European Review for Medical and Pharmacological Sciences 2018; 22: 5071-5076

Effects of HIF-1α on diabetic retinopathy angiogenesis and VEGF expression

D. ZHANG, F.-L. LV, G.-H. WANG

Department of Ophthalmology, Liaocheng People's Hospital, Liaocheng, Shandong, China

Abstract. – OBJECTIVE: To investigate the effect of hypoxia inducing factor (HIF)-1α on the expression of vascular endothelial growth factor (VEGF) and angiogenesis in diabetic retinopathy.

PATIENTS AND METHODS: 8-week healthy SD rats were used for the experiments. Under systemic anesthesia condition, control rats received a saline injection into the left ocular body (control A group), and 2 μl antisense oligonucleotides (ASODN) (10 μmol/L) into right eye (control B group). Model rats received a saline injection into the left eye (model A group), and 2 μl ASODN (10 μmol/L) into the right eye (model B group). Rats received an intraocular injection of HIF-1α ASODN for 2, 4, and 6 weeks (A1, A2, A3, B1, B2, B3, respectively). Retinal vessel development was observed by ADP staining. Vascular endothelial cells penetrating retinal inner membrane were counted. Immunohistochemistry was used to detect expressions of VEGF and HIF-1α proteins in the retina.

RESULTS: Prominent angiogenesis and hyperplasia were found in model A group. Relatively fewer newly formed vessels were shown in model group B. However, no significant change of retinal vascular morphology was presented in control group. Of note, the vascular endothelial cell counts, VEGF and HIF-1α contents were significantly increased in model group ($p < 0.05$). After treatment with HIF-1α ASODN, lower endothelial cell counts was found in model B group ($p < 0.05$ comparing to model A). VEGF expression in model B group was significantly decreased, among which, model B3 was observed with lower cell counts than model B1 or B2 ($p < 0.05$ comparing to model A). Injection of HIF-1α ASODN significantly suppressed HIF-1α level in model B in a time-dependent manner.

CONCLUSIONS: Retinal angiogenesis is closely related with increasing level of HIF-1α. Inhibition of HIF-1α suppressed VEGF expression and deterred angiogenesis in a time-dependent manner. This provided novel insights for treating diabetic retinopathy.

Key Words:
Hypoxia inducing factor, Antisense oligonucleotide, Vascular endothelial growth factor, Angiogenesis, Diabetic retinopathy.

Introduction

Diabetic retinopathy (DR) refers to the condition of retinal angiogenesis pathology caused by chronic ischemia in retinal tissues[1]. Currently, it is believed that DR is closely correlated with glucose metabolism and microvascular status. In specific, body glucose metabolic disorder, alternation of microvessels and blood flow blockade lead to microcirculation dysfunction, hypoxia, and ischemia status of the retina, and occurrence of retinopathy[2,3]. Currently, major treatment approaches for DR include drugs, laser surgery, and vitreous resection. These methods not only effectively preserve patient's residual vision, but also cause multiple complications such as visual field loss or refractive error[4]. Major pathological features of proliferative DR consist of retinal angiogenesis. Various clinical studies[5-7] demonstrated that vascular endothelial growth factor (VEGF) exhibited important values in normal retinal vascular development. Hypoxia-inducible factor (HIF)-1α is the core regulatory factor on the induction of hypoxia factor and repair of intracellular oxygen niche. The persistent elevation of HIF-1α level enhances body glycolysis and erythrocytosis, weakens mitochondrial metabolism and causes blood thickening[8]. Rigiracciolo et al[9] confirmed that, under hypoxia condition, HIF-1α regulated transcription activation of VEGF gene. In a study[10] on neonatal retinopathy, we found that hypoxia induced up-regulation of HIF-1α, which further increased VEGF expression, facilitated angio-

Corresponding Author: Dan Zhang, MD; e-mail: gainai00469@163.com

genesis, thus accelerating disease onset and progression. Therefore, we aimed to investigate the effect of HIF-1α on VEGF expression and angiogenesis in DR, in order to provide novel insights and theoretical grounds for DR treatment.

Patients and Methods

Experimental Animals

A total of 54 health SD rats (8 weeks old) were assigned into control group (N=27) and model group (N=27) for generating proliferative DR model, with half males and half females. Body weight ranged between 80 and 250 g. No significant difference was found in body weight or sex ratio between control and model group. All rats were purchased from Laboratory Animal Center of Shangdong University (Jinan, Shandong, China). This investigation was approved by the Ethical Committee for Animal Experiment.

Experimental Reagent and Equipment

HE staining kit was purchased from Boster Bioengineer Corp (Brooklyn, NY, USA). Strept Avidin-Biotin Complex (SABC) staining kit was obtained from Beyotime Biotech (Beijing, China). HIF-1α ASODN was synthesized by Sango (Shanghai, China).

Generation of Proliferative DR Rat Model

Using previously documented methods[11,12], proliferative DR rat model was generated according to the following steps. Streptozotocin (STZ, 0.01 mmol/L) (Sigma-Aldrich, St. Louis, MO, USA) was injected into the peritoneal cavity of a total of 27 healthy 8-week aged SD rats, at 60 mg/kg daily dosage for 4 consecutive weeks. Rat blood glucose level was measured, and those higher than 13.9 mmol/L were assigned to diabetic rats. After 4 weeks of STZ injection, 0.05 μg/L VEGF was injected into the bilateral vitreous body of diabetic rats under systemic anesthesia to generate proliferative DR rat model.

Experimental Methods

Under systemic anesthesia condition, control rats received a saline injection into the left ocular body (control A group), and 2 μl antisense oligonucleotides (ASODN) (10 μmol/L) into right eye (control B group). Model rats received a saline injection into the left eye (model A group), and 2 μl ASODN (10 μmol/L) into right eye (model B group). Local eye inflammation was treated by norfloxacin dropping. At 2, 4, and 8 weeks after treatment, rats were sacrificed for observation. In the meantime, all groups were further assigned into model A1 (2 weeks), model A2 (4 weeks), model A3 (8 weeks), model B1, model B2, model B3, control A1, control A2, control A3, control B1, control B2, and control B3 groups.

Observation of Retinal Vascular Development and Proliferation

Three rats were selected from each group and were sacrificed. ADP enzymatic histochemistry staining was used on retinal slices.

Vascular Endothelial Cell Nuclei Count and Assay for VEGF and HIF-1α

Six rats in each group were sacrificed at all time points. The eyeball was removed for preparation of tissue slices. Hematoxylin-eosin (HE) staining was performed to count the number of vascular endothelial cells penetrating the inner membrane. By SABC method, VEGF and HIF-1α contents were measured. Three fields were randomly selected from each slide (X400) to measure averaged optical density (OD) value of cells. Brown-yellow staining of nuclei was denoted as positive staining.

Statistical Analysis

All data were processed by SPSS 20.0 software (IBM, Armonk, NY, USA), and were presented as mean ± standard deviation (SD). Data were compared by student *t*-test. The one-way ANOVA with the Newman-Keulspost-test was applied in comparison among groups. The percentage data were compared by chi-square test. A statistical significance was defined when $p < 0.05$.

Results

Retinal Vascular Development and Proliferation Condition

Retinal slices were shown in Figure 1. Similar results at all time points were presented among model A group rats, with large amounts of newly formed vessels. In contrast, significantly fewer newly formed vessels were observed model B group ($p < 0.05$), among which, more prominent change appeared in group B3. Neither significant change was found between control A and control B groups, nor did any change of retinal vascular morphology.

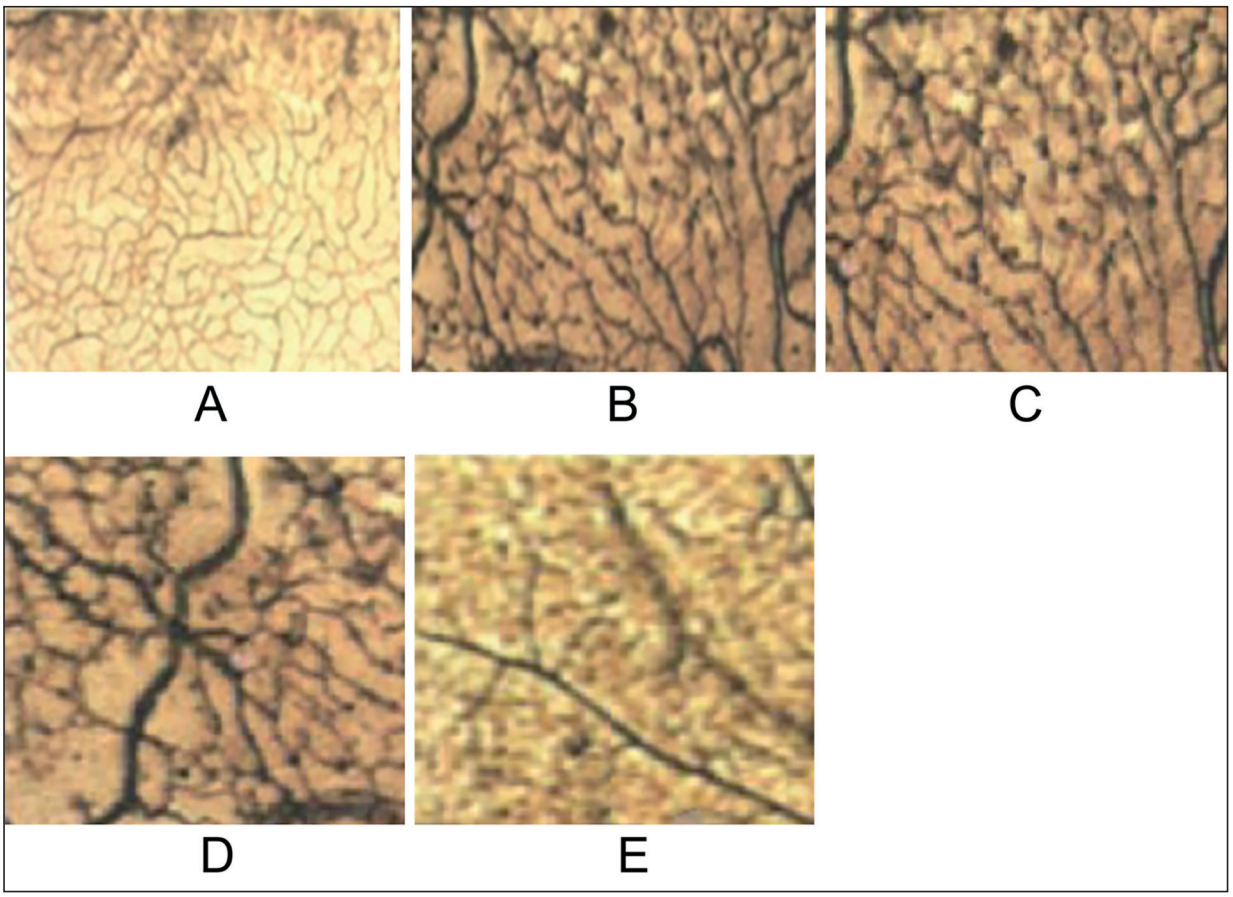

Figure 1. Retinal slide staining images. **A,** Control group; **B,** Model group A; **C,** Model group B1; **D,** Model group B2; **E,** Model group B3.

Vascular Endothelial Cell Count and Assay for VEGF and HIF-1α

The amount of vascular endothelial cells in model group was significantly increased compared to that of control group ($p < 0.05$). After treatment of HIF-1α ASODN, the number of vascular endothelial cells in model B group was significantly decreased compared to the control ($p < 0.05$). As the treatment of HIF-1α ASODN extended, the vascular endothelial cells gradually reduced. Notably, cells count in model B3 group (26.53 ± 0.87) was significantly lower than model B1 (28.90 ± 0.91) and B2 (28.00 ± 0.73) ($p < 0.05$) (Table I).

Comparison of VEGF Content Among All Groups

The VEGF expression in model group was significantly higher than that of control group ($p < 0.05$) (Figure 2a). After HIF-1α level was suppressed by ASODN, VEGF expression in model B group was remarkably decreased (Figure 2c) compared to that in model A group ($p < 0.05$) (Figure 2b). As the treatment time of HIF-1α ASODN extended, VEGF expression was further reduced. Particularly, the level of VEGF in model group B3 (0.30 ± 1.22) was significantly lower than that of model B1 (0.32 ± 1.16) or group B2 (0.32 ± 1.17) ($p < 0.05$) (Table I).

HIF-1α Contents Across All Groups

HIF-1α expression in model group was significantly elevated compared to that in control group ($p < 0.05$) (Figure 3a). However, HIF-1α ASODN significantly down-regulated HIF-1α expression in model B group compared to that in model group A ($p < 0.05$) (Figure 3b, 3c). As the processing time of HIF-1α ASODN went by, VEGF expression level was further downregulated in model B3 group (23.78 ± 0.89), comparing to that in model B1 (25.13 ± 0.93) and model B2 (25.07 ± 0.91) ($p < 0.05$) (Table I).

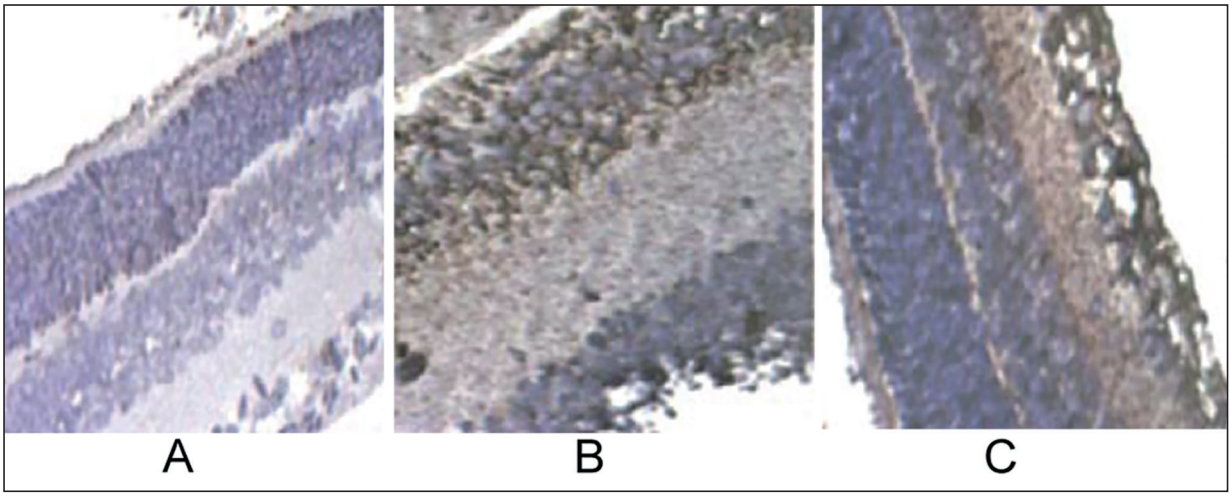

Figure 2. VEGF expression in all groups (SABC approach, X400). **A,** VEGF expression in control group; **B,** VEGF expression of model group A; **C,** VEGF expression in model group B.

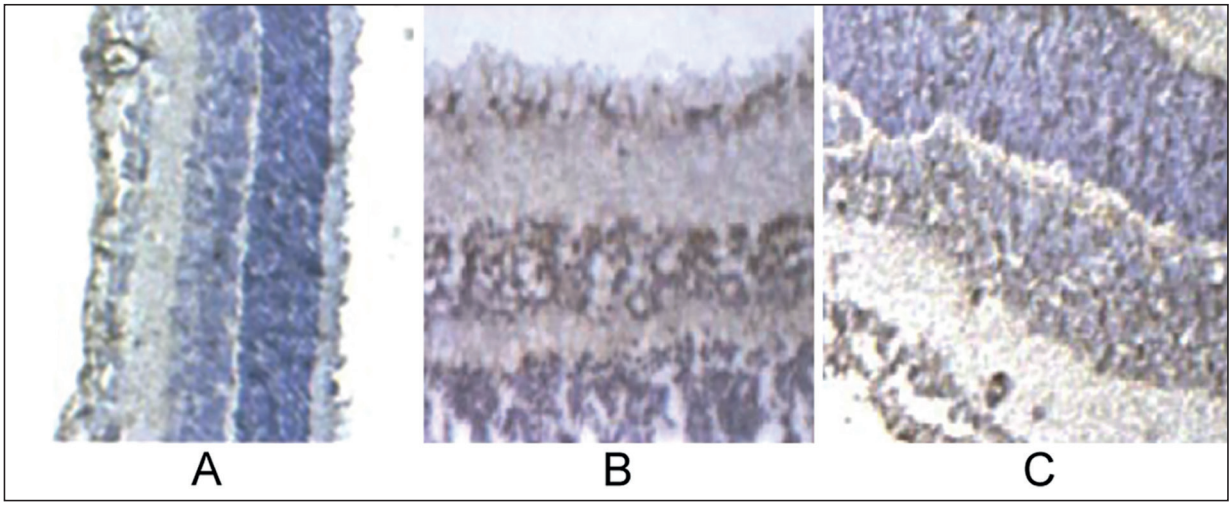

Figure 3. HIF-1α expression among all groups (SABC approach, X400). **A,** HIF-1α expression in control group; **B,** HIF-1α expression of model group A; **C,** HIF-1α expression in model group B.

Table I. Vascular endothelial cell count and contents of VEGF and HIF-1α between two groups.

Group		Vascular endothelial cell count	VEGF	HIF-1α
Control	A1	2.78 ± 0.65	0.28 ± 1.40	0
	A2	2.75 ± 0.72	0.28 ± 1.39	0
	A3	2.74 ± 0.88	0.28 ± 1.37	0
	B1	2.69 ± 0.93	0.28 ± 1.42	0
	B2	2.71 ± 0.73	0.28 ± 1.38	0
	B3	2.70 ± 0.78	0.28 ± 1.41	0
Model	A1	32.83 ± 0.91[a]	0.57 ± 2.13[a]	55.74 ± 1.78[a]
	A2	32.80 ± 0.79[a]	0.56 ± 2.12[a]	55.70 ± 1.69[a]
	A3	32.81 ± 0.72[a]	0.57 ± 2.14[a]	55.73 ± 1.89[a]
	B1	28.90 ± 0.91[a,c]	0.32 ± 1.16[a,c]	25.13 ± 0.93[a,c]
	B2	28.00 ± 0.73[a,c]	0.32 ± 1.17[a,c]	25.07 ± 0.91[a,c]
	B3	26.53 ± 0.87[a,b]	0.30 ± 1.22[a,b,c]	23.78 ± 0.89[a,b,c]

Note: [a]$p < 0.05$ comparing to control group; [c]$p < 0.05$ comparing to model group A; [b]$p < 0.05$ comparing to model group B1 and B2.

Discussion

As the major pathological feature of DR, the examination of retinal angiogenesis is of critical importance. Huang et al[13] showed that in addition to its function on normal vascular development, VEGF was also involved in abnormal angiogenesis. When tissues or cells are under hypoxia condition, HIF is probably produced. HIF-1α subtype regulates multiple gene expressions besides VEGF, which are all essential for angiogenesis in retina[14,15]. HIF-1α can up-regulate VEGF receptor expression, and facilitate angiogenesis[16,17]. The antisense oligonucleotide is one type of artificially synthesized short nucleotide sequence and can bind with specific DNA or RNA sequence. After antisense oligonucleotide enters the target cell, it can bind with specific mRNA sequence of the target gene, and selectively inhibit the transcription or translation of target gene[18]. In the previous study, we selected HIF-1α as the target. The treatment of HIF-1α ASODN locally into vitreous cavity significantly blocked HIF-1α expression.

The result of retinal slide staining showed abundant newly formed vessels in model group A rat retinal tissues, along with prominent hyperplasia. However, in model B group, newly formed vessels were significantly decreased, whilst in control group, no significant change of retinal vascular morphology was found. Significantly larger amount of vascular endothelial cells, with higher levels of VEGF and HIF-1α were observed in model B group. The HIF-1α positive cell was merely expressed in model group, suggesting that DR pathogenesis was correlated with retinal tissue hypoxia. Under hypoxia stress, HIF-1α upregulated the VEGF expression to induce prominent angiogenesis in DR pathology, and this was consistent with previous studies[19,20]. As HIF-1α level was inhibited in model group B, vascular endothelial cell count was remarkably suppressed in a time-dependent manner. Concomitantly, VEGF expression in model group B was also reduced. After retina hypoxia, positive expression of HIF-1α was observed in retinal ganglion cell layer, and is positively correlated with local VEGF expression. HIF-1α and VEGF presented significant function in the occurrence and development of ovarian cancer[21]. Moreover, our result showed that HIF-1α and VEGF expression presented consistent trends with retinal angiogenesis, and indicated that under DR condition, retinal hypoxia can affect VEGF expression via the HIF-1α pathway, thus modulating retinal angiogenesis. In this work, we found that the downregulation of HIF-1α further deterred expressions of HIF-1α and VEGF, resulting in the reduction of retinal newly formed vessels. However, a limitation of this report is that the stability and safety trials with HIF-1α ASODN require further evaluation.

Conclusions

Our data demonstrated that, during the process of retinal angiogenesis and hypoxia, HIF-1α expression was significantly enhanced. The decrease of HIF-1α in vitreous cavity reduced VEGF expression and inhibited angiogenesis, which offers an academic basis for the future prevention and treatment of DR.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Gong CY, Lu B, Sheng YC, Yu ZY, Zhou JY, Ji LL. The Development of Diabetic Retinopathy in Goto-Kakizaki Rat and the Expression of Angiogenesis-Related Signals. Chin J Physiol 2016; 59: 100-108.
2) Cheung N, Wong IY, Wong TY. Ocular anti-VEGF therapy for diabetic retinopathy: overview of clinical efficacy and evolving applications. Diabetes Care 2014; 37: 900-905.
3) Song SJ, Han K, Choi KS, Ko SH, Rhee EJ, Park CY, Park JY, Lee KU, Ko KS, Task Force Team for Diabetes Fact Sheet of the Korean Diabetes A. Trends in diabetic retinopathy and related medical practices among type 2 diabetes patients: Results from the National Insurance Service Survey 2006-2013. J Diabetes Investig 2018; 9: 173-178.
4) Stitt AW, Curtis TM, Chen M, Medina RJ, McKay GJ, Jenkins A, Gardiner TA, Lyons TJ, Hammes HP, Simo R, Lois N. The progress in understanding and treatment of diabetic retinopathy. Prog Retin Eye Res 2016; 51: 156-186.
5) Ozdemir G, Ergun Y, Bakaris S, Kilinc M, Durdu H, Ganiyusufoglu E. Melatonin prevents retinal oxidative stress and vascular changes in diabetic rats. Eye (Lond) 2014; 28: 1020-1027.
6) Chintala H, Krupska I, Yan L, Lau L, Grant M, Chaqour B. The matricellular protein CCN1 controls retinal angiogenesis by targeting VEGF,

Src homology 2 domain phosphatase-1 and Notch signaling. Development 2015; 142: 2364-2374.

7) Lenhartova N, Matasova K, Lasabova Z, Javorka K, Calkovska A. Impact of early aggressive nutrition on retinal development in premature infants. Physiol Res 2017; 66: S215-S226.

8) Goda N, Kanai M. Hypoxia-inducible factors and their roles in energy metabolism. Int J Hematol 2012; 95: 457-463.

9) Rigiracciolo DC, Scarpelli A, Lappano R, Pisano A, Santolla MF, De Marco P, Cirillo F, Cappello AR, Dolce V, Belfiore A, Maggiolini M, De Francesco EM. Copper activates HIF-1alpha/GPER/VEGF signalling in cancer cells. Oncotarget 2015; 6: 34158-34177.

10) Fu Z, Chen D, Cheng H, Wang F. Hypoxia-inducible factor-1alpha protects cervical carcinoma cells from apoptosis induced by radiation via modulation of vascular endothelial growth factor and p53 under hypoxia. Med Sci Monit 2015; 21: 318-325.

11) Gong CY, Lu B, Hu QW, Ji LL. Streptozotocin induced diabetic retinopathy in rat and the expression of vascular endothelial growth factor and its receptor. Int J Ophthalmol 2013; 6: 573-577.

12) Yang W, Yu X, Zhang Q, Lu Q, Wang J, Cui W, Zheng Y, Wang X, Luo D. Attenuation of streptozotocin-induced diabetic retinopathy with low molecular weight fucoidan via inhibition of vascular endothelial growth factor. Exp Eye Res 2013; 115: 96-105.

13) Huang L, Zhao S, Zhang JH, Sun X. Hydrogen saline treatment attenuates hyperoxia-induced retinopathy by inhibition of oxidative stress and reduction of VEGF expression. Ophthalmic Res 2012; 47: 122-127.

14) Chen X, Liu J, He B, Li Y, Liu S, Wu B, Wang S, Zhang S, Xu X, Wang J. Vascular endothelial growth factor (VEGF) regulation by hypoxia inducible factor-1 alpha (HIF1A) starts and peaks during endometrial breakdown, not repair, in a mouse menstrual-like model. Hum Reprod 2015; 30: 2160-2170.

15) Rahim M, Gibbon A, Hobbs H, van der Merwe W, Posthumus M, Collins M, September AV. The association of genes involved in the angiogenesis-associated signaling pathway with risk of anterior cruciate ligament rupture. J Orthop Res 2014; 32: 1612-1618.

16) Sappayatosok K, Maneerat Y, Swasdison S, Viriyavejakul P, Dhanuthai K, Zwang J, Chaisri U. Expression of pro-inflammatory protein, iNOS, VEGF and COX-2 in oral squamous cell carcinoma (OSCC), relationship with angiogenesis and their clinico-pathological correlation. Med Oral Patol Oral Cir Bucal 2009; 14: E319-324.

17) Demirovic A, Tomas D, Tomic K, Spajic B, Ibukic A, Cupic H, Kruslin B. Correlation of vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression with pathological renal artery changes in patients with renal cell carcinoma. Scand J Urol 2014; 48: 34-40.

18) Chen C, Liu R, Wang J, Yan Z, Qian S, Zhang W. RNAi knockdown of hypoxia-inducible factor-1alpha decreased the proliferation, migration, and invasion of hypoxic hepatocellular carcinoma cells. Cell Biochem Biophys 2015; 71: 1677-1684.

19) Mastropasqua R, Di Antonio L, Di Staso S, Agnifili L, Di Gregorio A, Ciancaglini M, Mastropasqua L. Optical coherence tomography angiography in retinal vascular diseases and choroidal neovascularization. J Ophthalmol 2015; 2015: 343515.

20) Gaoli X, Lili W, Zhiwu W, Zhiyuan G. [Research progress of mechanism of hypoxia-inducible factor-1alpha signaling pathway in condylar cartilage growth and remodeling]. Hua Xi Kou Qiang Yi Xue Za Zhi 2016; 34: 639-642.

21) Shen W, Li HL, Liu L, Cheng JX. Expression levels of PTEN, HIF-1alpha, and VEGF as prognostic factors in ovarian cancer. Eur Rev Med Pharmacol Sci 2017; 21: 2596-2603.