• Basic Research •

Inhibition of β-elemene on the expressions of HIF-lα, VEGF and iNOS in diabetic rats model

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Abstract

• AIM: To evaluate the effect of β-elemene on the expressions of hypoxia-inducible factor (HIF)-lα, vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) in a streptozotocin (STZ) induced diabetic Sprague-Dawley (SD) rat model.

• METHODS: SD rats were administered an abdominal injection of STZ and induced to a diabetic model. After 6wk course of diabetes, the treatment groups were given β-elemene through periocular and intravitreous injection separately and the control groups were given blank emulsion injection. HE staining was used to observe the morphology of retina. The mRNA expressions of HIF-1α, VEGF and iNOS was assayed by real-time polymerase chain reaction (PCR) and the protein expression was measured by Western blot and immunocytochemistry methods.

• RESULTS: The results indicated that the protein and mRNA expressions of HIF-1α, VEGF and iNOS after treated by β-elemene periocularly and intravitreally injections were all found to be reduced compared with the levels in the diabetic rats group (*P*<0.05). The inhibitory effect of intravitreal injection was more remarkable.

• CONCLUSION: The results show β-elemene protect the retina of diabetic rats from high glucose damage by downregulating the expression of HIF-1α, VEGF and iNOS.

• KEYWORDS: β-elemene; hypoxia-inducible factor-1α; vascular endothelial growth factor; inducible nitric oxide synthase; diabetic rat

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INTRODUCTION

Diabetic retinopathy (DR) is one of the most common and severe microvascular complications of diabetes. Its main causes of blindness are repeated vetreous hemorrhage and tractive retinal detachment. Recently, researches of DR become a hot spot of global public healthy research[1]. The most important procedure of DR is hyperglycemia state, resulting in hypoxia and a series of pathological changes. A number of studies have shown that hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) play an important part in the pathogenic process of DR[2-5].

Elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane) is extracted from the traditional Chinese medicinal herb Rhizoma zedoariae. It is studied *in vivo* and *in vitro* as an anticancer agent with gratifying results[6]. Elemene agent extract is a mixture of three isoforms: α, β, and δ[7]. Among the three isoforms, β-elemene is the active component of elemene and accounting for 60%-72% of the total extract, has been reported to be useful against amount types of cancers for instance in lung, gastric, and glioblastoma cancers[6-7]. The possible mechanisms of β-elemene have been reported including the following aspects: inhibition of tumor cell proliferation, induction of tumor cell apoptosis, cell cycle arrest, and antiangiogenesis[8-10]. However, new research revealed inhibiting the expression of VEGF becomes an important procedure. But, the direct targets and signal transduction pathways and the specificity of β-elemene were all unknown, so we need further detailed studies. Our research group had already proved that β-elemene can hold back the occurrence and development of retinal neovascularization, meanwhile, the effect of intravitreous injection was better than periocular injection[11]. As far as we know, no previous researches have studied whether β-elemene inhibits the expression of HIF-1α, VEGF and iNOS *in vivo*. So, our study focused on finding the role of β-elemene in a diabetic Sprague-Dawley (SD) rat model, and detecting its effect on the expression of HIF-1α, VEGF and iNOS of the retina. This effect may be a potential therapeutic target for patients with DR.

MATERIALS AND METHODS

Ethical Approval All animals were treated according to the statement of the Society for Vision and Ophthalmology on

the use of animals for ophthalmic and visual research. The experiment was approved by the Animal Ethics Committee of China Medical University and strictly abide by the National Institutes of Health guidelines for the care and use of laboratory animals.

Chemicals and Reagents β-elemene (10 mg/mL) and blank emulsion were provided by Yuan da Pharmaceuticals (Dalian, China). Antibodies against HIF-1α and VEGF were purchased from Abcam Biotechnology (Cambridge, London, UK). β-actin, antibody against iNOS, horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from SantaCruz Biotechnology (Dallas, Texas, USA).

Animals Male SD rats (10 weeks old, *n*=100) were purchased from the Beijing Vital River Animal Resources, each weighing 198±21 g. The rats were housed at a temperature of 24℃±1℃ and relative humidity of 40%-50% in a clean enviroment under 12:12 light and dark cycle. No eye disease was found in these animals by slit lamp inspection or indirect ophthalmoscopy.

Animal Model The rats were randomly divided into 6 groups: A) nomal control group; B) diabetic rats group; C) periocular compare group; D) periocular treat group; E) intraocular compare group; F) intraocular treat group, fifteen rats per group. The rats were fasted overnight (12h) and not limited to drinking water. Of 1% streptozotocin in freshly prepared sodium citrate buffer (pH 4.5) was injected into the peritoneal cavity once in a dose of 60 mg/kg to induce diabetes (groups B-F). Rats in group A were injected with the same volume of sodium citrate buffer. Diabetes was confirmed after 72h of streptozotocin injection and again on weekly basis during the experiment. Only the rats with glucose levels higher than 16.67 mmol/L were confirmed diabetic.

Therapeutic Agents and Treatment Schedule At the time point of six weeks duration of diabetes, periocular injections were performed with a 5 μL syringe (Hamilton, Reno, NV, USA) and a 33-gauge needle. Both eyes of the rats were injected periocularly with 5 μL of blank emulsion and 5 μL of β-elemene in group C and group D, respectively. Intravitreal injections were performed just posterior to the pars plana with a 5 μL syringe and a 33-gauge needle. Both eyes of the rats were injected into the vitreous at the pars plana with 5 μL of blank emulsion and 5 μL of β-elemene in group E and group F, respectively. The injections were repeated once every another day, three times a course of treatment. Rats with any kind of postoperative complication (*e.g.* cataract) were excluded from analysis. The rats were executed and the eyeballs were then fixed, embedded, cut into sections and stained with hematoxylin and eosin (HE), and the pathological morphology of retina was observed. The expression of HIF-1α, VEGF and iNOS protein and mRNA was determined using immunohistochemistry, Western blot technique and real-time polymerase chain reaction (PCR) technique.

Table1 The sequences of HIF-1α, VEGF and iNOS primers

Gene name	Sequence (5'-3')	Size
VEGF F	CCCGACAGGGAAGACAAT	131
VEGF R	TCTGGAAGTGAGCCAACG	
HIF-1α F	CCTACTATGTCGCTTTCTTGG	198
HIF-1α R	GTTTCTGCTGCCTTGTATGG	
iNOS F	CACCTTGGAGTTCACCCAGT	135
iNOS R	ACCACTCGTACTTGGGATGC	
β-actin F	GGAGATTACTGCCCTGGCTCCTAGC	155
β-actin R	GGCCGGACTCATCGTACTCCTGCTT	

Real-time Polymerase Chain Reaction The retina was removed from the rats and total RNA was extracted with Trizol (Invitrogen Inc. AQ4) as described by the manufacturer. Total RNA (1 μg) was reverse transcribed using reverse transcriptase (Superscript II; Invitrogen-Gibco, USA) and oligo-dT primers according to the manufacturer's instructions. Rat HIF-1α, VEGF and iNOS primers are listed in Table 1. PCR reactions of HIF-1α, VEGF, iNOS and β-actin genes. AQ5 was performed in a total volume of 20 μL under the same conditions using a SYBR Green PCR Core Kit (Applied Biosystems, Foster City, California, USA) according to the supplier's instructions and an ABI 7900HT (Applied Biosystems) real-time PCR instrument. The expression levels of HIF-1α, VEGF and iNOS were corrected by the expression level of β-actin as an endogenous control. The cycling conditions were 95℃ for 10min (AmpliTaq Gold), 40 cycles of 95℃ for 10s (denaturation) and 60℃ for 20s, 72℃ for 30s (annealing and extension). The CT values of the samples were calculated and the relative levels of HIF-1α, VEGF and iNOS mRNA were determined using the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicate.

Immunohistochemistry Immunohistochemical staining was performed as described previously. Briefly, the retinas were sectioned using a microtome, each having a thickness of 3 μm; dewaxed with xylene, rehydrated; and subjected to immunohistochemical staining. The activity of endogenous peroxidase is quenched by the application of hydrogen peroxide and then subjected to antigen retrieval. Slides were incubated with the appropriate primary antibody (*i.e.*, antibodies specific for HIF-1α, VEGF and iNOS) overnight at 4℃. The next day, tissue sections were incubated for 15min at room temperature in the corresponding secondary antibodies. Immunological tissue activity was visualized using diaminobenzidine and photographed by light microscopy at the indicated magnification. The primary antibody was replaced with PBS as a negative control. The final result was photographed under a microscope at a magnification of 400×.

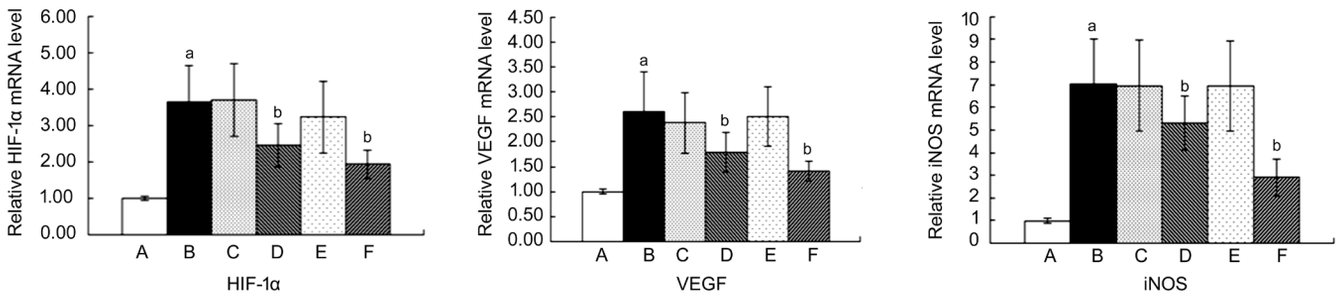

Figure 1 Inhibitory effect of β-elemene on the mRNA levels of HIF-1α, VEGF and iNOS in diabetic rats A: Normal control group; B: Diabetic rats group; C: Periocular compare group; D: Periocular treat group; E: Intraocular compare group; F: Intraocular treat group. [a]P<0.05 *vs* control group; [b]P<0.05 *vs* diabetic rats group.

Western Blot Western blot assays were performed using conventional methods. Briefly, the procedure was as follows. The retina was taken out from the rat and protein was extracted using radioactive immunoprecipitation buffer (China University of Biotechnology). Protein concentration was measured using the BCA assay. Total protein (70 μg) was separated on a 12% SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane (0.45 μm; Amersham, AQ6, USA), and the primary antibody against rabbit HIF-1α was probed with a probe. Probing (1:2000 dilution), VEGF (1:2000 dilution) and iNOS (1:1000 dilution) were carried out overnight at 4°C. The next day, the protein bands were washed 3 times with TBS+Tween (TBST) for 10min each and then incubated with goat anti-rabbit HRP secondary antibody (dilution 1:10 000) for 15min at room temperature. Clean the strips in the same way. Protein bands were detected using the Millipore ECL (Electro-Chemi-Luminescence) system and scanned using ImageJ2x software. Experiments were performed in triplicate.

Statistical Analysis All statistical analyses were performed using SPSS 17.0 software. First, the software is used to test the homogeneity of the variance test. Then, each group of values was evaluated by one-way analysis of variance. Differences in P values less than 0.05 were considered statistically significant.

RESULTS

Inhibitory Effects of β-elemene on the mRNA Expressions of HIF-1α, VEGF and iNOS HIF-1α, DR, VEGF and iNOS are known to play an important role in diabetes retinopathy. To investigate the effect of β-elemene on the expression of HIF-1α, VEGF and iNOS mRNA, real-time PCR analysis was performed. The primers used are shown in Table 1. The results indicate that the mRNA levels of HIF-1α, VEGF and iNOS in diabetic rats (group B) were obviously elevated compared with the control group (group A; Figure 1, P<0.05 *vs* control group), meanwhile, β-elemene significantly inhibited the mRNA levels of HIF-1α, VEGF and iNOS compared with the group B (Figure 1, P<0.05 *vs* diabetic rats group). These results indicate that β-elemene can protect the retina from damage under high glucose by down-regulating HIF-1α, VEGF and iNOS at the mRNA level.

Inhibitory Effects of β-elemene on the Protein Expression of HIF-1α, VEGF and iNOS To further investigate the relationship between β-elemene and HIF-1α, VEGF and iNOS, immunocytochemistry and Western blot analyses were used to detect the protein expression levels of HIF-1α, VEGF and iNOS. Using immunocytochemistry, observe the locations of the expressions of HIF-1α, VEGF and iNOS and the differences. The protein expressions of HIF-1α, VEGF and iNOS were all found to be reduced compared with the levels in the diabetic rats group (Figure 2; P<0.05). The results from Western blot analysis were consistent with the immunocytochemistry results.

Immunocytochemistry results Group A, less HIF-1α positive expression located in ganglial cell layer, outer plexiform layer; Group B, positive expression mostly located in ganglial cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and layer of rods and cones. Groups D, F, positive expression was less than group B, especially in group F. Groups C, E have no obvious differences between group B.

Group A, weak VEGF positive expression observed; Group B, positive expression located in inner layers of retina. Groups D, F, positive expression was less than group B, especially in group F. Groups C, E have no obvious differences between group B.

Group A, no iNOS positive expression observed; Group B, positive expression mostly located in ganglial cell layer and layer of rods and cones. Groups D, F, positive expression was less than group B, especially in group F. Groups C, E have no obvious differences between group B.

Western blot results The protein expressions of HIF-1α, VEGF and iNOS after treated by β-elemene periocularly and intravitreally injections were all found to be reduced compared with the levels in the diabetic rats group (Figure 3; P<0.05). The inhibitory effect of intravitreal injection was more remarkable.

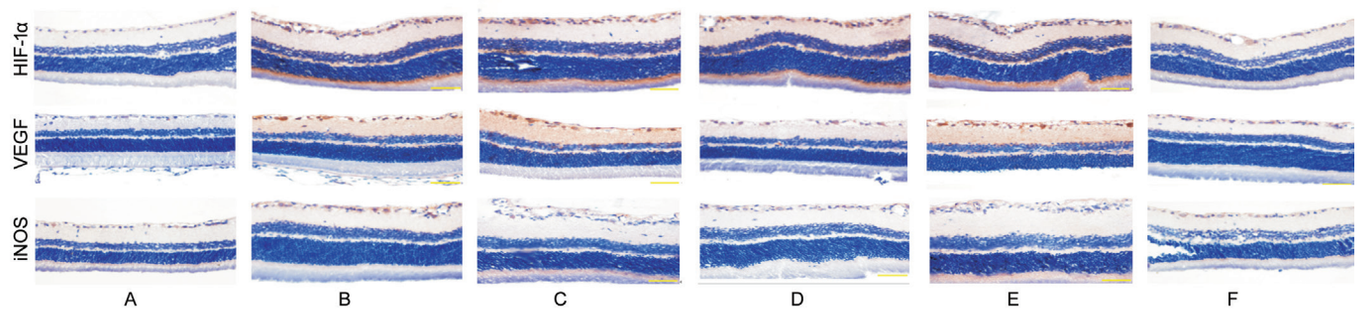

Figure 2 Expression of HIF-1α, VEGF and iNOS protein of the retina of rats examined by immunocyto-chemistry method (400×)
A: Normal control group; B: Diabetic rats group; C: Periocular compare group; D: Periocular treat group; E: Intraocular compare group; F: Intraocular treat group.

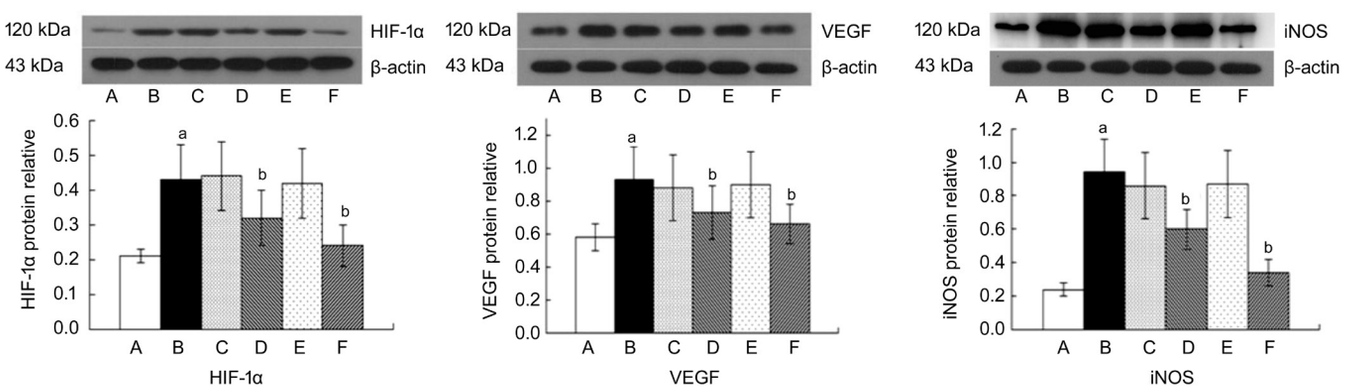

Figure 3 The expression of HIF-1α, VEGF and iNOS detected by Western blot analysis A: Normal control group; B: Diabetic rats group; C: Periocular compare group; D: Periocular treat group; E: Intraocular compare group; F: Intraocular treat group. [a]$P<0.05$ *vs* control group; [b]$P<0.05$ *vs* diabetic rats group.`

The results showed that the expression of HIF-1α, VEGF and iNOS in diabetic rats were significantly elevated comparing with the control group ($P<0.05$ *vs* control group). β-elemene could inhibit the expression of HIF-1α, VEGF and iNOS both by periocular injection and intraocular injection ($P<0.05$ *vs* diabetic rats group), while the effect was more obvious by intraocular injection.

DISCUSSION

In normal eye tissues, the stability of angiogenesis is controlled by the balance of the stimulus and inhibitory substances. Mostly, the inhibitory factors are dominant and it keeps the vessels quiet in normal tissues. VEGF is found to be the strongest angiogenesis promoting factor[12]. The expression of VEGF is increased in the visual cells of diabetic patients, and the concentration of VEGF is elevated in the aqueous humor and vitreous of the patients with DR.

Recent years, studies found that in the VEGF signal regulating pathway of hypoxia, HIF-l plays a role of central link. And its function concludes not only increasing VEGF mRNA stability and increasing the transcription activity of VEGF. A large number of animal experiments confirmed that HIF-lα played an important role in the occurrence of DR. HIF-lα is the functional subunit of HIF-1. Under the condition of hypoxia, HIF-lα is activated, combined with a common base sequence (5'-RCGTG-3') of a variety of target genes and then activates the transcription activity of target genes. It corresponds to the formation of the transcription initiation complexes which can activate the transcription and expression of related hypoxia-response genes. These main hypoxia response genes encode the following products: PDGF, TGF, EGF, EGFR, VEGF, COX-2, ET-l, iNOS, IGF-2, GAPDH, EPO, *etc*. The function exertion of HIF-1 is a multi-step process, including protein expression, polymerization, combining with the DNA target sequence and transcription activation, *etc*[13]. Several studies showed that in the early stage of DR, excessive nitric oxide (NO) was formed and microvascular lesions were caused by its cytotoxic effect[14]. The generation of NO and iNOS are closely related. Several studies revealed β-elemene may have strong inhibitory effect on inflammatory response progression[15-20]. It can also inhibit VEGF expression in several diseases[21-22]. Several studies have confirmed that in the process of DR development, HIF-1α, VEGF, iNOS play important role in different stages[23-25]. One new study demonstrated that β-elemene reduced retinal neovascularization in mouse oxygen-induced retinopathy models *via* microRNA-27a upregulation, leading to reduced VEGF expression[22]. HIF-1α is a crucial factor

regulating oxygen metabolism under anaerobic conditions of the cells [26-28]. At all stages of diabetic course of rats, the expression of HIF-lα, VEGF and iNOS were increased[29]. Therefore, the elevated expression of HIF-lα, VEGF and iNOS is an important step in the occurrence and development of DR.

In our experiment, we established a diabetic rats model, took a β-elemene injection in two ways: periocular injection and intravitreous injection. According to the results, we got fine effects in both two ways of injection by paying precise attention to the microscope operations. β-elemene is a kind of emulsion. In order to observe the effect of the emulsion on the retina, we set a blank emulsion control in our experiment. We proved that emulsion had no toxicity response on the retina, and it was safe to be used in diabetic rats. And we also compared the differences between two ways of injection. Studies have revealed that at the time point of one month of diabetic course, the HE staining of retina had no obvious changes, but at the time point of three months of diabetic course, the ganglial cell layer had obvious changes: the number of cells decreased with loosely arranged positions and fuzzy boundaries. At the time point of 2wk of diabetic rats course, HIF-1α, VEGF mRNA and protein expression was elevated, and at 8 to 10wk, HIF-1α expression reached peak, while at 12wk the expression began to decline. The VEGF expression was continued rising[29]. There is no research to observe the retinal form of diabetic rats for the duration of 6wk. Due to diabetic rats course of late mortality rate is high, the cost is too high, and all the comprehensive factors, this study adopted a diabetic rats for six weeks of disease course, and then we conducted local injections of β-elemene and blank emulsion.

In this study, HE staining in diabetic rats for the duration of 6wk revealed that in diabetic rats group, retinal cells appeared arranging disorder, fuzzy boundaries, and sparse nucleus could be observed sometimes, nucleus dyeing quality appeared fuzzy, and in periocular and intraocular treat groups, retinal cells were arrayed better than diabetic rats group, while in periocular and intraocular compare groups, we did not see any improvement. Therefore, we deduced β-elemene had non-toxic reactions to the retina of diabetic rats at 6wk of diabetic course, and the morphology of the retina could be improved to a certain extent. Immunocytochemistry results showed in diabetic rats without β-elemene injection, positive expression of HIF-1α mostly located in ganglial cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and layer of rods and cones while positive expression of VEGF located in inner layers of retina and also positive expression of iNOS mostly located in ganglial cell layer and layer of rods and cones. After injection, the expression of HIF-1α, VEGF, iNOS in the retina of diabetic rat had dropped especially in the group of intraocular injection. So whether the protective effects of β-elemene to the retina was related to the inhibition of HIF-1α, VEGF, iNOS expression? We detected the expression of HIF-1α, VEGF, iNOS in the retina of diabetic rats using real-time PCR method and Western Blot method respectively from the mRNA and protein levels. The results confirmed that β-elemene can inhibit the expression of HIF-1α, VEGF and iNOS in early diabetic rats course, and the effect of intravitreous injection is much better than periocular injection. But the definite mechanism is unknown, so we need a forward mechanism research.

In summary, this study proves that injection of β-elemene periocularly and intraocularly can protect the retinal form and can inhibit the expression of HIF-1α, VEGF and iNOS of diabetic rats in the course of 6wk. And the effect of intraocular injection is better *in vitro*. In the future work, we will focus on whether this downregulation effect of HIF-1α, VEGF and iNOS expression occurs *in vivo*, as well as the probable signaling pathway involved.

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Conflicts of Interest: Zhou Y, None; **Liu Y,** None; **Chen J,** None; **Sun YZ,** None; **Li LH,** None; **Chen L,** None.

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