European Review for Medical and Pharmacological Sciences 2020; 24: 1460-1467

Estrogen and estrogen receptor affects MMP2 and MMP9 expression through classical ER pathway and promotes migration of lower venous vascular smooth muscle cells

M.-Y. ZHAO[1], T. ZHAO[2], Q.-Y. MENG[1], L. ZHAO[3], X.-C. LI[1]

[1]Department of Vascular Surgery, [2]Intensive Care Unit, and [3]Department of Anesthesiology; The Third Affiliated Hospital of Qiqihar Medical University, Qiqihar, Heilongjiang, China

Abstract. – OBJECTIVE: To explore the role of estrogen and estrogen receptors in the migration of vascular smooth muscle cells in varicose lower-extremity veins.

PATIENTS AND METHODS: Tissue samples of normal lower extremity vein (56 cases) and varicose lower extremity vein (47 cases) were collected. Western blot and real-time fluorescent qPCR were performed to measure the expression of Estrogen receptor α (ERα) in tissues. Two cell co-culture systems were established for human umbilical vein endothelial cells (HUVECs) and human umbilical vein smooth muscle cells (HUVSMCs). One system was incubated under normal oxygen conditions (normal oxygen group), and the other was under oxygen-poor conditions (hypoxia group). The two systems were treated with 10-7 mM Estrogen E2, 10-7 mM BSA-conjugated Estrogen E2-BSA, 10-7 mM Estrogen E2+10-3 mM Tamoxifen (TAM), respectively for 24 h. The treated cells were subjected to cell scratch assay, transwell assay, and Western blot analysis of MMP2 and MMP9 protein expression.

RESULTS: The expression of ERα in varicose lower extremity vein was significantly up-regulated compared with that in normal lower extremity vein. The cell migration rate and the number of migrating cells in untreated hypoxia group and E2-treated normal oxygen group were comparable ($p>0.05$) to those in untreated normal oxygen group. The cell migration rate and the number of migrating cells were significantly increased ($p<0.05$) in E2-treated hypoxia group, compared with E2-treated normal oxygen group and untreated hypoxia group. The cell migration rate, the number of migrating cells, and expression levels of MMP2 and MMP9 were significantly decreased in E2/TAM-treated hypoxia group, compared with those in E2-treated hypoxia group.

CONCLUSIONS: In summary, E2 can promote the migration of vascular smooth muscle cells and induce varicose veins of the lower extremities, which may be related to the promotion of MMP2 and MMP9 expression through the classical pathway of ER.

Key Words:

Varicose lower extremity vein, Estrogen, Estrogen receptor classical pathway, Vascular smooth muscle cells, Cell migration

Introduction

Varicose vein in the lower extremities is a common peripheral vascular disease. Superficial veins are enlarged, dilated, and twisted. The symptoms include pain, edema, eczema, and ulceration in severe cases. Although the disease is not life-threatening, varicose veins in the lower extremities and associated complications seriously affect patients' physical activities and quality of life. In severe cases, ulceration occurs, which is difficult to treat, bringing significant economic burden to patients and their families[1]. The pathogenic mechanism of varicose veins in the lower extremities has not yet been fully elucidated. It was reported that varicose veins in the lower extremities were closely associated with hypoxia and increased venous pressure[2]. During hypoxic conditions and when the venous pressure increases, endothelial cells are damaged, recruiting white blood cells and monocytes to attach to their surface. Then, the leukocytes infiltrate the venous wall and release pro-inflammatory cytokines, resulting in extracellular matrix deposition. Vascular smooth muscle cells switch from secretory phenotype to synthetic phenotype and start migration to the endothelium, resulting in

Corresponding Author: Xi-Chun Li, Ph.D; e-mail: zmylxc806@126.com

stiffness and reduced contractility of the venous wall. The above is the pathophysiological process of vascular remodeling[3,4]. High expression of matrix metalloproteinases (MMPs) was also found in varicose veins in the lower extremities, suggesting that vascular remodeling of varicose veins may be associated with MMPs[5].

Fischer et al[6] reported that the synthesis and transformation of extracellular matrix proteins are closely associated with the activity of sex hormones. Epidemiological studies[7] showed that the incidence of varicose lower extremity veins in females was significantly higher than that in males, suggesting that sex hormones may play a role in the onset of varicose lower extremity veins. The structure of normal veins contains primarily three components: vascular endothelial cells, vascular smooth muscle cells, and extracellular matrices. Vascular smooth muscle cells play a major role in vascular remodeling of varicose lower extremity veins. Under the action of hypoxia, inflammatory cytokines, and collagen, these cells transform into the synthetic phenotype and migrate to the endothelium[6]. As far as we know, multiple studies[8-10] have confirmed that sex hormones can up-regulate MMPs in various tissues. Based on the findings in the literature mentioned above, we were interested in investigating the association of varicose lower extremity veins with the action of estrogen/estrogen receptor. In this study, cell co-culture systems were established for human umbilical vein endothelial cells (HUVECs) and human umbilical vein smooth muscle cells (HUVSMCs), aimed to investigate the role of estrogen/estrogen receptor in migration of vascular smooth muscle cells and the underlying mechanism.

Patients and Methods

Clinical Data

Lower extremity veins were collected from patients who were treated in the Third Affiliated Hospital of Qiqihar Medical College from April 2016 to June 2018. Normal lower extremity veins were collected from 56 patients, aged (47.8±4.9) years, who underwent bypass surgery or suffered from lower extremity trauma or car accident injuries, including 36 males and 20 females. Varicose lower extremity veins were collected from 47 patients, aged (48.3±6.8) years, who underwent varicose vein stripping surgery, including 18 males and 29 females. Patients who had the following conditions were excluded from this study: diabetes mellitus, lower extremity thrombosis, ischemia, and cardiovascular and cerebrovascular diseases. All patients were aware of the intended use of their tissue samples and signed an informed consent form. This investigation was approved by the Medical Ethics Committee of the Third Affiliated Hospital of Qiqihar Medical College.

Materials and Reagents

Endothelial Cell Medium (ECM) (cat. #1001) and smooth muscle cell medium (SMCM) (cat. #1101) cell culture media from ScienCell Research Laboratories; fetal bovine serum (FBS) from Gibco (Rockville, MD, USA); 17β-estradiol (E2), BSA-estradiol conjugates (E2-BSA), and estrogen receptor antagonist tamoxifen (TAM) from Sigma-Aldrich (St. Louis, MO, USA); TRIzol reagent from Invitrogen (Carlsbad, CA, USA); quantitative fluorescent polymerase chain reaction (PCR) dyes from Bio-Rad (Hercules, CA, USA); reverse transcription kit from Toyobo (Osaka, Japan); bicinchoninic acid (BCA) kit (cat. #P0009) from Beyotime Biotechnology Co., Ltd.; matrix metalloproteinase 2 (MMP2) mAb (cat. #40994), matrix metalloproteinase 9 (MMP9) mAb (cat. #13667) and estrogen receptor α (ERα) mAb (cat. #13258) from CST (Danvers, MA, USA); internal reference β-actin (cat. #20536-1-AP) and horseradish peroxidase (HRP)-labeled secondary antibody (cat. #SA00001-2) from Proteintech.

Establishment of Cell Co-Culture Systems

Human umbilical vein endothelial cells (HUVECs) and human umbilical vein smooth muscle cells (HUVSMCs) were cultured in ECM and SMCM media with 10% FBS, respectively. The cells were routinely cultured in an incubator with 5% CO_2 and at 37°C. At 80-90% confluency, the cells were digested with trypsin containing 0.25% ethylenediamine tetraacetic acid (EDTA). The cell co-culture systems were established using a transwell system (0.4 μm pore size membrane). Cells were seeded either in the upper or the lower chamber based on the experiment design. A mixture of ECM and SMCM at a volume ratio of 1:1 was used as the culture media of the co-culture systems. A co-culture system was established after 24 h of culture. Fetal bovine serum treated with activated carbon (Gibco, Rockville, MD, USA) was used to exclude any potential effect of hormones in the serum on the experimental data.

Experimental Grouping and Treatment

Tissue samples were divided into normal vein group and varicose vein group. Cell co-culture systems were divided into normal oxygen group and hypoxia group. Cells in the normal oxygen co-culture system were cultured at 37°C with 5% CO_2, while cells in the hypoxia co-culture system were cultured at 37°C with 5% CO_2 and 95% N_2. Stock solutions of E2, E2-BSA, and tamoxifen (TAM) (all 10 mM) were prepared using dimethyl sulfoxide (DMSO) as solvent. When treating cells, these stock solutions were diluted with the mixed media into a final concentration of 10^{-7} mM, 10^{-7} mM, and 10^{-3} mM, respectively. Then, the cells were added to the normal oxygen or hypoxia co-culture system in accordance with the experimental design.

Total RNA Extraction and qPCR

RNA was extracted from liquid nitrogen-ground freshly harvested lower extremity veins using trizol. Total RNA concentration was measured on a spectrophotometer. About 1 μg of RNA was used to synthesize cDNA via reverse transcription in accordance with the protocol of the reverse transcription kit. In this qPCR assay, βactin was used as internal reference. The primer sequences were as follows: for βactin, the forward sequence was CAT GTA CGT TGC TAT CCA GGC, and the reverse sequence was CTC CTT AAT GTC ACG CAC GAT; for ERα, the forward sequence was GGG AAG TAT GGC TAT GGA ATC TG, and the reverse sequence was TGG CTG GAC ACA TAT AGT CGTT; for pS2, the forward sequence was CCC CGT GAA AGA CAG AAT TGT, and the reverse sequence was GGT GTC GTC GAA ACA GCAG. The PCR reaction was performed as follows: pre-denaturation at 95°C for 5 min, and 40 cycles of 95°C for 5 s (denaturation), 60°C for 30 s (anneal), and 72°C for 30 s (extension), followed by extension at 72°C for 5 min at the end of the cycles. The experiment was repeated three times. The relative expression level of target gene was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct=Ct_{target\ gene}-Ct_{internal\ reference}$, and $\Delta\Delta Ct=\Delta Ct_{experimental\ group}-\Delta Ct_{control\ group}$.

Protein Extraction and Western Blot

HUVSMCs were seeded in the lower chamber of a six-well plate at 10^5 cells/well, and HUVECs were seeded in the upper chamber of the co-culture system at $1x10^5$ cells/well. A final concentration of 10^{-7} mM E2, 10^{-7} mM E2-BSA, and 10^{-7} mM E2+10^{-3} mM TAM was added to the chambers, respectively. The co-culture systems were incubated in a tri-gas incubator for 24 h under hypoxia condition, after which cells were collected. Appropriate amounts of RIPA lysis buffer and protease inhibitor were added, followed by sonication on ice for 5 min. After complete lysis, cells were centrifuged at 12,000 g for 15 min at low temperature. The supernatant was collected, 10 μL of which was used for concentration measurement using the BCA method. The remaining supernatant was mixed with 5× loading buffer at a volume ratio of 4:1, followed by heating in a 100°C water bath for 10 min. Samples containing equivalent amount of total protein were loaded onto gels containing 5% stacking gel and 10% separation gel for electrophoretic analysis. The gels were run at a constant voltage of 80 V until bromophenol blue entered the stacking gel, when the voltage was changed to 120 V until the target bands were separated. The protein bands were transferred from gel to Polyvinylidene difluoride (PVDF) membrane by wet transfer method under a constant current of 275 mA for 80 min. After the membrane was blocked with 5% milk at room temperature for 2 h, corresponding diluted MMP2, MMP9 or βactin antibody (dilution factor:1000) was added, followed by incubation at 4°C overnight. After washing, the secondary antibody diluted with 2% milk (1: 10,000) was added, followed by incubation at room temperature for 1 h. After development, the image was analyzed using Image J software to obtain gray values of the bands. βactin was used as an internal reference. The ratio of gray value of the target protein to gray value of βactin was regarded as the expression level of that protein.

Cell Scratch Assay

HUVSMCs were seeded in the lower chamber of a 6-well plate at a density of 10^5 cells/well. HUVECs were seeded in the upper transwell chamber (pore size 0.4 μm) at a density of $1 x 10^5$ cells/well. When cells were 100% confluent, the medium was removed and replaced with serum-free medium. After starvation for 24 h, at least three even scratches were created at the bottom of each well using a pipette tip in a perpendicular way. The plate was rinsed three times with phosphate-buffered saline (PBS) to remove detached cells, followed by adding serum-free medium and incubation for 24 h. Images were taken at 0 h and 24 h under a microscope to measure the widths of

the scratches with image Pro plus software. Cell migration rate was calculated by (scratch width at 0 h-scratch width at 24 h)/scratch width at 0 h.

Transwell Assay

HUVECs and HUVSMCs were resuspended in the mixed media and diluted to the following concentration: 10^4 cells per 100 μL for HUVECs and $1.0x10^4$ cells per 100 μL for HUVSMCs. HUVECs (600 μL) were seeded in the lower chamber of a 24-well plate. HUVSMCs (300 μL) were seeded in a transwell chamber (pore size 8.0 μm), which was later inserted into the 24-well plate. After the system was incubated for 24 h, cells attached at the inner surface of the upper chamber were wiped out using a cotton swab, followed by immersing the chamber in 4% paraformaldehyde for 10 min. After fixation, the fixed cells were stained with 0.1% crystal violet dye. Excessive dye was washed away under running water. After drying at room temperature, the migrated cells were photographed and counted under a microscope and counted with image Pro plus software.

Statistical Analysis

Statistical analysis was performed using SPSS 23 software. Each test was repeated more than three times. Data were expressed as mean ± standard deviation. A normality test was first performed. If data followed a normal distribution, *t*-test was performed between two groups. If data did not follow a normal distribution, Mann-Whitney's test was then performed. A difference was statistically significant if $p<0.05$.

Results

ERĐ Expression in Varicose Lower Extremity Vein

The expression levels of ERα in normal lower extremity vein and varicose lower extremity vein were measured and compared. It was found that both the ERα mRNA level measured by qPCR and the ERα protein level measured by Western blot were significantly up-regulated ($p<0.0001$, $p<0.0001$) in varicose lower extremity vein (Figure 1).

Effects of Hypoxia and Estrogen E2 on Migration of Vascular Smooth Muscle Cells

As shown in Figure 2a, the migration rate, and migration number of HUVSMCs in untreated hypoxia group and E2-treated normal oxygen group were comparable ($p>0.05$) to those in untreated normal oxygen group. Under hypoxia conditions, the migration rate and migration number of vascular smooth muscle cells all increased ($p<0.05$) in the presence of E2. Compared with untreated hypoxia group, E2 treatment significantly increased the migration rate and migration number of HUVSMCs under hypoxia conditions ($p<0.05$).

TAM Inhibited Migration of HUVSMCs

As shown in Figure 2b, under hypoxia conditions, E2 significantly increased the migration rate and the migration number of HUVSMCs ($p<0.05$). However, E2-BSA had no effect on

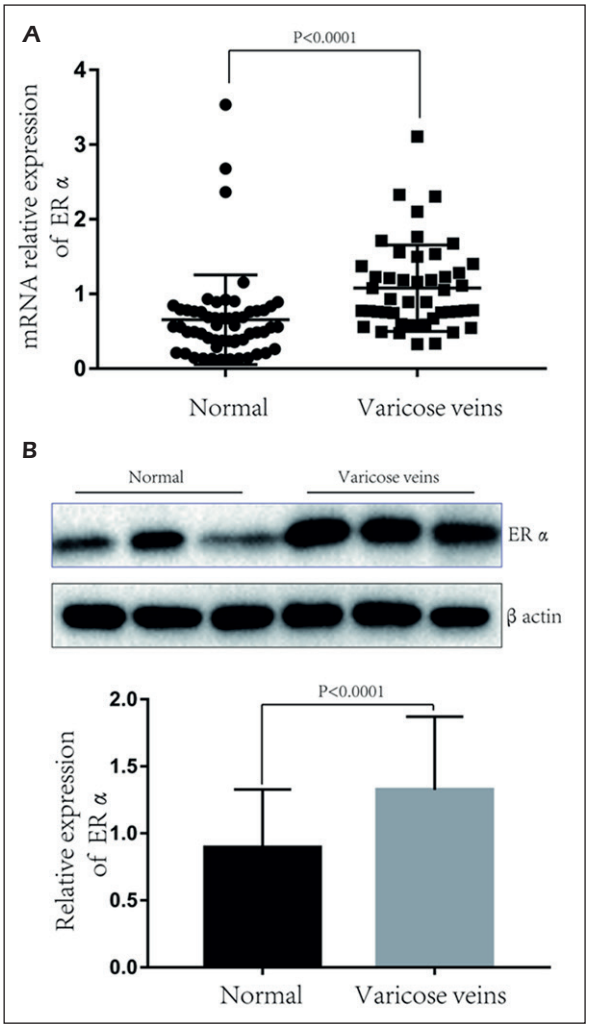

Figure 1. ERα expression in varicose lower extremity vein. **A,** ERα mRNA relative expression levels. **B,** ERα protein levels measured by western blot. Relative ERα expression level was the ratio of the gray values of the target protein to the internal reference βactin. Mann-Whitney'a test was used.

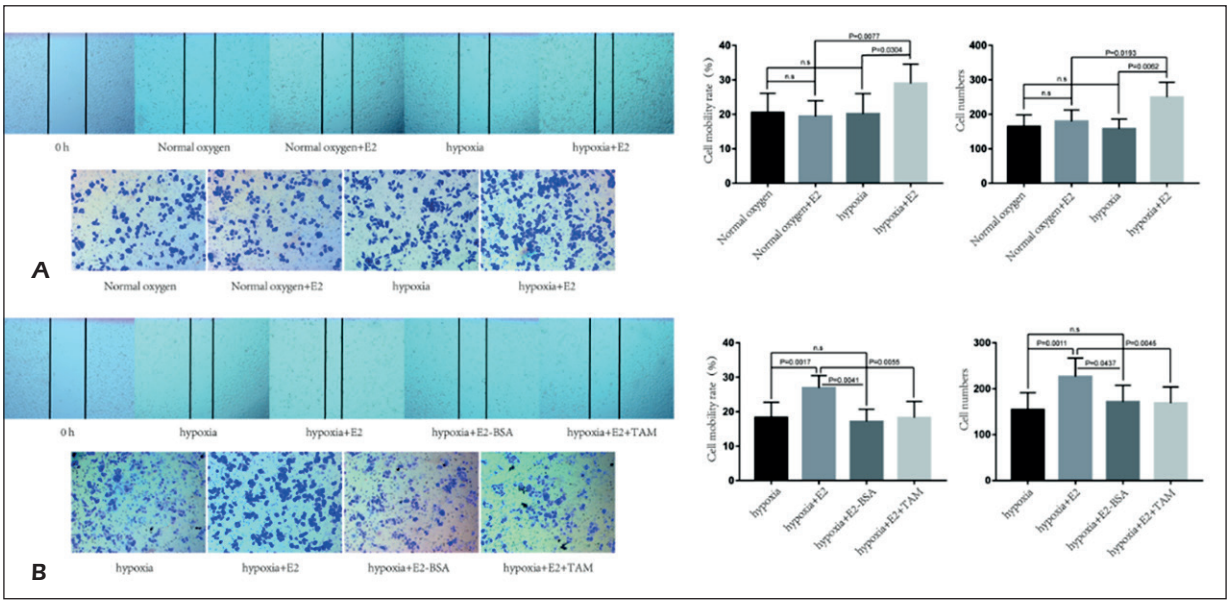

Figure 2. Cell scratch assay and transwell assay. **A,** Hypoxia and E2 promoted migration of HUVSMCs (10×); **B,** TAM inhibited migration of HUVSMCs (10×). In cell scratch assay, HUVSMCs were seeded into the lower chamber, while HUVECs were seeded into the upper chamber. They were reversed in transwell assay. *t*-test was used.

migration of HUVSMCs ($p>0.05$) due to its inaccessibility to cells. Compared with E2-treated hypoxia group, the estrogen receptor antagonist TAM not only reduced ERα expression (Figure 3), but also significantly reduced the migration rate and migration number of HUVSMCs.

E2 Regulation of MMP2 and MMP9 Expression Through the Classical Pathway

As Western blot results showed, the levels of MMP2 and MMP9 in E2-treated hypoxia group were significantly higher than those in untreated hypoxia group. However, these levels in E2-BSA-treated hypoxia group were comparable to those in untreated hypoxia group ($p>0.05$). Compared with E2-treated hypoxia group, treatment with the estrogen receptor antagonist TAM significantly reduced levels of MMP2 and MMP9 ($p<0.05$, Figure 4).

Discussion

Varicose lower extremity vein is a common vascular disease. It is widely accepted that vascular remodeling plays a role in the onset of varicose lower extremity vein. Venous vascular remodeling is a process characterized mainly by changes in vascular smooth muscle cells and extracellular matrices. This process represents the compensatory mechanism for the veins to adapt to pathological conditions such as hypoxia and increased venous pressure[11]. To investigate the hypothesis that gender may be associated with the onset of varicose lower extremity vein, histological analysis was performed on tissues of normal

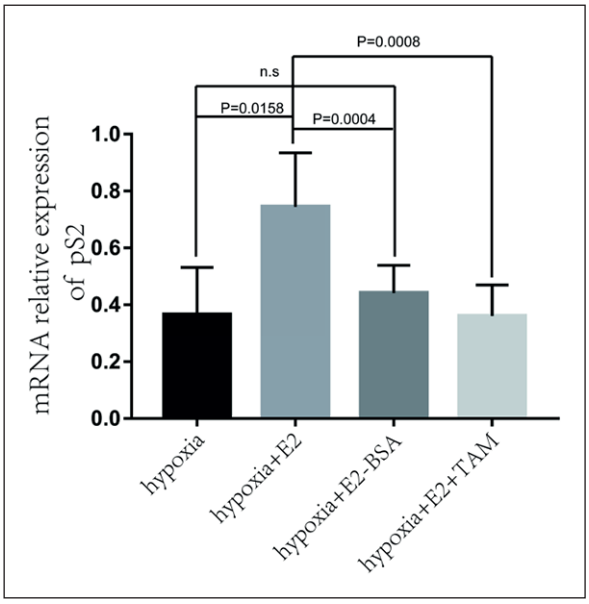

Figure 3. Expression of estrogen-responsive pS2 gene. *t*-test was used.

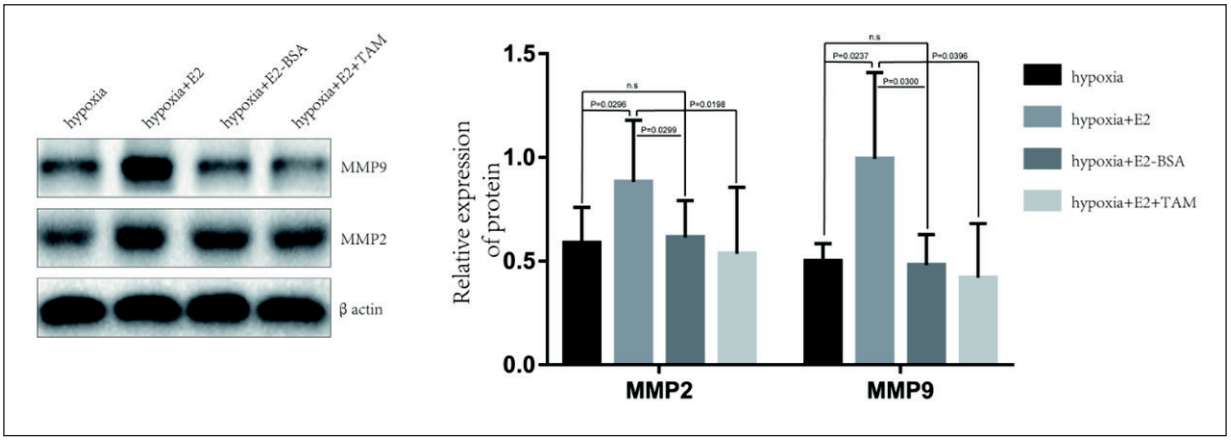

Figure 4. Western blot analysis of MMP2 and MMP9 expression. HUVSMCs were seeded in the lower chamber of a 6-well plate, while HUVECs were seeded in the upper chamber. Cells incubated under hypoxia conditions were randomly divided into untreated hypoxia group, E2-treated hypoxia group (E2 concentration: 1×10^{-7} mM), E2-BSA-treated hypoxia group (E2-BSA concentration: 1×10^{-7} mM) and E2/TAM-treated hypoxia group (E2/TAM concentrations: 1×10^{-7} mM/1×10^{-3} mM). *t* test was used.

lower extremity vein and varicose lower extremity vein. It was found that ERα was up-regulated in varicose vein tissue, suggesting ERα may play a role in the onset and progression of varicose lower extremity vein.

When varicose lower extremity vein occurs, blood stagnates in the distorted blood vessels. Slower blood flow not only causes insufficient oxygen uptake in the tissue, but also leaves vascular endothelial cells and smooth muscle cells in hypoxic state[12]. In view of the state of endothelial cells and smooth muscle cells in varicose vein, a co-culture system was established for human umbilical vein endothelial cells (HUVECs) and human umbilical vein vascular smooth muscle cells, aimed at simulating the *in vivo* environment to explore the effect of hypoxia on smooth muscle cells. It was found that the migration of vascular smooth muscle cells increased only when hypoxia and E2 were both present, suggesting that the onset of varicose lower extremity vein was associated with synergistic action of hypoxia and E2. The incidence of varicose lower extremity veins is higher in women than that in men, implicating that estrogen might be a player in the onset of the disease. Fischer et al[13] reported that estrogen played an important role in the synthesis of extracellular matrices, and abnormal synthesis and transformation of extracellular matrices were involved in vascular remodeling in varicose veins. When the vein is in an oxygen-deficient state, the endothelial cells are damaged, resulting in an increase of pro-inflammatory cytokines in the blood. Pro-inflammatory cytokines stimulate the synthesis of extracellular matrices, promoting migration of vascular smooth muscle cells[3]. As far as we know, synergistic action of hypoxia and estrogen in varicose vein has not been reported.

Estrogen is a steroid hormone. Its modes of action on cells include classical mechanism and non-genomic (non-classical) mechanism. The classical mechanism involves estrogen entering the cell and binding to estrogen receptor (ER), after which the receptor dimerizes and binds to estrogen response elements (EREs) on the genome. Thus, it is also called genomic mechanism. The non-classical mechanism involves estrogen binding to the G protein-coupled estrogen receptor (GPER) on the cell membrane, triggering changes in intracellular signaling pathways[14]. In the non-classical mechanism, estrogen does not need to enter the cell to exert its action. Estrogen binds to the estrogen receptor on the surface of the cell membrane to trigger rapid intracellular response. In the classical pathway, ER is bound to heat shock protein 90 (HSP90), stabilizing the receptor in an inactive state, when estrogen is absent. When estrogen enters the cell, it binds to ER, inducing formation of ER homodimer or heterodimer. At the same time, HSP90 dissociates, and the dimers enter the nucleus and activate transcription of the target genes. TAM can compete with estrogen for binding to ER, preventing transcription of target genes[15]. To further explore

the mode of action of E2 on HUVSMCs, the cells were treated with membrane impermeable BSA-conjugated E2 (E2-BSA) and estrogen receptor antagonist TAM. It was found that the estrogen responsive pS2 gene was upregulated in the E2-treated hypoxia group, downregulated in the E2/TAM-treated hypoxia group, and had no significant change in the E2-BSA-treated hypoxia group. The above findings suggested TAM significantly inhibited migration of HUVSMCs possibly through the classical pathway of estrogen action.

In this study, it was also found that migration of HUVSMCs was associated with the expression levels of MMP2 and MMP9 (Figures 2 and 4). This is related to the decomposition of extracellular matrices by MMPs. Matrix metalloproteinases are a class of enzymes that break down extracellular matrices. Multiple evidence has showed abnormality of MMPs expression in varicose veins. Badier-Commander et al[16] and Gillespie et al[17] reported that the TIMP-1/MMP-2 ratio in varicose veins was three times as large as that in the control group. Woodside et al[18] and Xu et al[19] reported that MMP-1 and MMP9 were mainly expressed in fibroblasts, smooth muscle cells and endothelial cells in varicose veins, and compared with normal veins they were highly expressed in smooth muscle cells. In this study, elevated expression levels of MMP2 and MMP9 were observed when HUVSMCs were treated with E2. However, there were no significant changes in the expression levels of MMP2 and MMP9 when treated with E2-BSA. This finding implicated that E2 may regulate the expression of MMPs through the classical pathway of estrogen action. To further validate this hypothesis, HUVSMCs were treated with the estrogen receptor antagonist TAM in combination with E2. It was found that expression levels of MMP2 and MMP9 were significantly decreased after TAM inhibited the activity of ER.

Conclusions

We found that E2 can promote the migration of vascular smooth muscle cells and induce varicose veins of the lower extremities, which may be related to the promotion of MMP2 and MMP9 expression through the classical pathway of ER. In this work, a cell model of varicose lower extremity vein was established for the first time by co-culture of vascular endothelial cells and vascular smooth muscle cells under hypoxic conditions. The model will provide an alternative platform for the study of varicose lower extremity vein. Of course, certain shortcomings were realized in the design of this study. First, hypoxia is not the only player in the onset of varicose veins. Inflammation, oxidative stress, and apoptosis are all involved in the disease. Secondly, the sample size in this study was not large enough, which may lead to some biased findings. The conclusion still needs support from a large sample size. Lastly, although E2 regulation of MMPs by the ER classical pathway was investigated in this study, it is necessary to explore the mechanism from experiments.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

This study was supported by Qiqihar Science and Technology Project (SFZD2017007).

References

1) Ascher E, Jacob T, Hingorani A, Gunduz Y, Mazzariol F, Kallakuri S. Programmed cell death (Apoptosis) and its role in the pathogenesis of lower extremity varicose veins. Ann Vasc Surg 2000; 14: 24-30.
2) Michiels C, Arnould T, Remacle J. Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions. Biochim Biophys Acta 2000; 1497: 1-10.
3) Sakata N, Kawamura K, Takebayashi S. Effects of collagen matrix on proliferation and differentiation of vascular smooth muscle cells in vitro. Exp Mol Pathol 1990; 52: 179-191.
4) Janowski K, Sopinski M, Topol M. Changes in the wall of the great saphenous vein at consecutive stages in patients suffering from chronic vein disease of the lower limbs. Folia Morphol (Warsz) 2007; 66: 185-189.
5) Raffetto JD, Khalil RA. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. Biochem Pharmacol 2008; 75: 346-359.
6) Fischer GM, Swain ML. Effects of estradiol and progesterone on the increased synthesis of collagen in atherosclerotic rabbit aortas. Atherosclerosis 1985; 54: 177-185.
7) Criqui MH, Denenberg JO, Bergan J, Langer RD, Fronek A. Risk factors for chronic venous disease:

the San Diego Population Study. J Vasc Surg 2007; 46: 331-337.

8) Suzuki T, Sullivan DA. Estrogen stimulation of proinflammatory cytokine and matrix metalloproteinase gene expression in human corneal epithelial cells. Cornea 2005; 24: 1004-1009.

9) Potier M, Elliot SJ, Tack I, Lenz O, Striker GE, Striker LJ, Karl M. Expression and regulation of estrogen receptors in mesangial cells: influence on matrix metalloproteinase-9. J Am Soc Nephrol 2001; 12: 241-251.

10) Zylberberg C, Seamon V, Ponomareva O, Vellala K, Deighan M, Azzarolo AM. Estrogen up-regulation of metalloproteinase-2 and -9 expression in rabbit lacrimal glands. Exp Eye Res 2007; 84: 960-972.

11) Surendran S, K SR, Suresh A, Binil Raj SS, Lakkappa RK, Kamalapurkar G, Radhakrishnan N, C Kartha C. Arterialization and anomalous vein wall remodeling in varicose veins is associated with upregulated FoxC2-Dll4 pathway. Lab Invest 2016; 96: 399-408.

12) Flore R, Ponziani FR, Gerardino L, Santoliquido A, Di Giorgio A, Lupascu A, Nesci A, Tondi P. Biomarkers of low-grade inflammation in primary varicose veins of the lower limbs. Eur Rev Med Pharmacol Sci 2015; 19: 557-562.

13) Moura MJ, Marcondes FK. Influence of estradiol and progesterone on the sensitivity of rat thoracic aorta to noradrenaline. Life Sci 2001; 68: 881-888.

14) Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol 2008; 70: 165-190.

15) Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA, Safe S. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 2000; 275: 5379-5387.

16) Badier-Commander C, Verbeuren T, Lebard C, Michel JB, Jacob MP. Increased TIMP/MMP ratio in varicose veins: a possible explanation for extracellular matrix accumulation. J Pathol 2000; 192: 105-112.

17) Gillespie DL, Patel A, Fileta B, Chang A, Barnes S, Flagg A, Kidwell M, Villavicencio JL, Rich NM. Varicose veins possess greater quantities of MMP-1 than normal veins and demonstrate regional variation in MMP-1 and MMP-13. J Surg Res 2002; 106: 233-238.

18) Woodside KJ, Hu M, Burke A, Murakami M, Pounds LL, Killewich LA, Daller JA, Hunter GC. Morphologic characteristics of varicose veins: possible role of metalloproteinases. J Vasc Surg 2003; 38: 162-169.

19) Xu HM, Zhao Y, Zhang XM, Zhu T, Fu WG. Polymorphisms in MMP-9 and TIMP-2 in Chinese patients with varicose veins. J Surg Res 2011; 168: e143-148.