF-1α/VEGF/NF-κB pathway, thus reducing the release of IL-1β and TNF-α in the retina. Our findings indicate that oral administration of berberine could be a promising anti-DR candidate agent for its high-efficient glucose/lipid-lowering effect and anti-angiogenesis activity.

4. Experimental

4.1. Animals and treatments

Mice were purchased from Jiangsu GemPharmatech Co., Ltd. (Nanjing, China, SCXK-2018-008). Berberine, HPLC≥98%, was purchased from Desite Biotech Company (Chengdu, China, lot No. 633-65-8). Eight male C57BL/6 mice were used as the control animals. After one week adaption, the mice in the metformin and berberine groups were administrated intragastrically with 200 mg/kg metformin, and 200 mg/kg berberine (dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na) solution), respectively, for the consecutive ten weeks. The control and model groups were treated with an equal volume of 0.5% CMC-Na 10 ml/kg. At the end of the experiment, all the mice were fasted for 10 h and anesthetized intraperitoneally by 350 mg/kg 10% chloral hydrate solution. Blood samples were collected *via* eye venous plexus and then the mice were sacrificed by bleeding to death. Left eyes of the mice were removed carefully and quickly fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4) and right eyes were collected as retina samples for further research.

All the animals were acclimated under standard laboratory conditions (ventilated room, 25±1 °C, 60±5 % humidity, 12 h light/dark cycle) and had free access to standard water and food (SYXK-2013-100). All procedures were conducted in accordance with the “Guiding Principles in the Care and Use of Animals” (China) and were approved by the Laboratory Animal Ethics Committee of Sichuan Academy of Chinese Medicine Sciences (P20190515-2).

4.2. Measurement of blood glucose, lipids and biomarkers of hepatotoxicity

The fasting blood glucose (FBG) was monitored every two weeks with a glucometer (Roche, Accu-Chek). The blood samples from eye venous plexus were centrifuged at 3000 r/min at 4 °C for 15 min, and then the serum was separated. The level of fasting glucose, total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were measured by the automatic biochemical analyzer (HITACHI 7020).

4.3. Haematoxylin and eosin (HE) staining of retinas

HE staining of the retinas was performed as described by the manufacturer’s protocols. Briefly, after deparaffinage, sections were incubated for 10 min with hematoxylin solution, rinsed and then incubated in HE (Solarbio, HE staining kit) solution for 20 min. The sections were washed with distilled water, dehydrated through an ethanol series, cleared with xylene, and coverslipped with permount™ mounting medium. Histomorphology of the retinas was evaluated by a light microscopy (OLYMPUS, CX21FS1) and the photos was processed using Leica image analysis system (LEICA DM1000).

4.4. Periodic acid-Schiff (PAS) staining of retinas

According to the manufacturer’s protocols, PAS staining of retinas was performed as following. Briefly, after deparaffinage, sections were incubated for 15 min with periodic acid solution, rinsed twice and then incubated darkly in Schiff solution for 30 min and rinsed with running water. After that, the sections were treated with haematoxylin solution for 2 min, and then washed with tap water, dehydrated and photographed just as the procedure described above.

4.5. Determination of IL-1β and TNF-α by ELISA

Under a microscope, the cornea was cut circle-wise along the dentate line, and the eye lens and vitreous body were removed carefully to obtain the retina samples. Retinal tissue homogenate was prepared in pre-cooled PBS (1 mg tissue: 9 ml PBS) by a Homogenizer (Bertin, Precellys 24). The supernatant of the retinal homogenate was separated after centrifugation at 3000 r/min, at 4 °C, for 15 min, and was used for the measurement of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels. A standard protocol was applied to perform the IL-1β and TNF-α assays by enzyme linked immunosorbent assay (ELISA) kits (Liankebio, EK201BHS-96, EK282HS-96) according to the manufacturer’s instruction. The optical density (OD) value was read with a micro-plate reader (Thermo, Varioskan Flash) at 450 nm.

4.6. Immunofluorescence assay for VEGF, VEGFR2, HIF-1α and NF-κB

To perform the protein expression analyses, the immunofluorescence assay for the eye retinas was applied. According to the manufacturer, the sections of the eye were deparaffined, antigen retrieved, spontaneously quenched by the fluorescence quencher, and then blocked with bovine serum albumin (BSA) solution for 30 min. Subsequently, these sections were incubated with VEGF (1:200, Abcam, ab39250), VEGFR2 (1:200, Proteintech, 26415-1-AP), HIF-1α (1:200, Abcam, ab16066) or NF-κB p65 (1:500, Servicebio, GB11142) protein primary antibodies overnight at 4 °C, followed by the corresponding fluorescent secondary antibody (1:5000, Abcam, ab6939) for 50 min at room temperature in the dark. Nuclei were stained with 4,6-diamidino-2-phenyindole, dihydrochloride (DAPI) solution (Abcam, ab104139) for 10 min, and then examined under a fluorescence microscope (NIKON ECLIPSE C1) and image analysis system (NIKON DS-U3) (Blue florescence labeled by DAPI, red florescence labeled by CY3).

4.7. Statistical analysis

All the values were expressed as mean±standard deviation. The differences between every group were compared with one-way ANOVA followed by Student’s t test using SPSS version 19.0 software. The value of significance was set at $P < 0.05$.

Ethics approval and consent to participate: All procedures were conducted in accordance with “Guiding Principles in the Care and Use of Animals” (China) and were approved by the Laboratory Animal Ethics Committee of Sichuan Academy of Chinese Medicine Sciences (P20190515-2).

Authors’ Contributions: Zhujun Yin and Ruirong Tan designed the study. Zhujun Yin, Li Li and Tianmin Yuan conducted the experiment. Zhujun Yin, Li Li, Shilong Chen, Yunyun Quan, Qing Hao and Jin Zeng contributed to initial data analysis and interpretation and drafted the initial manuscript. Li Li and Junning Zhao supervised all aspects of the study, critically reviewed and revised the manuscript, and approved the final manuscript as submitted.

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