Histol Histopathol (2022) 37: 757-767

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE **Open Access**

Effect of diabetes blood-stasis syndrome and Xuefu Zhuyu decoction on ERK1/2-VEGF signal pathway in rat retina Müller cells

Xiaofeng Ye[1], Hui Ren[1], Tingting Jiang[1], Ting Zhang[1] and Gang Li[2]

[1]Eye Institute and [2]Laboratory of Center, EENT Hospital, Fudan University, Shanghai, China

Summary. Aims. This research was aimed to investigate whether diabetic blood-stasis syndrome had a relationship with ERK1/2-VEGF signaling pathway in rat retina Müller cells and Traditional Chinese Drugs designed for promoting blood circulation to remove blood stasis had effectiveness for diabetic retinopathy (DR) treatment.

Methods. Immunofluorescence was applied to determine purity of Müller cells. Müller cells were stimulated by blood serum obtained from rats with blood-stasis syndrome and then treated by Xuefu Zhuyu decoction. Western blot analysis, RT-PCR and ELISA were used to measure the expression of VEGF. Western blot analysis was used to determine the phosphorylation of ERK1/2. The status of AP-1 DNA binding activity was monitored by electrophoretic mobility shift assay (EMSA).

Results. Stimulation of Müller cells by blood serum of rat with diabetic blood stasis increased the secretion of VEGF, activated ERK1/2 and AP-1 DNA-binding activity. And treatment of Xuefu Zhuyu decoction could weaken this phenomenon. What's more, ERK1/2 signaling pathway inhibitor U0126 also could inhibit the expression of VEGF.

Conclusions. Diabetic blood-stasis syndrome in theory of traditional Chinese Medicine has positive role in regulating ERK1/2-VEGF signaling pathway. Traditional Chinese drugs for promoting blood circulation to remove blood stasis would be an effective therapy to treat DR.

Key words: Diabetic retinopathy, Blood-stasis syndrome, VEGF, ERK1/2 signaling pathway, Müller cells

Corresponding Author: Xiaofeng Ye, Eye Institute, EENT Hospital, Fudan University, No. 83 Fengyang Road, Shanghai 200031, China. e-mail: yxf_kxy@126.com

DOI: 10.14670/HH-18-427

Introduction

Diabetic retinopathy (DR) has been identified as one of the most serious eye diseases that may cause blindness (Fong et al., 2003; Solomon et al., 2017). Traditional therapies have no distinct effectiveness to improve DR patients' vision because of various imperfections (Hernández et al., 2017; Stewart, 2017). Recently, clinical observation turned out that the development and progression of DR have close relationship with blood-stasis syndrome which is a diagnosis that indicates a very strong sense of traditional Chinese medicine. Consistently, traditional Chinese drugs for promoting blood circulation to remove blood stasis exhibited therapeutic effect for DR to some extent (Yin et al., 2009; Yue et al., 2017). Blood-stasis syndrome is usually related to syndromes of unsmooth blood circulation and subsequent dissipation of bleeding which were caused by deficiency and circulation stagnation of vital energy, congealing cold and blood-heat (Chen et al., 2016; Liao et al., 2016; Xu and Chen, 2017). Therefore, it is very important to explore the effect and corresponding molecular mechanisms of blood-stasis syndrome on DR, which can reveal specific clinical significance of this abstract concept, thus deepening the understanding of blood-stasis syndrome in theory of traditional Chinese Medicine and providing theoretical foundation for the pharmaceutical effect against DR of traditional Chinese drugs designed for promoting blood circulation to remove blood stasis.

VEGF has been recognized as a therapeutic target of DR (Li et al., 2017; Lu et al., 2017) and our previous research focused on the relationship between vascular endothelial growth factor (VEGF) and DR, but the relationship and mechanism between the blood-stasis syndrome and VEGF is largely unknown (Ye et al., 2010). Some studies reported that the traditional Chinese drugs for promoting blood circulation to remove blood stasis could decrease the over-expression of VEGF in eyes and inhibit the development and progression of DR (Yin et al., 2009; Xu and Chen, 2017). For example, it has been reported that Fu Fang Xue Shuan Tong capsule combined

with Calcium Dobesilate can attenuate elevated levels of oxidative stress in the retina and reduce the recurrence rate, reduce the levels of hs-CRP, VEGF and Insulin-like Growth Factor-1 (IGF-1), thus delaying the proliferation of blood vessels in the patients with DR (Fang et al., 2012). Moreover, Gegen Qinlian Decoction plus Salvia miltiorrhiza displayed preventive and therapeutic effect for DR and could increase perivascular cells, reduce thickness of basement membrane of blood vessel and stop blood vessel obstruction (Yang and Wang, 2011; Rui and Heng, 2015). These results suggested that the traditional Chinese drugs for promoting blood circulation to remove blood stasis may alleviate blood-stasis syndrome thus curing DR through down-regulation of VEGF which was closely related to DR.

It has been demonstrated that several cellular signaling pathways participated in regulation of the releasing process of VEGF in DR (Qin et al., 2016). However, plentiful researches showed that VEGF has the closest relationship with extracellular signal-regulated kinase (ERK)1/2 signaling pathway and the activation of its downstream substrate, activator protein-1 (AP-1) (Janknecht et al., 1993; Karin, 1995), is also involved in the abnormal expression of VEGF (Ye et al., 2012). Our previous research also proved that ERK1/2 signaling pathway mediated the over-expression of VEGF in DR and regulated the upstream of VEGF in Müller cells which were in simulative diabetes environment (Ye et al., 2010). Therefore, we were wondering whether the regulation of ERK1/2 signaling pathway is also the underlying mechanism of the modulation effect of DR by blood-stasis syndrome.

In this study, we chose the proper macroglia cells in the retina of rats, Müller cells, as the cell research model to study the effect and mechanism of diabetic blood-stasis syndrome injury (Reichenbach et al., 1995; Lorenzi and Gerhardinger 2001). This research model was established based on normal Müller cells through the stimulation by the serum of rats with induced diabetic blood-stasis syndrome. This method has been considered as an important and credible method for blood-stasis syndrome research in traditional Chinese Medicine. This work aimed to investigate the effect of diabetic blood-stasis syndrome on expression of VEGF and activation status of ERK1/2 signaling pathway in Müller cells. Moreover, the effect of Xuefu Zhuyu decoction, which is capable of promoting blood circulation and removing blood stasis, on expression of VEGF and activation status of ERK1/2 signaling pathway. It would provide experimental evidence for treating DR by Traditional Chinese drugs through promoting blood circulation to remove blood stasis in molecular level, and developing effective therapeutic treat DR in clinic.

Materials and methods

Materials

One hundred male Sprague-Dawley (SD) rats weighing 250±20 g were purchased from Shanghai Jiesijie Experimental Animals Co., Ltd (Shanghai, China). Streptozotocin and U0126 were obtained from Selleck Chemicals (Houston, Texas, TX, USA). The SYBR® Fast qPCR Mix Kit was purchased from Takara Biotechnology, Co., Ltd (Dalian, China). The ELISA kit was purchased from WuHan Boster Bio-Engineering Co., Ltd (Wuhan, China). The medicinal materials of Xuefu Zhuyu decoction were acquired from Shenzhen Resources Sanjiu Modern Chinese Medicine Co., Ltd (Shenzhen, Guangdong, China). The primary antibodies used in the western blotting such as anti-ERK1/2 monoclonal antibodies (mAb), anti-p-ERK1/2 mAb, anti-VEGF mAb and anti-GAPDH mAb were purchased from Cell Signaling Technology (Danvers, Massachusetts, MA, USA), as well as the secondary antibodies conjugated with HRP.

Experimental diabetes induction and screening for blood-stasis syndrome rats

This study was approved by the Ethic Committee of EYE & ENT Hospital and Eye Institute of Fudan University. One hundred male Sprague-Dawley (SD) rats weighing 250±20 g were assigned randomly to diabetes group or non-diabetes group (normal group), among them 10 normal rats as control in the normal group. Diabetic rat models (DM group) were established through a single injection of streptozotocin (60 mg/kg body weight) into the abdominal cavity, while citrate buffer (6 ml/kg) was injected into normal rats using the same method. Three days after injection, the rats with blood glucose levels above 16.7 mM were considered as diabetic models induced successfully. Twelve weeks after establishment, the diabetic rats with characterizations including obscure fur and feather, nigrescent ears and lips, deeply purple onyx and tails and hemangiectasis in cornea and ears were screened as the blood-stasis syndrome rats. Then, these blood-stasis syndrome rats were classified into four intervention groups: (1) low dosage Xuefu Zhuyu decoction group (8 g/10 ml) (low dose group); (2) middle dosage Xuefu Zhuyu decoction group (16 g/10 ml)(middle dose group); (3) high dosage Xuefu Zhuyu decoction group (32 g/10 ml) (high dose group); (4) Placebo group (1 ml/100 g of distilled water). Xuefu Zhuyu decoction and distilled water were lavaged to the blood-stasis syndrome rats. The recipe of Xuefu Zhuyu decoction was demonstrated as follow: 12 g of peach seed, 9 g of flos carthami, 9 g of angelica sinensis, 9 g of radix rehmanniae recen, 5 g of rhizoma chuanxiong, 6 g of red peony root, 9 g of achyranthes bidentate, 3 g of bupleurum, 5 g of radix platycodi, 6 g of fructus aurantii and 3 g of radix glycyrrhizae. These medicinal materials were steeped, boiled and concentrated into liquid pharmaceuticals of 2 g/ml, and then stored at 4°C after sterilization.

After observation for 4 weeks, the diabetic blood-stasis syndrome rats were sacrificed through anesthesia

of chloral hydrate, and the blood sample from the rats of different intervention groups was separated from blood in abdominal aorta and then centrifuged for 15 min at 3000 rpm at 4°C to obtain serum. Next, the serum was inactivated for 30 min at 56°C, filtered with 0.22 μm millipore filter and stored at -80°C.

Hematoxylin-Eosin Staining

After all rats were sacrificed through anesthesia of chloral hydrate, the pancreas of some rats in both diabetes and normal groups were acquired and then they were fixed with 10% paraformaldehyde (PFA). Subsequently, the paraffin-embedded sections were prepared for histological analysis. The slices were cut with 5 μm sections and next stained respectively in dye liquor of hematoxylin and eosin for histological evaluation. Finally, these slices were analyzed under a microscope (Olympus, Tokyo, Japan).

Primary Müller cell culture and treatment with the blood serum of blood-stasis syndrome rats and U0126

Retinas were isolated from the eyeball of new-born SD rats under a biological dissection microscope. Subsequently, the isolated retinas were digested with 1% trypsin for 5 min with phosphate-buffered saline (PBS) washing for 45 min and then the supernatant was removed, and the dissociated retinal cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and 5.5 mM glucose in a humidified atmosphere of 95% air plus 5% CO_2 at 37°C. Seven days later, the aggregates and cellular debris in the medium were dislodged and this promoted to generate a purified flat cell preparation. In the culture process, confluent cultures were passaged 3 times for all the experiments and the medium was replaced at 24 h before the treatment. The purity of these isolated cells was evaluated by immunofluorescence microscopy using glutamine synthetase (GS) antibody (abcam), Vimentin antibody (abcam) and 4', 6-diamidino-2-phenyl-indole (DAPI) staining. The positive cells for GS, Vimentin and DAPI were used three passages and to treat with the blood serum of blood-stasis syndrome rats and U0126.

The blood serum from different groups of rats was acquired as described above and added into DMEM at a concentration of 50 ml/l (5%), which was determined based on a series of pre-experiments. The stimulation time was 48 h. Furthermore, some groups were treated with U0126 (Cell Signaling Technology, Boston, Massachusetts, MA, USA) in dosage of 0.02 mM for 24 h.

Immunofluorescence

To evaluate the purity of the isolated Müller cells and these cells were treated in 30 mM glucose for 24 h. Then, the isolated cells were fixed in 4% paraformaldehyde (PFA) with 0.01 M PBS for 20 min. Subsequently, the Müller cells were permeabilized with 0.3% Triton X-100 for 30 min and 5% goat serum for 45 min, and next incubated with GS and Vimentin antibodies overnight at 4°C (1:200). Afterwards, the Müller cells were incubated in FITC conjugated secondary antibodies for 30 min. Eventually, the nuclei of these Müller cells were stained using DAPI. The final slides were observed under a confocal microscopy (Leica TCS SP2, Germany).

Western blot analysis

Cells were lysed by ice-cold Lysis Buffer, then total proteins were extracted. Proteins were respectively added to gel for electrophoresis, and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% non-fat milk at room temperature for 1 h, primary antibodies were bound overnight at 4°C. In the next day, the PVDF membrane was incubated with corresponding secondary antibodies at room temperature for 2 h. Each membrane was visualized with ECL-plusTM Western blotting system (Amersham, Buckinghamshire, UK), and were detected by X-ray imaging analyzer (Eastern Kodak, Rochester, NY, USA). Membranes were scanned with ImageJ to quantify band intensity.

Quantitative Real-Time PCR

The procedure of qRT-PCR was conducted as the instructions of Takara Biotechnology, Co., Ltd (Dalian, China). Briefly, the total RNA of Müller cells were extracted using a TRIzol® kit (Takara Biotechnology, Co., Ltd., Dalian, China). Then the cDNA Synthesis was similarly performed using a cDNA Synthesis kit according to the protocol of Takara Biotechnology, Co., Ltd. Subsequently, the qRT-PCR was performed using the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, California, CA, USA). Quantification of the gene expressions was done using the $2^{-\Delta\Delta CT}$ method, with β-actin as an endogenous control. The primer sequences used in this study were as listed in Table 1.

ELISA

The ELISA was performed to detect the protein concentration of VEGF and the VEGF concentration in the supernatants of Müller cells was assayed using a VEGF ELISA kit (WuHan Boster Bio-Engineering Co., Ltd., Wuhan, China) following the manufacturer's instructions. The optical density (OD) of each well within 10 min using a Thermo Scientific Varioskan Flash (Waltham, Massachusetts, MA, USA) at 450 nm.

Electrophoretic mobility shift assay

The Müller cells were suspended in 10 mM HEPES

(pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM PMSF. After incubated 15 min at 4°C, the Müller cells were vortexed with 0.5% NP-40. Subsequently, the buffer including 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF was used to extract the nuclear pellet of Müller cells through centrifugation of 15,000 rpm for 15 min at 4°C. Afterwards, the protein concentrations were determined using a TaKaRa BCA Protein Assay Kit (cat. no. T9300A). Next, AP-1 (5'-CGCTTGATGACTC AGCCGGAA-3') oligonucleotide, 10 × buffer of T4 polynucleotide kinase, (γ-32P) ATP, nuclear-free water and T4 polynucleotide kinase were mixed into 10μl of total reaction system and incubated for 30 min at 37°C. 1 μl of EDTA (0.5 M) was added into the total system to stop reaction. After adding 89 μl Tris-EDTA (TE) buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, the unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column. To go ahead, the nuclear extract was added into gel shift binding buffer. After incubation, the mixtures were electrophoresed by 6% PAGE. Eventually, the gels were exposed to an X-ray film. Membranes were scanned with ImageJ to quantify band intensity.

Statistical analysis

All experiments were repeated at least three times in this study. All data are shown as mean ± SD and GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, California, CA, USA) was used for statistical analysis. Differences between two groups were determined with paired or unpaired Student's t-tests. ANOVA was used for multiple comparisons. $P<0.05$ was considered as statistical significant.

Results

Successful establishment of diabetic blood-stasis syndrome rat models

After injection of streptozotocin (STZ), blood glucose and body weight of SD rats were measured to confirm whether diabetic rat models were induced successfully. As shown in Fig. 1, the blood glucose level was strikingly increased in rats of diabetes group compared with normal group even in the first week of treatment ($P<0.01$, $n\geq3$, Fig. 1A). Moreover, the mean body weight of normal group gradually increased while that in diabetes group slightly decreased and was always lower than that in normal group ($P<0.01$, $n\geq3$, Fig. 1B). A portion of the rats in diabetes group presented obvious cataract-related symptoms (Fig. 1C) and classic symptoms of blood-stasis syndrome such as obscure fur and feather, nigrescent ears and lips, deeply purple onyx and tails and hemangiectasis in cornea and ears. Moreover, the blood serums from these rats were feculent (Fig. 1D). These symptoms confirmed a successful screening for blood-stasis syndrome rats and the rest diabetic rats were excluded from this study.

Then we randomly eviscerated pancreas of some rats in both groups and hematoxylin-eosin (H&E) staining was used for evaluation. The results demonstrated that, compared with the normal group, diabetes group exhibited interstitial edema, necrosis, catheter expansion, incomplete cell structure accompanied by a small amount of hemorrhage (Fig. 1D, a vs b, d vs e) suggesting the successful establishment of diabetes rat model again. Noteworthy, only mild oedema of the glandular interstitium, mild fibrosis, mild catheter dilation accompanied by a small amount of hemorrhage could be observed in the Xuefu Zhuyu decoction treatment group (Fig. 1D,c,f), indicating the pharmaceutical effect of Xuefu Zhuyu decoction.

Determination of Müller cells in high purity

Müller cells of the third generation isolated from the retina of new-born SD rats were cultured in good condition (Fig. 2A). Then immunofluorescence using GS, Vimentin antibodies and DAPI staining was adopted to evaluate the purity of these Müller cells. DAPI staining results, as shown in Fig. 2B, demonstrated that the isolated Müller cells had high purity according to the blue fluorescence emitted from majority of the cells. Besides, an immunofluorescence observation with antibodies to GS and Vimentin showed that more than 95% Müller cells exhibited green fluorescence and this demonstrated that Müller cells had strong GS and Vimentin immunoreactivity.

Diabetic blood-stasis syndrome increased the secretion of VEGF in Müller cells

The expression level of VEGF in Müller cells was determined by ELISA and western blot analysis after stimulated by the serum from rats in different groups, followed by the treatment of different dosage of Xuefu Zhuyu decoction in vitro. As shown in Fig. 3, after stimulated by the serum of diabetic blood-stasis rats in placebo group, the protein levels of VEGF in Müller

Table 1. Primers used in this study.

Name	Forward primer	Reverse primer
VEGF	5'-CAATGATGAAGCCCTGGAGT-3'	5'-TTTCTTGCGCTTTCGTTTTT-3'
β-actin	5'-TGACGTGGACATCCGCAAAG -3'	5'-CTGGAAGGTGGACAGCGAGG-3'

cells were extremely increased compared to that stimulated by the serum of rats in normal group ($P<0.001$, $n\geq3$, Fig. 3A,B). On the other hand, compared with the placebo group, the Müller cells stimulated by the serum of diabetic blood-stasis rats treated with different doses of Xuefu Zhuyu decoction exhibited

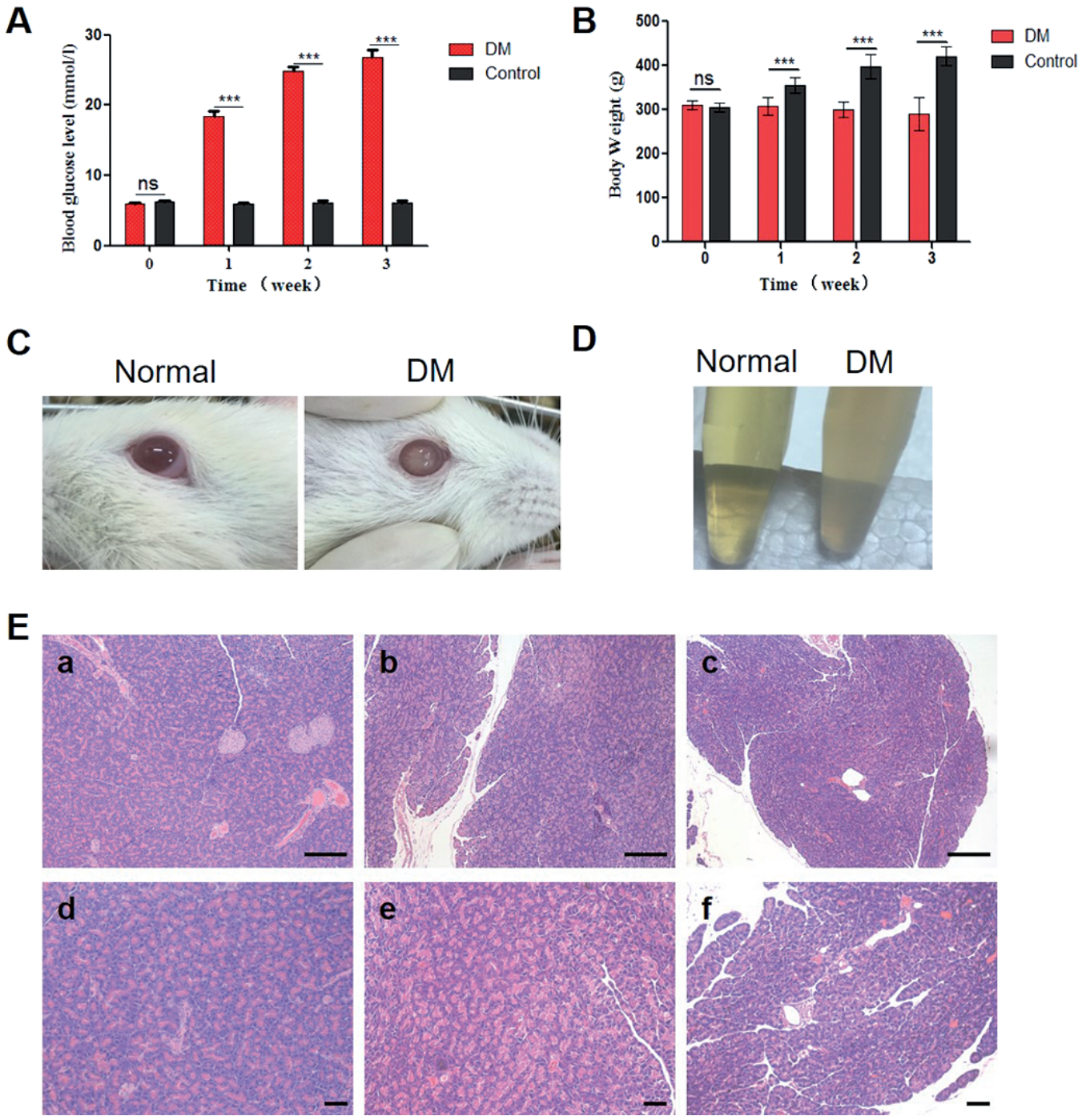

Fig. 1. Results of screening for diabetic blood stasis syndrome rats. After injection of STZ into the abdominal cavity of SD rats, the blood glucose levels **(A)** and body weight **(B)** of all SD rats were measured every week. Then, to screen for diabetic rats with blood-stasis syndrome, the cataract-related symptoms **(C)** and blood serum **(D)** were observed and photographed. **E.** The pancreas of some rats in normal (a&d), diabetes (b&e) and Xuefu Zhuyu decoction treatment (c&f) groups were acquired, the pancreas islets structure was inspected by H&E staining. The data were expressed as mean±SD, P value less than 0.05 was considered statistically significant, 'ns' indicates no significance, *P<0.05, **P<0.01, ***P<0.001. Scale bars: 100 μm.

obviously decreased VEGF output with slight concentration dependent manner. Specifically, in the middle dosage and high dosage Xuefu Zhuyu decoction groups, protein levels of VEGF were nearly equal to the normal serum group.

Otherwise, qRT-PCR results showed that the relative mRNA level of VEGF in Müller cells also increased by stimulation of the serum of diabetic blood-stasis rats in placebo group compared with the normal serum group (Fig. 3C), whereas the low dosage Xuefu Zhuyu decoction groups had an unexpected higher VEGF mRNA expression. Still, the middle and high dosage Xuefu Zhuyu decoction groups declined the VEGF mRNA expression compared with the placebo group ($P<0.001$). Collectively, these results indicated that diabetic blood-stasis syndrome could up-regulate the secretion of VEGF in Müller cells of rat which could be reversed by Xuefu Zhuyu decoction.

Diabetic blood-stasis syndrome activated ERK1/2 signaling pathway in Müller cells

In order to investigate the effect mechanism of blood-stasis syndrome on VEGF expression of Müller cells, the cells were treated with the serum from different groups of normal or blood-stasis syndrome rats for 48 h to determine whether the ERK1/2 signaling pathway was activated by blood-stasis syndrome in vitro. The results in Fig. 4 indicated that the phosphorylation of ERK1/2 in Müller cells was significantly enhanced by stimulation of serum obtained from diabetic blood-stasis rats in placebo group compared with the normal group. Moreover, the treatment of Xuefu Zhuyu decoction could reverse the effect, which is in consistence with the abovementioned results about expression of VEGF. These results demonstrated that diabetic blood-stasis syndrome plays critical role in the activation of ERK1/2 signaling pathway in Müller cells.

Diabetic blood-stasis syndrome increased AP-1 DNA-binding activity in Müller cells

To determine whether AP-1 DNA binding activity of Müller cells was increased by blood-stasis syndrome, the nuclear protein extracts from cultured Müller cells in vitro were analyzed by electrophoretic mobility shift assay (EMAS) after stimulated by the serum from different groups of rats. As shown in Fig. 5, the placebo group showed distinctly increased DNA binding activity of AP-1, visualized as a distinct band of labeling, while the Müller cells stimulated by serum of rats in normal group exhibited nearly no AP-1 DNA binding activity. Moreover, as expected, Xuefu Zhuyu decoction treatment of the serum source rats could inhibit the promotion of AP-1 DNA binding activity by blood-stasis syndrome. Therefore, similar results about the effect of blood-stasis syndrome and Xuefu Zhuyu decoction on Müller cells were also achieved in the respect of AP-1 DNA binding activity.

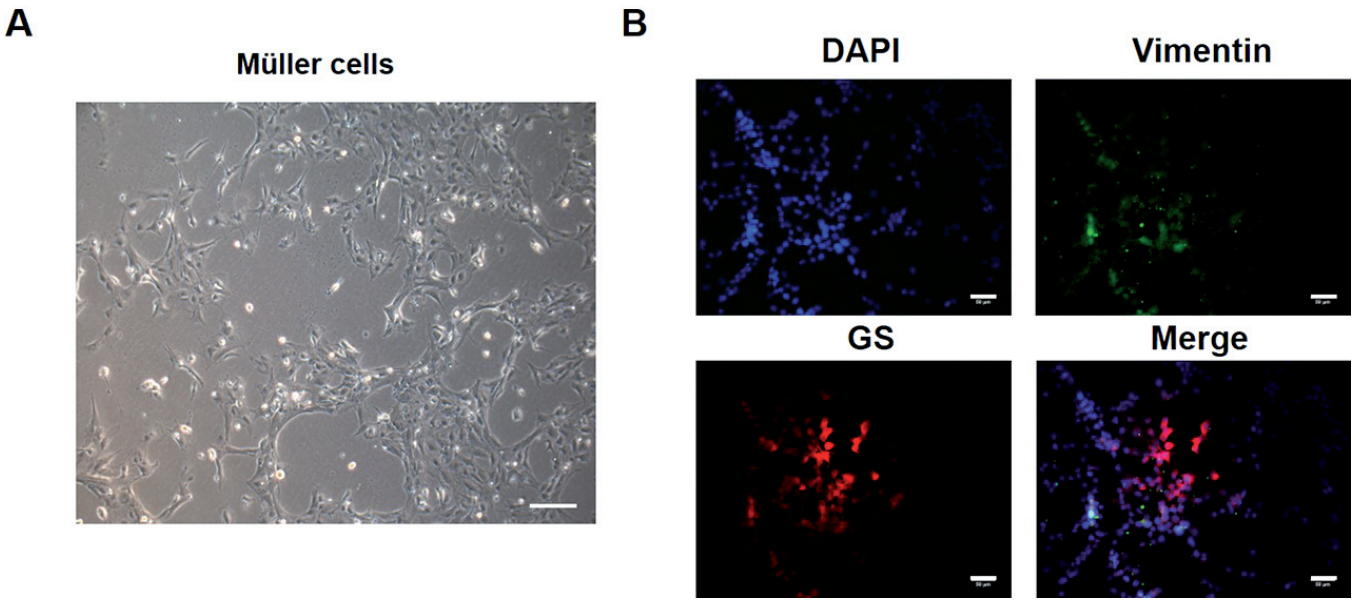

Fig. 2. Results of determining the purity of Müller cells. Morphology of Müller cells **(A)** in good condition was photographed and then the immunofluorescence **(B)** was performed to detect the purity of Müller cells: (DAPI) the DAPI staining for nucleus of Müller cells; (Vimentin) the green fluorescence of Vimentin in Müller cells; (GS) the red fluorescence of GS in Müller cells; (Merge) The merged images of DAPI channel, Vimentin channel and GS channel. Scale bars: 100 μm.

U0126 blocked the increase of VEGF secretion and activation of ERK1/2 signaling pathway in Müller cells

U0126 is an inhibitor of ERK1/2 signaling pathway and could effectively suppress the phosphorylation of ERK1/2. Western blot analysis showed that phosphorylation level of ERK1/2 increased by the serum of blood-stasis syndrome rats was significantly inhibited by U0126 ($P<0.05$, n=3, Fig. 6). In addition, the secretion of VEGF determined by western blot analysis also decreased in U0126 treated groups compared to untreated groups ($P<0.05$, n=3, Fig. 6).

These results showed that the blockage of ERK1/2 signaling pathway with U0126 could abolish the effect

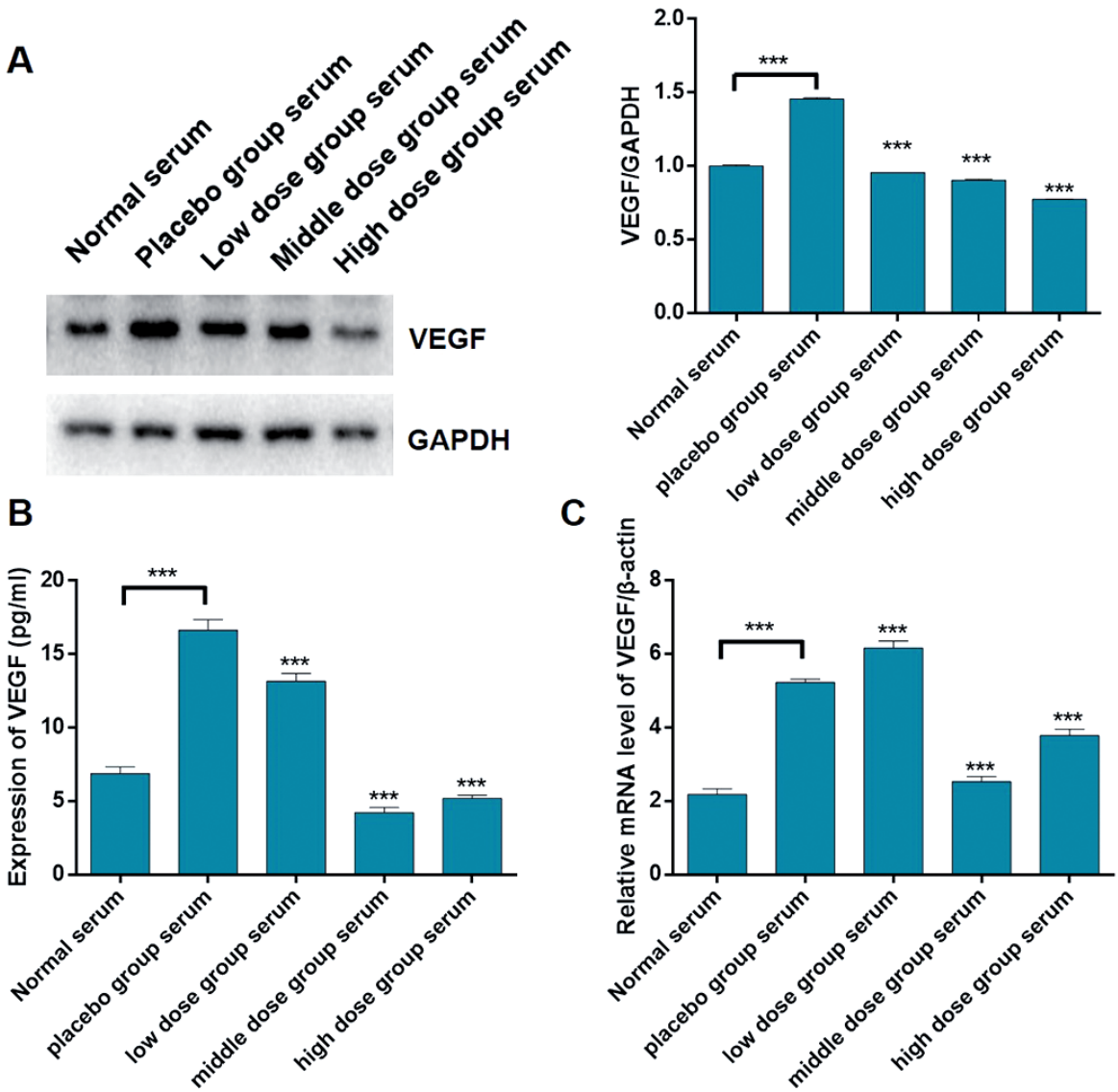

Fig. 3. Diabetic blood-stasis syndrome increased the secretion of VEGF in Müller cells. After stimulated by the serum from different groups of blood-stasis syndrome rats, western blot analysis **(A)** and ELISA **(B)** were used to detect the protein expression levels of VEGF in Müller cells, while the mRNA expression level was inspected via qRT-PCR **(C)**. The data were expressed as mean±SD, P value less than 0.05 was considered statistically significant, 'ns' indicates no significance, *P<0.05, **P<0.01, ***P<0.001. The placebo group was compared with normal group. Other groups were compared with placebo group.

of blood-stasis syndrome on not only the phosphorylation of ERK1/2 signaling pathway, but also VEGF expression in Müller cells. It indicates that the activation of ERK1/2 signaling pathway in Müller cells is necessary for blood-stasis syndrome to influence the expression of VEGF, thus the development and progression of DR.

Discussion

Diabetic retinopathy (DR) has become a major eye disease that could cause blindness and is one of the most serious complications of diabetes. However, there is still no effective treatment for those DR patients (Klein et al., 1984). Recent years, more and more clinical and basic research reported that Traditional Chinese Medicine had some certain effectiveness on DR. Especially, the effect of Chinese herbal medicine for promoting blood circulation thus removing blood stasis on DR has attracted many clinicians' attention. Therefore, it would have great clinical significance to illustrate the molecular mechanism of Chinese drugs of promoting blood circulation to remove blood stasis in curing DR.

Our previous researches indicated that Müller cells are the key in treatment of DR because Müller cells would increase the expression of VEGF during DR, and VEGF over-expression eventually results in blindness and is a dominated pathogenic factor and therapeutic target for DR (Ye et al., 2010). Based on these results, we inferred that diabetic blood-stasis syndrome, which is a theory proposed by Chinese medicine practitioners, have positive role in promoting VEGF expression. To verify this inference, we successfully isolated Müller cells and induced diabetic rat models and screened out blood stasis rat models (Figs. 1, 2). Results in Fig. 3 showed that blood-stasis syndrome increased protein and mRNA expression level of VEGF in Müller cells in vitro. What's more, this tendency was impaired upon the treatment of Xuefu Zhuyu decoction in different dosage.

The ERK1/2 signaling pathway could promote cell proliferation, cell differentiation and cell transformation (Cavaletti et al., 2007). Its activation needs phosphorylation on threonine and tyrosine residues of ERK1/2 kinase (Peng et al., 1998; Berra et al., 2000; Cavaletti et al., 2007; Teruel and Meyer 2000). We have turned out that ERK1/2 signaling pathway participates in regulation of expression level of VEGF in Müller cells of diabetic rats and activation of this pathway could increase expression of VEGF. In this study, phosphorylation level of ERK1/2 was increased in Müller cells after stimulated by blood stasis rat serum in vitro which could be reversed by treatment of Xuefu Zhuyu decoction for serum source rats (Fig. 4). We concluded that diabetic blood-stasis syndrome increase VEGF expression through activation of ERK1/2 signaling pathway, Chinese medicine used to remove blood stasis could inhibit ERK1/2 signaling pathway and subsequently the expression of VEGF in DR.

Although the promoted expression of VEGF by activation of ERK1/2 signaling pathway in Müller cells has been demonstrated, the underlying mechanisms of this process still need further investigation. It has been reported that activation of ERK1/2 signaling pathway could stimulate downstream transcription factors such as activator protein (AP-1) (Cho et al., 2002; Hoshi et al., 2002). Noteworthy, AP-1 binding consensus sequence is

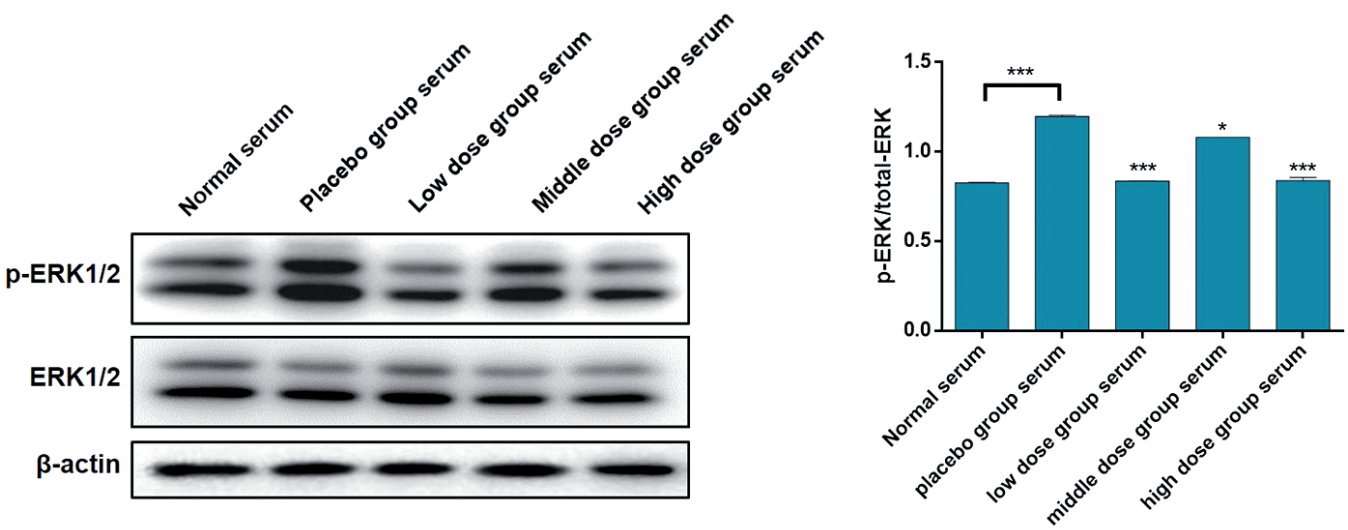

Fig. 4. Diabetic blood-stasis syndrome activated ERK1/2 signaling pathway in Müller cells. After stimulated by the serum from different groups of blood-stasis syndrome rats, western blot analysis was conducted to detect the expression levels of p-ERK1/2 and ERK1/2 in Müller cells, while β-actin was used to confirm the equal amount of proteins loaded in each lane. The data were expressed as mean±SD, P value less than 0.05 was considered statistically significant, *P<0.05, **P<0.01, ***P<0.001. The placebo group was compared with normal group. Other groups were compared with placebo group.

in the promoter region of the VEGF gene (Jia et al., 2016). Therefore, the expression of VEGF should be related to the AP-1 binding activity in Müller cells. In Fig. 5, our EMSA results proved that blood stasis rat serum enhanced AP-1 binding activity of Müller cells compared with the normal rat serum. Similarly, Xuefu Zhuyu decoction treatment of the blood-stasis syndrome rats could decreased AP-1 binding activity of Müller cells after stimulated by their serum.

In order to further confirm that diabetic blood-stasis syndrome could promote expression of VEGF through activating ERK1/2 signaling pathway, U0126, which is an inhibitor of ERK1/2 signaling pathway (Stepanenko et al., 2016), was used to treat the Müller cells. As shown in Fig. 6, U0126 simultaneously decreased protein level of VEGF and phosphorylation level of ERK1/2. So we believed that blood-stasis syndrome could regulate expression of VEGF through ERK1/2 signaling pathway in Müller cells and ERK1/2 signaling pathway may act as the therapeutic target for Chinese

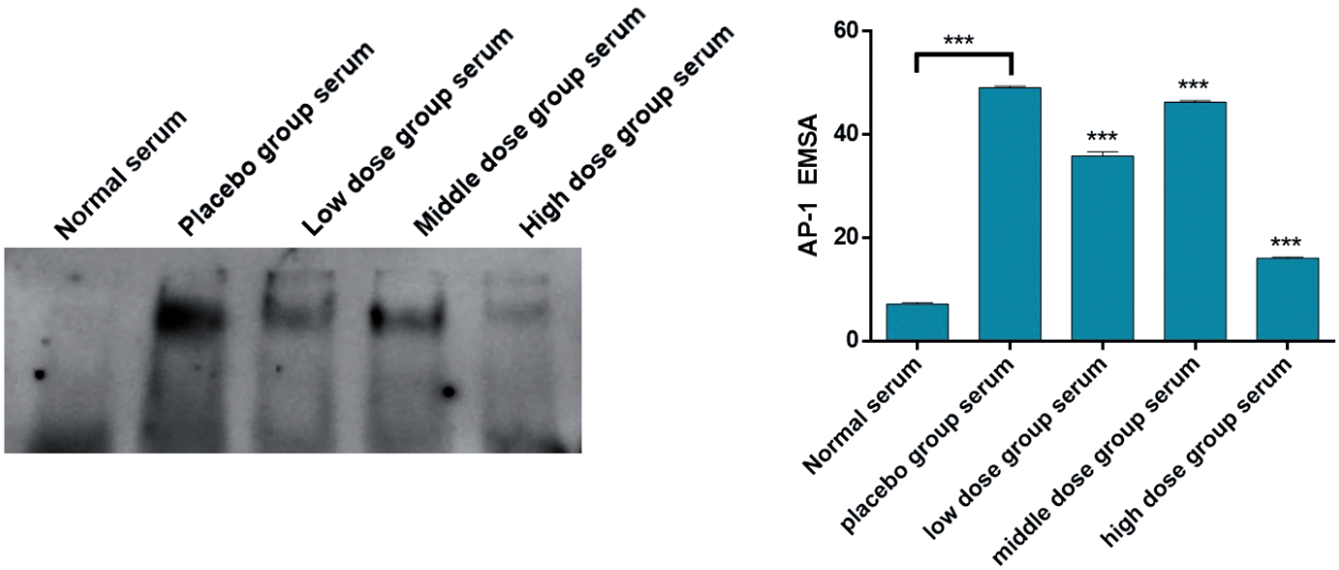

Fig. 5. Diabetic blood-stasis syndrome increased AP-1 DNA-binding activity in Müller cells. After stimulated by the serum from different groups of blood-stasis syndrome rats, the AP-1 DNA-binding activity of Müller cells was determined through EMSA. The data were expressed as mean±SD, P value less than 0.05 was considered statistically significant, *P<0.05, **P<0.01, ***P<0.001. The placebo group was compared with normal group. Other groups were compared with placebo group.

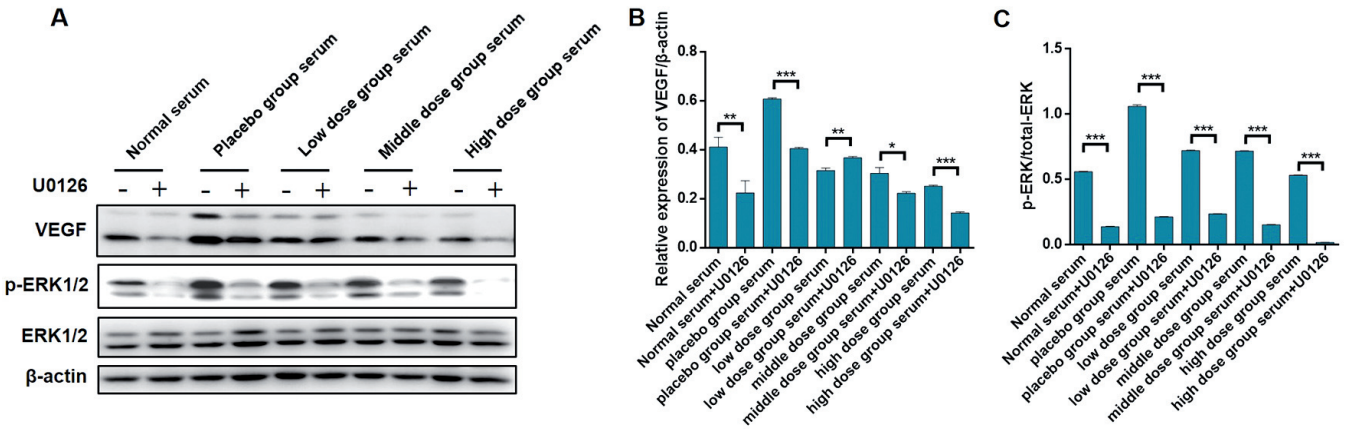

Fig. 6. U0126 blocked VEGF secretion, and activation of ERK1/2 signaling pathway in Müller cells. After stimulated by the serum from different groups of blood-stasis syndrome rats, U0126 was added into the culture medium of Müller cells and incubated for 24 h. Subsequently, western blot analysis was conducted to detect the expression levels of VEGF, p-ERK1/2 and ERK1/2. The data were expressed as mean±SD, P value less than 0.05 was considered statistically significant, *P<0.05, **P<0.01, ***P<0.001.

drugs that can remove the blood stasis such as Xuefu Zhuyu decoction in the treatment of DR.

In conclusion, our results indicated that Diabetic blood-stasis syndrome could activate the ERK1/2 signaling pathway, thus increasing the expression of VEGF, and the AP-1 DNA binding activity in Müller cells, while Xuefu Zhuyu decoction, a traditional Chinese drug, could decline the effect of diabetic blood-stasis syndrome on Müller cells.

Acknowledgements. This study was supported by National Natural Science Foundation of China (Grant Number 81300805 and 81403245).

Author contributions. XY made substantial contributions to the concept and design of the present study. XY, HR and TZ conducted the experiments. HR, TJ and GL conducted data analysis. XY and GL produced the manuscript. All authors read and approved the final manuscript.

Conflict of interest. The authors declare no conflict of interest.

References

Berra E., Pagès G. and Pouysségur J. (2000). MAP kinases and hypoxia in the control of VEGF expression. Cancer Metastasis Rev. 19, 139-145.

Cavaletti G., Miloso M., Nicolini G., Scuteri A. and Tredici G. (2007). Emerging role of mitogen-activated protein kinases in peripheral neuropathies. J. Peripher. Nerv. Syst. 12, 175-194.

Chen K.J., Shi D.Z., Fu C.G., Gao Z.Y., Xu H., Lv S.Z., You S.J. and Huang L. (2016). Diagnostic criterion of blood stasis syndrome for coronary heart disease: Activating blood circulation committee of chinese association of integrative medicine. Chin. J. Integr. Med. 22, 803-804.

Cho M.L., Cho C.S., Min S.Y., Kim S.H., Lee S.S., Kim W.U., Min D.J., Min J.K., Youn J., Hwang S.Y., Park S.H. and Kim H.Y. (2002). Cyclosporine inhibition of vascular endothelial growth factor production in rheumatoid synovial fibroblasts. Arthritis Rheum. 46, 1202-1209.

Fang D., Wan X., Deng W., Guan H., Ke W., Xiao H. and Li Y. (2012). Fufang Xue Shuan Tong capsules inhibit renal oxidative stress markers and indices of nephropathy in diabetic rats. Exp. Ther. Med. 4, 871-876.

Fong D.S., Aiello L., Gardner T.W., King G.L., Blankenship G., Cavallerano J.D., Ferris F.L. 3rd and Klein R. (2003). Diabetic retinopathy. Diabetes Care 26, 226-229.

Hernández C., Simó-Servat A., Bogdanov P. and Simó R. (2017). Diabetic retinopathy: new therapeutic perspectives based on pathogenic mechanisms. J. Endocrinol. Invest. 40, 925-935.

Hoshi S., Nomoto K., Kuromitsu J., Tomari S. and Nagata M. (2002). High glucose induced VEGF expression via PKC and ERK in glomerular podocytes. Biochem. Biophys. Res. Commun. 290, 177-184.

Janknecht R., Ernst W.H., Pingoud V. and Nordheim A. (1993). Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J. 12, 5097-5104.

Jia J., Ye T., Cui P., Hua Q., Zeng H. and Zhao D. (2016). AP-1 transcription factor mediates VEGF-induced endothelial cell migration and proliferation. Microvasc. Res. 105, 103-108.

Karin M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. 270, 16483-16486.

Klein R., Klein B.E., Moss S.E., DeMets D.L., Kaufman I. and Voss P.S. (1984). Prevalence of diabetes mellitus in southern Wisconsin. Am. J. Epidemiol. 119, 54-61.

Li X., Zarbin M.A. and Bhagat N. (2017). Anti-vascular endothelial growth factor injections: The new standard of care in proliferative diabetic retinopathy?. Dev. Ophthalmol. 60, 131-142.

Liao J., Liu Y. and Wang J. (2016). Identification of more objective biomarkers for Blood-Stasis syndrome diagnosis. BMC Complement. Altern. Med. 16, 371.

Lorenzi M. and Gerhardinger C. (2001). Early cellular and molecular changes induced by diabetes in the retina. Diabetologia 44, 791-804.

Lu Q., Lu L., Chen W. and Lu P. (2017). Expression of angiopoietin-like protein 8 correlates with VEGF in patients with proliferative diabetic retinopathy. Graefes Arch. Clin. Exp. Ophthalmol. 255, 1515-1523.

Peng M., Li Y., Luo Z., Liu C., Laties A.M. and Wen R. (1998). Alpha2-adrenergic agonists selectively activate extracellular signal-regulated kinases in Müller cells *in vivo*. Invest. Ophthalmol. Vis. Sci. 39, 1721-1726.

Qin B., Liu J., Liu S., Li B. and Ren J. (2016). MiR-20b targets AKT3 and modulates vascular endothelial growth factor-mediated changes in diabetic retinopathy. Acta Biochim. Biophys. Sin. (Shanghai), 48, 732-740.

Reichenbach A., Siegel A., Rickmann M., Wolff J.R., Noone D. and Robinson S.R. (1995). Distribution of Bergmann glial somata and processes: implications for function. J. Hirnforsch. 36, 509-517.

Rui P. and Heng G. (2015). Clinical effects and hs-CRP VEGF and IGF-1 levels of Xueshuantong capsule combined with Calcium Dobesilate in treatment of early diabetic retinopathy. Modern J. Integrated Traditional Chinese and Western Med.

Solomon S.D., Chew E., Duh E.J., Sobrin L., Sun J.K., VanderBeek B.L., Wykoff C.C. and Gardner T.W. (2017). Diabetic retinopathy: A position statement by the american diabetes association. Diabetes Care 40, 412-418.

Stepanenko A.A., Andreieva S.V., Korets K.V., Mykytenko D.O., Baklaushev V.P., Chekhonin V.P. and Dmitrenko V.V. (2016). mTOR inhibitor temsirolimus and MEK1/2 inhibitor U0126 promote chromosomal instability and cell type-dependent phenotype changes of glioblastoma cells. Gene 579, 58-68.

Stewart M.W. (2017). A review of ranibizumab for the treatment of diabetic retinopathy. Ophthalmol. Ther. 6, 33-47.

Teruel M.N. and Meyer T. (2000). Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. Cell 103, 181-184.

Xu H. and Chen K.J. (2017). Practical diagnostic criterion of blood stasis syndrome: Activating blood circulation committee of Chinese association of integrative medicine. Chin. J. Integr. Med. 23, 243-244.

Yang M.M. and Wang J. (2011). Preventive and therapeutic effect of Salvia Miltiorrhiza for diabetic retinopathy. J. Harbin Med. University.

Ye X., Ren H., Zhang M., Sun Z., Jiang A.C. and Xu G. (2012). ERK1/2 signaling pathway in the release of VEGF from Müller cells in diabetes. Invest Ophthalmol. Vis. Sci. 53, 3481-3489.

Ye X., Xu G., Chang Q., Fan J., Sun Z., Qin Y. and Jiang A.C. (2010). ERK1/2 signaling pathways involved in VEGF release in diabetic rat retina. Invest Ophthalmol. Vis. Sci. 51, 5226-5233.

Yin D.H., Liang X.C. and Y. and Piao Y.L. (2009). Analysis of Chinese medicine syndrome pattern in patients with type 2 diabetes mellitus

and its relationship with diabetic chronic complications. Zhongguo Zhong Xi Yi Jie He Za Zhi, 29, 506-510. (in chinese).

Yue S.J., Xin L.T., Fan Y.C., Li S.J., Tang Y.P., Duan J.A., Guan H.S. and Wang C.Y. (2017) Herb pair Danggui-Honghua: mechanisms underlying blood stasis syndrome by system pharmacology approach. Sci. Rep. 7, 40318.

Accepted February 3, 2022