Effects of SU5416 on angiogenesis and the ERK-VEGF/MMP-9 pathway in rat endometriosis.

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Abstract. SU5416 is a small molecule vascular endothelial growth factor (VEGF) receptor signal transduction inhibitor, which can block the VEGF receptor autophosphorylation and inhibit receptor tyrosine kinase signal transduction, thereby reducing VEGF activity. However, there are few reports about the correlation of SU5416 to the occurrence and angiogenesis in endometriosis. In this study, we observed the effects of VEGF receptor inhibitor SU5416 on angiogenesis in endometriosis in rats. Thirty female specific-pathogen-free Sprague-Dawley rats were randomly divided into sham operation group (SOG), model group (MG), and SU5416 group (n=10 for each group). In the SOG, only the uterus was cut and sutured, and endometriosis models were established in the MG and SU5416 group by autologous transplantation. The SU5416 group was injected with 15 mg/kg SU5416 intraperitoneally, and the SOG and MG were intraperitoneally injected with an equal volume of normal saline for 6 weeks. The volume of ectopic lesions was lower in the SU5416 group at 42 d postoperatively than in the MG ($p<0.05$). The proportion of CD31-positive cells in the endometrial tissue of the SU5416 group was lower than that of the MG ($p<0.05$); angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), laminin-5γ2 (LN-5γ2) and phosphorylation of ERK (P-ERK), VEGF, matrix metalloproteinase (MMP)-2, and MMP-9 protein expressions were lower in the SU5416 group than in the MG ($p<0.05$). VEGF receptor inhibitor SU5416 can inhibit endometriosis angiogenesis and reduce inflammatory response in rats, and its mechanism of action may be related to the down-regulation of the ERK-VEGF/MMP-9 pathway expression.

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Efecto del SU5416 sobre la angiogenesis y la via ERK-VEGF/MMP-9 en la endometriosis de ratas.

Resumen. SU5416 es un inhibidor de la transducción de señales del receptor del factor de crecimiento endotelial vascular (VEGF), una molécula pequeña, capaz de bloquear la autofosforilación del receptor VEGF e inhibir la transducción de señales de la tirosina quinasa del receptor, reduciendo así la actividad del VEGF. Sin embargo, existen escasos informes acerca de la correlación entre SU5416 y la aparición y angiogénesis de la endometriosis. En este estudio, hemos observado los efectos del inhibidor del receptor del VEGF, SU5416, sobre la angiogénesis en la endometriosis en ratas. Treinta ratas Sprague-Dawley hembra, libres de patógenos específicos, fueron divididas aleatoriamente en un grupo de operación simulada (SOG), un grupo de modelo (MG) y un grupo de SU5416 (n=10 en cada grupo). En el SOG, solo se realizó una incisión en el útero y se suturó, mientras que en los grupos MG y SU5416 se establecieron modelos de endometriosis mediante trasplante autólogo. Al grupo SU5416 se le inyectaron 15 mg/kg de SU5416 por vía intraperitoneal, y tanto el SOG como el MG recibieron una inyección intraperitoneal de un volumen igual de solución salina normal durante 6 semanas. El volumen de lesiones ectópicas fue menor en el grupo SU5416 a los 42 días después de la operación en comparación con el MG ($p<0,05$); la proporción de células CD31 positivas en el tejido endometrial del grupo SU5416 fue inferior a la del MG ($p<0,05$); las expresiones de las proteínas angiopoyetina-1 (Ang-1), angiopoyetina-2 (Ang-2), laminina-5γ2 (LN-5γ2) y la fosforilación de ERK (P-ERK), VEGF, metaloproteinasa de matriz (MMP)-2 y MMP-9 fueron menores en el grupo SU5416 que en el MG ($p<0,05$). El inhibidor del receptor del VEGF, SU5416, puede inhibir la angiogénesis de la endometriosis y reducir la respuesta inflamatoria en ratas, y su mecanismo de acción puede estar relacionado con la regulación a la baja de la expresión de la vía ERK-VEGF/MMP-9.

INTRODUCTION

Endometriosis (EMs) is a benign morphological manifestation, but the biological behaviors such as implantation invasion, aggressive growth and distant metastasis are similar to those of malignant tumors, which can induce painful intercourse, dysmenorrhea, chronic pelvic pain, and infertility up to 25%-35% [1,2]. The specific etiology of this disease has not been elucidated, and it is mostly thought to be related to genetic factors, endometrial implantation, retrograde menstruation, implantation, and immune regulation [3,4]. However, both endometrial implantation and menstrual reflux implantation depend on adequate blood supply, so angiogenesis plays a key role in the occurrence and development of EMs.

Vascular endothelial growth factor (VEGF) is an autocrine and paracrine growth factor that can improve vascular permeability, damage the tight junctions of vascular endothelial cells, induce endothelial cell proliferation, and promote extracellular fluid accumulation, vascular leakage, and neovascularization [5,6]. Matrix metalloproteinase-9 (MMP-9), one of the important protein hydrolases in the family of MMPs, disrupts basement membrane integrity and promotes neovascularization and vascular endothelial cell outgrowth, thus playing an important role in the ectopic implantation, adhesion, and growth of endometrial cells [7]. Therefore, downregulation of VEGF and MMP-9 expression is particularly critical in inhibiting angiogenesis and blocking signaling in vascular endothelial cells. SU5416 is a small molecule VEGF receptor signaling inhibitor, which can block VEGF receptor autophosphorylation, inhibit receptor tyrosine kinase signaling, and reduce VEGF activity [8]. It was found that SU5416 in a rat pulmonary hypertension model reduced pulmonary inflammatory response, inhibited intimal proliferation of small pulmonary arteries, and promoted pulmonary vascular remodeling [9]. However, there are few reports on the relevance of SU5416 on the development of EMs and angiogenesis worldwide. The objective of this study was to analyze the effects of SU5416, a VEGF receptor inhibitor, on angiogenesis and ERK-VEGF/MMP-9 signaling pathway in EMs in rats.

MATERIAL AND METHODS

Experimental animals

The experiment procedures conformed to the relevant requirements of the Regulations of the People's Republic of China on the Administration of Laboratory Animals; 30 female specific-pathogen-free (SPF) Sprague-Dawley (SD) rats weighting 180-200 g were purchased from (purchased from Beijing Viton Lihua Laboratory Animal Technology Co., Ltd; animal Use License), Animal Use License No.: SYXK (Beijing) 2018-0015, Laboratory Animal Production License No.: SCXK (Beijing) 2018-0022. Rats were housed at a temperature of (24±1) °C, relative humidity of 50%, and noise<80 db. The researcher changed the bedding, and cleaned and disinfected the rat cages regularly.

Drugs, reagents and instruments

VEGF receptor inhibitor SU5416 (Shanghai Hengfei Biotechnology Co., Ltd., China), Two-steps IHC detection kits for rat tissues (Shanghai Qi Ming Biotechnology Co., Ltd., China), Western blot electrophoresis instrument (Bio-Rad Inc., USA), BCA protein concentration assay kit (Beijing Solaibao Technology Co. Ltd., China), Hematoxylin Eosin Staining Kit (Wuhan PhD Bioengineering Co., Ltd., China), angiopoietin (Ang)-1, Ang-2, laminin-5γ2 (LN-5γ2), phosphorylation of ERK (P-ERK), VEGF, MMP-2, MMP-9, GAPDH antibodies (CST Biotechnology Co., Ltd., USA), Horseradish Peroxidase (HRP)-Labeled Goat Anti-Rabbit Immunoglobulin (Ig) G (Solepow Technology Co., Ltd., China), VEGF, MMP-9, and GAPDH primers (synthesized by Sangon Biotech (Shanghai) Co., Ltd.), fluorescent quantitative PCR kit (Lot. No. 639519, Takara Bio Inc., Japan), Trizol kit (Thermo Fisher Science, USA), RNA extraction kit (Nanjing Novozymes Biotechnology Co., Ltd., China), reverse transcription kit (Genecopoeia, Inc., USA), 5415D high-speed centrifuge (Eppendorf, Germany), and CX21 optical microscope (Olympus, Japan), Light Cycler 2.0 Real-time PCR instrument (Roche Equipment Ltd., Switzerland), -80℃ ultra-low temperature refrigerator (SANYO, Japan), enzyme immunoassay analyzer (Shanghai Kunke Instruments Co., Ltd., China), surgical instruments (Beijing Youcheng Jiaye Biotechnology Ltd., China), VEGF, MMP-9, GAPDH primers (Shanghai Bioengineering Co., Ltd., China), vernier calipers (Shanghai YuYan Scientific Instruments Co., Ltd., China), etc.

METHODS

Model establishment and grouping

Thirty female SPF SD rats were randomly divided into sham operation group (SOG), model group (MG), and SU5416 group (n=10 for each group). In the SOG, only the uterus was cut and sutured. The EMs models were established in the MG and SU5416 group by autologous transplantation, that is, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate at a dose of 300 mg/kg, and the rats were placed in the supine position and fixed on the operating table, disinfected. A 2 cm incision was made in the middle of the lower abdomen, the uterus and endometrium were separated, and two 5mm × 5mm fragments were taken from the left uterine horn, which were quickly transplanted into the rat mesenteric artery with abundant blood vessels. The abdominal cavity was washed and sutured layer by layer, and the postoperative anti-infection was performed for 3 days. The success criteria for model establishment: the graft was opened 14 d postoperatively, and the volume of the graft was observed visually to be increased, with a light red, round or oval vesicle with internal fluid accumulation, and the surface was covered with a large number of blood vessels and closely adhered to the surrounding tissues. The SU5416 group was injected intraperitoneally with 15 mg/kg SU5416 twice a week, and the sham operation and MGs were injected intraperitoneally with equal volume of saline for 6 weeks. Every procedure was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Fujian Medical University.

Morphology of normal and ectopic endometrial tissue and volume of ectopic lesions in rats

At 42 d after surgery, morphological changes of endometrial tissue in rats were observed under optical microscope; at 14 d and 42 d after surgery, the width and length of ectopic lesions were measured using vernier calipers, and the volume of lesions was calculated = length×width^2×0.5.

Specimen collection

After 24h after the last administration, 3mL of tail vein blood was collected from rats, left for 15min, centrifuged at 2000×g for 15min, and the supernatant was stored in an ultra-low temperature refrigerator at -80℃. After anesthesia, the rats were executed and dissected. In the MG and SU5416 group, ectopic endometrium was removed, and in the SOG, normal endometrium was removed. Each specimen was immediately cut into three parts and rinsed with saline, and 1g of tissue was cut off and stored in an ultra-low temperature refrigerator at -80℃, which were used for real-time quantitative PCR and western blot assay. The remaining endometrium tissue was fixed with 4% paraformaldehyde, dehydrated in gradient alcohol, transparent in xylene, embedded in paraffin, and routinely pathologically sectioned.

Immunohistochemical staining method

CD31 is a platelet endothelial cell adhesion molecule expressed at the tight junctions between endothelial cells, which regulates the process of angiogenesis and reflects the microvessel density (MVD), and can therefore be used as a marker for the measurement of microangiogenesis. In this study, the number of CD31 positive cells in endometrial tissues was mainly detected by immunohistochemical detection. Paraffin sections were dewaxed, soaked in alcohol from high to low gradient, rinsed with distilled water, incubated with 0.3% H_2O_2 for 30 min, blocked with 5% serum for 2 h, incubated with CD31 primary antibody at 4℃ overnight, rinsed with TBST, immunohistochemical staining, rinsed with distilled water, dehydrated in gradient alcohol, transparent in xylene, and sealed. The staining was observed using light microscopy, and any five fields of view of each section were photographed. The number of CD31 positive cells and the total number of cells were counted,

and the proportion of CD31 positive cells was calculated. Proportion of CD31 positive cells = number of CD31 positive cells/total number of cells × 100%.

Enzyme-linked immunosorbent assay (ELISA)

The spare serum was taken, and the levels of serum VEGF, MMP-9, interleukin (IL)-1, IL-2, IL-6 and tumor necrosis factor (TNF)-α were determined according to the instructions of the ELISA kit. Blank wells (no sample and enzyme reagents were added to the blank control wells, the rest of the procedure was the same), standard wells and test sample wells were set respectively. Standard (50 μL) was accurately added on the ELISA coated plate. Sample diluent (40 μL) was added to the test sample wells, and then 10 μL of test sample was added (the final sample dilution was five times). The sample was added to the bottom of the well of ELISA plate without touching the wall of the well, and was gently shaken and mixed. After sealing the plate with sealing film, the sample was incubated at 37 °C for 30 min. The 30 times concentrated washing solution was diluted with 30 times distilled water for further use. After carefully removing the sealing film, the solution was discarded, and the sample was shaken dry. Each well was filled with washing solution, and after leaving for 30 s, the solution was discarded, repeating this for 5 times and patting dry. Enzyme reagent (50 μL) was added to each well, except blank wells. The plate was the sealed with sealing film, and the sample was incubated at 37° C for 30 min. After washing the plate as the above method, Chromogen solution A (50 μL) was added to each well first, and then Chromogen solution B (50 μL) was added to each well, and the mixture was gently shaken and mixed for chromogenic reaction at 37 °C for 15 min. The stop solution (50 μL) was added to each well to stop the reaction (at this time, the blue immediately turned to the yellow). The blank well was set to zero, and the optical density (OD) of each well at 450 nm wavelength was measured.

Quantitative real-time fluorescence (qRT-PCR)

Spare uterine tissue was taken and total RNA from uterine tisuue extracted according to the instructions of Trizol kit and total RNA extraction kit, and use reverse transcription kit to reverse-transcribe the total RNA into cDNA. Using the reverse transcribed cDNA as a template, the expression of VEGF and MMP-9 was detected by Real-time PCR instrument. The total reaction volume was 20 μL, GAPDH was used as the internal reference, and the relative expression of genes in each group was calculated by the 2-ΔΔCt method.

Western blot

The spare remaining uterine tissue was taken, followed by the BCA method for protein quantification, 12% SDS-polypropylene gel electrophoresis, wet transfer to PVDF membrane, treatment with a closure solution for two h at room temperature in a shaker, primary antibody (1:500 dilution) (Ang-1, Ang-2, LN-5γ2, P-ERK, VEGF, MMP-2, MMP-9, GAPDH) was added, incubated overnight at 4 °C, and the membrane was washed three times with TBST for 15 minutes each time. HRP-labeled Goat Anti-Rabbit IgG secondary antibody (1:2000 dilution) was added, incubated for two hours at room temperature, and the membrane was washed three times with TBST for 15 minutes each time. Enhanced chemiluminescence (ECL) reagents were used to develop the color and quantitative analysis was performed using Image J software. GAPDH (1:1000 dilution) was used as the internal reference, and the ratio result indicated the relative concentration of the target protein.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 24.0 statistical analysis software was used, and the measurement data conforming to normal distribution were ex-

pressed as $\overline{x} \pm s$ and compared using one-way analysis of variance (ANOVA) while least significant difference (LSD)-t test was used for two-way comparison, $p<0.05$ was considered statistically significant difference.

RESULTS

Volume of ectopic lesions

The volume of ectopic lesions in the MG and SU5416 group at 42 days after operation was higher than that at 14 days after operation ($p<0.05$), but there was no significant difference in the volume of ectopic lesions between the MG and SU5416 group at 14 days after operation ($p>0.05$); the volume of ectopic lesions in the SU5416 group was lower than that in the MG at 42 days after operation ($p<0.05$), indicating that SU5416 could effectively inhibit the increase in the volume of ectopic lesions in rats (Fig. 1).
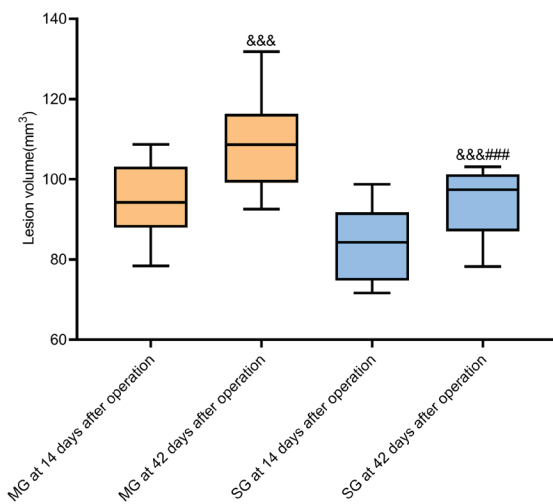

Fig. 1. Comparison of ectopic lesion volume in each group.

Shows that there was no significant difference in the volume of ectopic lesions between the model group and SU5416 group at 14 days after operation, while the volume of ectopic lesions in the SU5416 group was lower than that in the model group at 42 days after operation. Note: MG: model group; SG: SU5416 group. Compared within the group at 14 days after operation, &&&p<0.001; compared with the model group at 42 days after operation, ###p<0.001.

Morphological changes of normal and ectopic endothelial tissues in rats under light microscopy

Since the rats in the sham-operation group did not develop EMs and there was no presence of ectopic endometrial tissue, the comparison was made between normal endometrial tissue and ectopic endometrial tissue from the other groups. At 42 days after operation, the normal endometrial epithelial cells and glandular epithelium of the rats in the sham-operation group were arranged in a columnar shape with a complete structure, and the glands, blood vessels and interstitial cells of the lamina propria were neatly arranged and structurally complete. The ectopic endometrial epithelial cells and the glandular epithelium of the rats in the MG were high columnar; the endometrial structure was circular sawtooth closed, the numbers of lamina propria glands, stromal cells and blood vessels were large, and the nucleus oval was deeply stained. The ectopic endometrial epithelial cells of the SU5416 group showed atrophic changes, the structure of some epithelial cells is incomplete, the lamina propria gland epithelial cells are incomplete, the mesenchymal cells become smaller, the number of blood vessels is reduced and the arrangement is disordered (Fig. 2).

CD31-positive cell expression in endometrial tissue

Since the rats in the sham-operation group did not develop EMs and there was no presence of ectopic endometrial tissue, the comparison was made between normal endometrial tissue and ectopic endometrial tissue from the other groups. The staining of CD31 positive cells and the proportion of positive cells were detected by immunohistochemical method, which could show the changes of MVD of endometrium. The proportion of MVD of ectopic endometrial tissue (that is, CD31-positive cell expression) in the MG and SU5416 group was higher than that of normal endometrial tissues in the SOG ($p < 0.05$); The proportion of CD31-positive cell
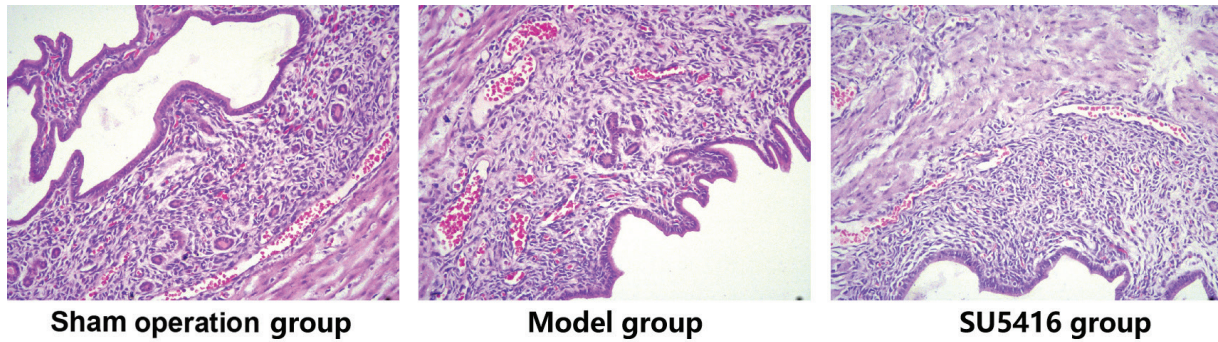
Sham operation group   **Model group**   **SU5416 group**

Fig. 2. Morphological changes of normal and ectopic endometrial tissues of rats under light microscopy (200×). Shows that there was no endometriosis lesion in the sham-operation group, so the comparison was made between normal endometrial tissue and ectopic endometrial tissue from the other groups. At 42 days after operation, the normal endometrial epithelial cells and glandular epithelium of the rats in the sham-operation group were arranged in a columnar shape with a complete structure. The ectopic endometrial epithelial cells and the glandular epithelium of the rats in the model group were high columnar; the endometrial structure was circular sawtooth closed, the numbers of lamina propria glands, stromal cells and blood vessels were large, and the nucleus oval was deeply stained. The ectopic endometrial epithelial cells of the SU5416 group showed atrophic changes.

expression of ectopic endometrial tissues was lower in the SU5416 group than in the MG ($p<0.05$), which showed that SU5416 could effectively reduce the MVD of endometrial tissue in rats (Fig. 3).

Endometrial angiogenesis-related protein expression

Ang-1, Ang-2, and LN-5γ2 protein expression of ectopic endometrial tissues in the MG and SU5416 group were higher than those of normal endometrial tissues in the SOG ($p<0.05$); Ang-1, Ang-2, and LN-5γ2 protein expression in ectopic endometrial tissues of the SU5416 group were lower than that of the MG ($p<0.05$), indicating that SU5416 could effectively inhibit endometrial angiogenesis-related protein expression in rats (Fig. 4).

VEGF, MMP-9

The serum VEGF and MMP-9 levels and the expression of VEGF messenger RNA (mRNA) and MMP-9 mRNA in ectopic endometrial tissues of the MG and SU5416 group were higher than those of normal endometrial tissues in SOG ($p<0.05$); The serum VEGF and MMP-9 levels and the expression of VEGF mRNA and MMP-9 mRNA in ectopic endometrial tissues of the SU5416 group were lower than those of the MG ($p<0.05$), which showed that SU5416 could effectively inhibit the serum expression of VEGF and MMP-9 and endometrial tissues of rats (Fig. 5).

ERK-VEGF/MMP-9 pathway-related protein expression

The protein expression of P-ERK, VEGF, MMP-2 and MMP-9 of ectopic endometrial tissues in the MG and SU5416 group were higher than those of normal endometrial tissues in the SOG ($p<0.05$); The protein expression of P-ERK, VEGF, MMP-2 and MMP-9 in ectopic endometrial tissues of the SU5416 group were lower than that of the MG ($p<0.05$). It is evident that SU5416 can effectively inhibit the activation of ERK-VEGF/MMP-9 pathway in rats (Fig. 6).

Inflammatory factors

The levels of serum IL-1, IL-2, IL-6 and TNF-α were higher in the MG and SU5416 group than in the SOG ($p<0.05$); The levels of serum IL-1, IL-2, IL-6 and TNF-α were
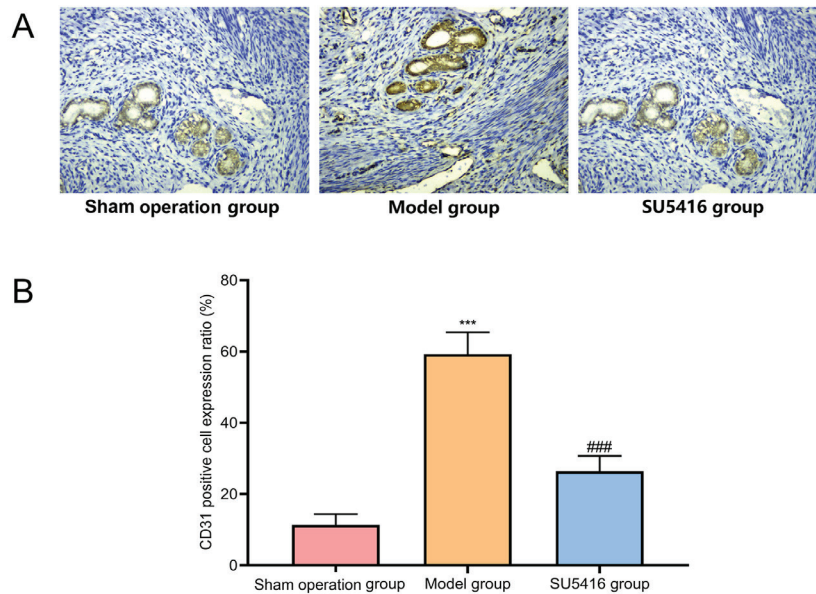
Fig. 3. Comparison of CD31-positive cell expression in endometrial tissues of rats in each group.

Shows that there was no endometriosis lesion in the sham-operation group, so the comparison was made between normal endometrial tissue and ectopic endometrial tissue from the other groups. (A) Staining of CD31-positive cells detected by immunohistochemistry (200×); (B) proportion of CD31-positive cell expression. Note: Compared with the sham operation group, ***$p<0.001$; Compared with the model group, ###$p<0.001$. Proportion of CD31-positive cell expression = number of CD31 positive cells/total number of cells × 100%.
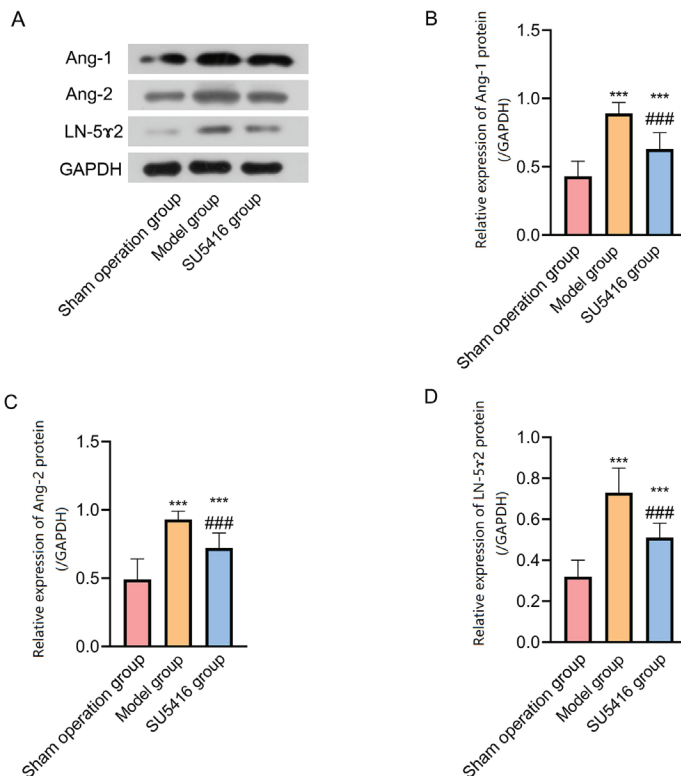

Fig. 4. Comparison of endometrial angiogenesis-related protein expression.

Shows that (B) Ang-1, (C) Ang-2, and (D) LN-5γ2 protein expressions in endometrial tissues of the SU5416 group were lower than those of the model group. Note: Ang-1: angiopoietin-1; Ang-2: angiopoietin-2; LN-5γ2: laminin-5γ2. Compared with the sham operation group, ***$p<0.001$; Compared with the model group, ###$p<0.001$.
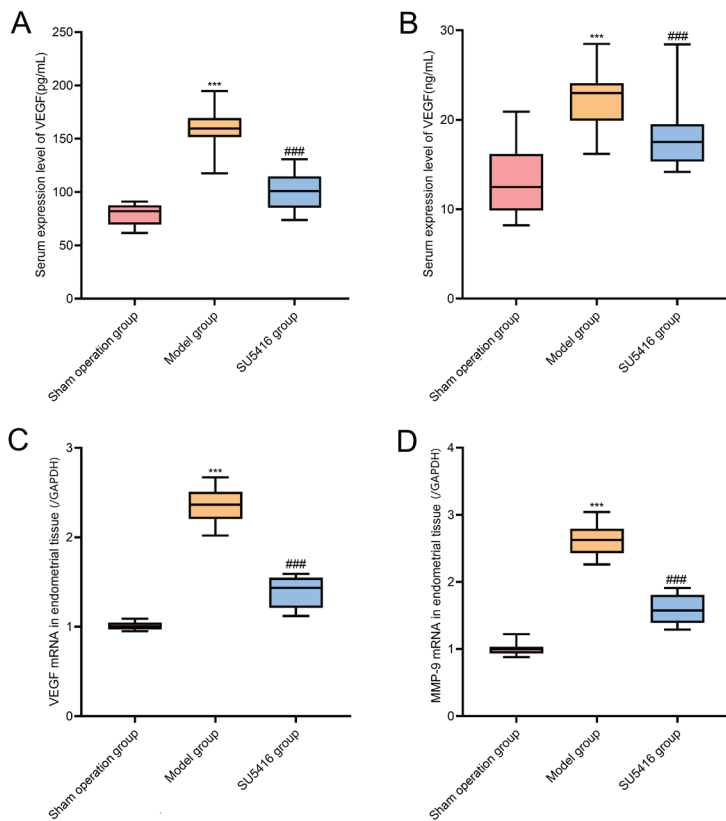
Fig. 5. Comparison of VEGF and MMP-9 expression in rats in each group.

Shows that the protein expression levels of serum (A) VEGF and (B) MMP-9 and the mRNA expression levels of (C) VEGF and (D) MMP-9 in endometrial tissue of the SU5416 group were lower than those of the model group. Note: VEGF: vascular endothelial growth factor; MMP-9: matrix metalloproteinase-9. Compared with the sham operation group, ***$p<0.001$; Compared with the model group, ###$p<0.001$.
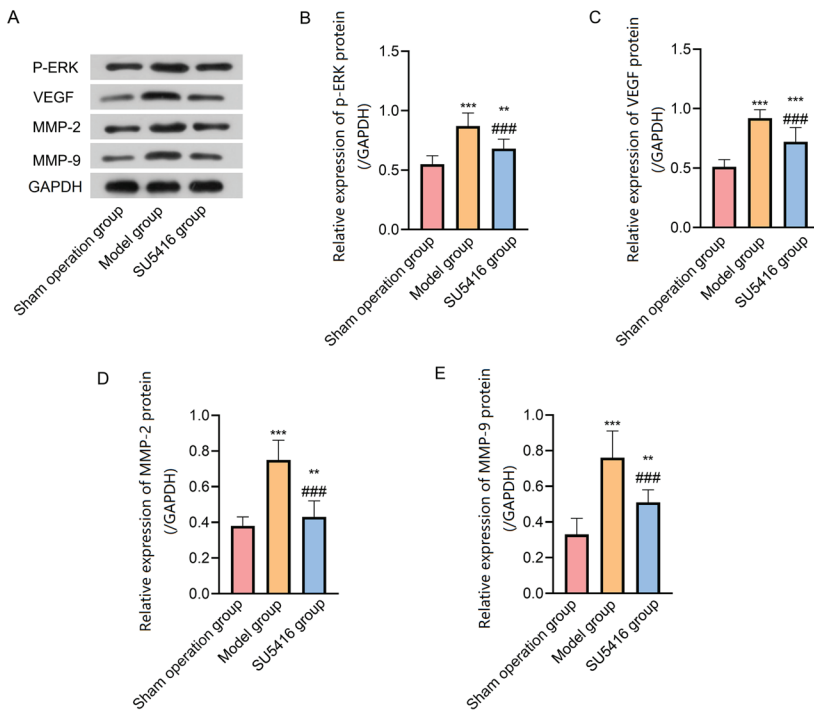

Fig. 6. Comparison of ERK-VEGF/MMP-9 pathway-related protein expression.

Shows that the expression levels of (B) P-ERK, (C) VEGF, (D) MMP-2, and (E) MMP-9 proteins in the endometrial tissue of the SU5416 group were lower than those of the model group. Note: P-ERK: phosphorylation of ERK; VEGF: vascular endothelial growth factor; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9. Compared with the sham operation group, **$p<0.01$, ***$p<0.001$; Compared with the model group, ###$p<0.001$.

lower in the SU5416 group than in the MG ($p<0.05$), which showed that SU5416 could effectively reduce the inflammatory response in rats (Fig. 7).

DISCUSSION

The etiology and mechanism of EMs are complex and diverse. The implantation theory of reverse flow of menstrual blood proposed by Sampson in 1921 is the most supported, that is, the exfoliated endometrial fragments flow back into the pelvic and abdominal cavity with the menstrual blood, and are implanted in the ovary and adjacent pelvic and abdominal cavity, causing a local inflammatory response in the peritoneum, leading to the establishment of a local microenvironment and angiogenesis, thereby providing a continuous angiogenesis stimulus for vascular remodeling [10-13]. However, either theory involves neovascularization

and degradation and reconstruction of the extracellular matrix. The reason for the initiation of pathological angiogenesis lies in the unbalanced regulation of vascular inhibitory factors and promoting factors, among which the increase of pro-angiogenic factors such as VEGF and matrix metalloproteinase (MMP) is the main cause [14]. Therefore, anti-angiogenesis is of great importance in the prevention and treatment of EMs. SU5416 is a lipid-soluble small molecule VEGF receptor signal transduction inhibitor, which can block the interaction between VEGF and its receptor and inhibit angiogenesis [15]. However, there are no reports on the effect of SU5416 in EMs. In this experiment, an EMs model was established by autologous transplantation. The results showed that compared with the MG, the volume of ectopic lesions, the proportion of CD31 positive cells and the level of serum inflammatory factors in the SU5416 group were reduced
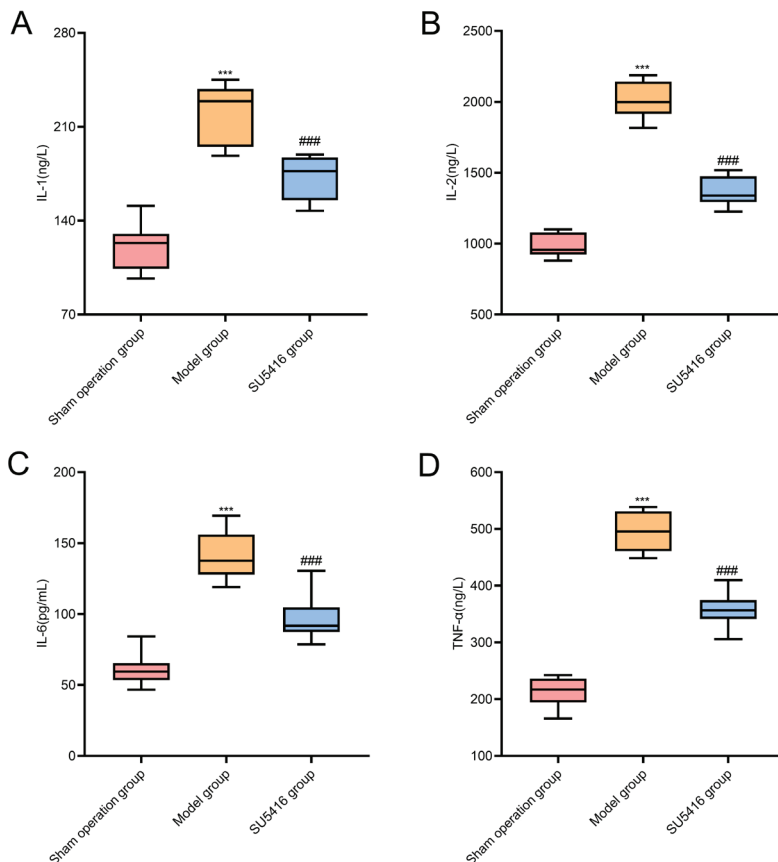

Fig. 7. Comparison of serum inflammatory factor levels in rats of each group.

Shows that the expression levels of (A) IL-1, (B) IL-2, (C) IL-6 and (D) TNF-α in endometrial tissue of the SU5416 group were lower than those of the model group. Note: IL-1: interleukin-1; IL-2: interleukin-2; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α. Compared with the sham operation group, ***$p<0.001$; Compared with the model group, ###$p<0.001$.

at 42 days after operation. Membrane epithelial cells showed atrophic changes, mesenchymal cells became smaller, and the number of blood vessels decreased. It can be seen that SU5416 can reduce the volume of ectopic lesions and prevent the inflammatory response by inhibiting cell proliferation and angiogenesis.

The ERK signaling pathway is one of the mitogen-activated protein kinase (MAPK) pathways. Activated ERK can activate downstream targets such as the 90kD ribosomal S6 protein kinase family (RSKs), and promote the translocation of RSK1/2 and pERK1/ pERK2 into the nucleus, activate early and immediate gene transcription, thereby regulating cell survival, apoptosis, proliferation, metabolism, transcription and other biological behaviors [16,17]. VEGF is one of the endothelial cell-specific vascular-derived proteins, which can promote fibrinogen exudation, increase trophoblast, endometrial and meconium permeability, promote endothelial cell proliferation and subperitoneal vascular network formation, thus inducing lesion growth and endothelial implantation; Moreover, VEGF can bind to relevant receptors on endothelial cells and initiate paracrine mechanisms via signaling pathways, which play an important role in endothelial implantation and placenta formation [18,19]. MMP-9 can degrade the main components of extracellular matrix such as collagen type IV, collagen V, and gelatin, destroy the integrity of the basement membrane, and promote the formation of new blood vessels and the sprouting of vascular endothelial cells [20]. Meanwhile, the degraded extracellular matrix protein fragments could regulate apoptosis, migration, and invasion of epithelial cells, leading to invasion into other parts of the eutopic endometrium [21]. Chen [22] *et al.* found that the ERK-VEGF/MMP-9 signaling pathway is closely related to angiogenesis, and down-regulating the expression of this signaling pathway can inhibit cell proliferation, invasion and

angiogenesis. Guo [23] *et al.* found that inhibition of extracellular ERK activation downregulated MMP-9 and VEGF expression and signaling, thereby slowing down the rate of vascular invasion and growth. Yilmaz [24] *et al.* found increased expression of P-ERK, VEGF, and MMP-9 proteins in rats with EMs, while blocking cytokine binding to surface receptors and intercellular signaling pathways could control abnormal endometrial proliferation and angiogenesis. The results of this study revealed that the expression of P-ERK, VEGF, MMP-2, and MMP-9 proteins in the endometrial tissues of the MG was higher than that of the SOG, which was consistent with the above findings and again confirmed that the activation of ERK-VEGF/MMP-9 signaling pathway might be related to the development of EMs. The expression of P-ERK, VEGF, MMP-2, MMP-9 and Ang-1, Ang-2, LN-5γ2 proteins were reduced in the endometrial tissues of rats after SU5416 treatment, which showed that the VEGF receptor inhibitor SU5416 could reduce the synthesis and secretion of extracellular signal-regulated kinases and inhibit the activation of ERK-VEGF/MMP-9 signaling pathway in ectopic endometrial tissues, thus preventing angiogenesis and the growth, implantation and adhesion of abnormal proliferating cells in the pelvic and abdominal peritoneum.

In summary, the VEGF receptor inhibitor SU5416 inhibited angiogenesis and reduced inflammatory response in rat EMs, and its mechanism of action may be related to the downregulation of ERK-VEGF/MMP-9 pathway expression. This experiment confirmed the effects of SU5416 on angiogenesis, signaling pathway and inflammatory response in rats with EMs, providing new ideas for the clinical treatment and the development of new drugs. However, further in vivo experiments are needed to verify the therapeutic effects and specific mechanism of action of the VEGF receptor inhibitor.

Conflicts of interest

Funding

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Authors Contribution

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

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