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Research article

Xue Ping Tablets treat abnormal uterine bleeding via VEGF-ERK1/2 pathway

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ABSTRACT

Objective: To investigate the protective effects against abnormal uterine bleeding (AUB) and possible mechanisms of Xue Ping tablets (XPT) using a rat model.
Methods: A total of 58 unmated female and 25 male SPF SD rats aged 8–9 weeks were selected. Eight unmated female rats were selected as the blank control group according to the complete random method. The other 50 rats were mated in a female/male ratio of 2:1. In the morning after mating, vaginal smears were collected. Presence of vaginal plug or sperm was regarded as the first day of pregnancy. All pregnant rats were given 8.3 mg/kg of mifepristone by gavage at 8:00 a.m. and 100 μg/kg misoprostol by gavage at 6:00 p.m. on the seventh day of pregnancy to induce incomplete abortion, thereby establishing a rat model of AUB. Forty rats were randomly divided into model, low- (220 mg/kg), medium- (441 mg/kg), high-dose (882 mg/kg) XPT, and positive control groups. The positive group was given 130 mg/kg Gong Xue Ning (GXN). The model group and the blank group were given an equal amount of distilled water.
Results: Compared with the model group, the volume of bleeding in the positive and middle- and high-dose XPT groups decreased ($P < 0.05$). Moreover, compared with the model group, the progesterone levels in the positive and XPT groups were significantly increased. Immunohistochemistry showed that XPT significantly decreased the expression levels of VEGF, *p*-ERK, NF-κB, SAA, MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3. WB results showed that XPT significantly decreased the expression levels of *p*-ERK, MMP-9, NF-κB, MMP-2 and VEGF. QRT-PCR results showed that XPT significantly decreased the expression levels of VEGF, NF-κB, SAA, MMP-2, TIMP-1, TIMP-2 and TIMP-3 ($P < 0.05$).
Conclusions: XPT could reduce AUB by inhibiting the inflammatory factors involved in the VEGF-ERK1/2 pathway.

1. Introduction

Abnormal uterine bleeding (AUB) refers to any abnormality in frequency, regularity, length and bleeding volume during a menstrual cycle [1]. Hemostasis disorder, endometrial damage and inflammatory overreaction in uterine tissues are the main pathological factors of AUB [2–6]. The mechanism of AUB involves the VEGF-ERK1/2 pathway [7–16].

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Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, are mainly expressed on the glandular epithelium, stroma and vascular basement membrane of the endometrium. MMPs can decompose extracellular matrix (ECM) during such processes as angiogenesis, autoimmunity, tissue remodeling, cancer metastasis and invasion. This function of MMPs can be inhibited by tissue inhibitor of metalloproteinase (TIMP), the upregulation of which can activate MMPs to degrade ECM excessively, then, the content of type IV collagen decreases, the contact between vascular endothelium and basement membrane weakens, and the fragility of blood vessels increases, all contributing to prolonged uterine bleeding and increased bleeding volume.

MMP-2/9, a type IV collagenase, shows a hydrolytic activity enhanced in AUB patients. MMP-2/9 overexpression promotes the degradation of vascular basement membrane and the infiltration of inflammatory cells, then, with the destruction vascular integrity and arousal of inflammatory reaction, endometrial repair is disrupted, leading to AUB. The role of inflammatory response in AUB has received increasing attention. Recent studies have shown that cells undergo hyperinflammation during AUB. In addition, the binding of VEGF with its receptor can increase the expression level of MMP, thereby degrading collagen fibers, increasing vascular fragility, further leading to fibrinolysis, decreased hemostasis, and abnormal bleeding. VEGF mediates angiogenesis in the development and repair of endometrium, probably relying on the activation of extracellular regulated kinase (ERK) 1/2. Knocking down vascular endothelial growth factor (VEGF) can effectively improve AUB. ERK has also been proven as a key indicator of AUB. The up-regulation of VEGF promotes the phosphorylation of ERK1/2 to increase MMP-2/9 expression and uterine bleeding, suggesting that VEGF and ERK could be potential targets for AUB prevention.

Oral drugs, such as progesterone, short-term contraceptives, and levonorgestrel, are currently recommended for the treatment of AUB, but its recurrence rate after drugs discontinuation is high, and intermittent breakthrough bleeding during medication is great concern. In recent years, Xue Ping Tablets (XPT), a preparation of traditional Chinese medicine (TCM), has achieved significant therapeutic efficacy and safety in the prevention and treatment of AUB. XPT are produced by Jiangxi Puzheng Pharmaceutical Co., Ltd., and its main ingredients include seven kinds of Chinese herbal medicines, including raw Rehmannia, wine Rhubarb, Sanguisorba officinalis, Notoginseng, Cuttlebone, Madder, Pollen Typhae Parch. It has the effects of clearing heat, removing blood stasis, stopping bleeding and regularizing menstruation. Clinical reports also have obvious effects on preventing postpartum hemorrhage [17]. Our study aimed to investigate how XPT treat AUB by inhibiting the inflammatory response mediated by the VEGF-ERK1/2 pathway.

Fig. 1. Experimental research flow chart.

In this study, a rat model of AUB induced by incomplete abortion was constructed. The effects of XPT on uterine bleeding-related markers, including serum estradiol (E2) and progesterone, VEGF, *p*-ERK, MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3 and NF-κB, were observed, and the regulatory mechanism was elucidated. The experimental research design is shown in Fig. 1.

2. Materials and methods

2.1. Animals

A total of 58 (25 male) specific pathogen-free (SPF) Spraque Dawley (SD) unmatched female rats (age 8–9 weeks, weight 200–250 g, license number SCXK [Beijing] 2021-0011) were provided by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., and fed at the Pharmacological Experiment Center of Jiangsu Provincial Hospital of Chinese Medicine in 12 h/24 h light-dark cycles at room temperature (24 ± 3 °C) and a humidity of 55–65 %, with free accesses to food and water. This experiment was approved by the Ethics Committee of Experimental Animal Research of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Animal Ethics No.: 2021 DW-39-02).

2.2. Modeling

After one week of routine feeding and acclimatization, the female rats were examined with vaginal pictures to confirm that they had a normal ovulation cycle. Eight unmatched female rats were selected as the blank control group, according to the complete randomization method. Except for the blank group, the other rats were mated according to in a male/female ratio of 2:1. The next morning, vaginal smears were examined to find the vaginal plug or sperm as the first day of pregnancy. All pregnant rats were given mifepristone 8.3 mg/kg by gavage at 8:00 a.m. and 100 μg/kg misoprostol by gavage at 6:00 p.m. on the seventh day of pregnancy to induce incomplete abortion, by which a rat model of AUB was established [4,5]. The quantitative cotton ball (70–80 mg) was rubbed into a slender cotton strip, and placed into the vagina after administration. The vaginal cotton strip was taken out the next morning, and the model was successful if the cotton strip was stained with blood. Then, 40 rats were randomly averaged into model, low-, medium- and high-dose XPT, and positive control groups, with eight rats in each group. The intervention was started on the 8th day after the model was established. The drugs were dissolved in double distilled water and prepared into suspensions. According to the Pharmacological Experimental Methodology edited by Xu Shuyun, the dose for the rats was converted by the body surface area ratio between humans and animals, and administered by gavage. Correspondingly, the dose for rats was 6.3 times that of humans when the body weight of a woman was 60 kg. Therefore, the XPT dose was set as follows: 220 mg/kg in low dose group, 441 mg/kg in medium dose group, 882 mg/kg in high dose group; The positive group was given Gongxuening 130 mg/kg [18]. The model group and blank group were given equal amount of distilled water.

2.3. Determination of uterine bleeding volume

The rats were infused with misoprostol at 6 p.m. on the 7th day of pregnancy, and then a quantitative cotton sliver (cotton ball weight 70–80 mg) was inserted into the vagina of the rats, taken out at 8:00 a.m. the next day, replaced with a new cotton sliver, which was then taken out at 5:00 p.m. and replaced with a new cotton sliver at the day. Finally, the cotton sliver was taken out, put into a plastic bag, and stored in a refrigerator at 4 °C. This operation was performed daily, until the seventh day of administration. Afterward, every 20 μ of rat orbital venous blood was collected and dissolved with 4 mL of 5 % NaOH. The cotton sliver of each rat was put into a beaker, soaked using 50 g/L NaOH solution, squeezed and scrubbed. The cotton sliver was first soaked with a small amount of blood, then with a plenty of blood. Then, the soaking solution was combined, mixed well, and centrifuged at 800r/min for 5min. The absorbance was determined at 546 nm wavelength of the enzyme marker. Vaginal bleeding volume (mL)=(venous blood volume 0.02 mL) × (Absorbance of sliver washing solution × V2)/(venous blood absorbance × V1). Among them, V1 is the amount of NaOH solution used to dilute venous blood (4 mL), and V2 is the amount of NaOH solution used to extract uterine bleeding cotton ball.

2.4. H&E staining

Rat uterine tissue was extracted from 4 % paraformaldehyde, embedded in paraffin, sliced, dewaxed with xylene I and II, and subjected to high to low concentrations of ethanol. Finally, the tissue was placed in distilled water and stained with hematoxylin solution for 5 min. The differentiation solution was differentiated for 20 s, washed with double distilled water, dyed with eosin staining solution for 3 min, rinsed with double distilled water again, dehydrated with gradient ethanol, made transparent twice with xylene, and finally sealed with neutral gum.

2.5. Immunohistochemistry

Immunohistochemical sections were prepared as did in H&E staining. The tissue sections were placed in EDTA antigen repair buffer for 15 min and washed with PBS for 5 min and 3 times. Then, the sections were incubated at room temperature in a 3 % hydrogen peroxide solution for 25 min and washed with PBS for 5 min and 3 times. At room temperature, BSA was sealed for 30 min. Then, one antibody was added, 4 °C, overnight, washed with PBS for 5 min and 3 times. Incubate with secondary antibody for 50 min and wash with PBS for 5 min × 3 times. DAB color solution was added and the sections were re-dyed with hematoxylin for 3 min. Finally, the

sections were dehydrated and sealed.

2.6. Western blot

Rat uterine tissue was added with RAPI lysate to extract the total protein. The protein concentration was determined by BCA method. The tissue samples were loaded, transferred to PVDF membrane after SDS-PAGE electrophoresis, incubated with the primary antibody overnight at 4 °C, then with the secondary antibody (1:4000) at room temperature for 1 h, developed with chemiluminescence solution. The gel imaging system was used to collect images to obtain expression bands of VEGF, *p*-ERK, ERK, MMP-2, MMP-9, NF-κB, and GAPDH proteins.

2.7. Quantitative real-time PCR (qRT-PCR) analysis

Every 10 mg of uterine tissue was placed into 1 ml of Trizol with 3 small iron beads, ground at 60HZ for 60 s and three times, allowed to stand at room temperature for 5 min, further added with 0.2 ml of chloroform. The EP tube was covered, shaken vigorously at 4 °C, and centrifugated 12000 at rpm for 15 min. The upper aqueous phase was gently transferred to a new EP tube, added with 0.5 ml of isopropanol and mixed thoroughly, allowed to stand at room temperature for 10 min, centrifugated at 4 °C, 12000 rpm, for 15 min. The supernatant was discarded, added with 1 ml of 75 % ethanol, gently mixed, centrifugated at 4 °C and12000 rpm for 5 min. The supernatant was discarded. The remaining was allowed to dry naturally, added with 20 μ l enzyme-free water, shaken, dissolved, and detected with a spectrometer. Then qRT-PCR was performed. The primers were listed in Table 1. The ratio of the mRNA expression of target gene to that of β-actin was defined as $2^{-\triangle\triangle Ct}$.

2.8. Kits for estradiol and progesterone

Levels of estradiol and progesterone were measured using assay kits (Nanjing Yifeixue Biotechnology Co., Ltd, Nanjing, China) according to the instruction of manufacturer.

2.9. Molecular docking

The crystal structure of VEGF was obtained from RCSB Protein Data Bank. Then protein structure was shown in Discovery Studio 2016 Client software. Corresponding small molecular structures were downloaded from TCMSP. Small molecules and proteins were dehydrated and hydrogenated, and then Autodock Vina was used for molecular docking.

2.10. Statistical analysis

SPSS 20 software was used to statistical analysis. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. XPT reduced blood volume and increased progesterone level in AUB in vivo

Compared with the control group, the blood volume in the model group significantly increased ($P < 0.01$). Compared with the model group, the blood volumes in the positive control model, middle-dose, high-dose groups significantly decreased ($P < 0.05$). Compared with the control group, the progesterone level in the model group significantly decreased ($P < 0.01$). XPT and GXN significantly increased the progesterone level ($P < 0.05$). There was no significant difference in serum E2 hormone level among groups ($P > 0.05$) (Fig. 2A and B).

Table 1
Primers used in Real-Time Quantitative PCR analysis.

Target Gene	Forward Primer	Reverse Primer
Rat MMP2	AGTATGGGAACGCTGATGGC	TTGTAAGAGGTGCCCTGGAAG
Rat MMP9	GCAAACCCTGCGTATTTCCATT	GCGATAACCATCCGAGCGAC
Rat TIMP1	CCTGGTTCCCTGGCATAATC	GATCGCTCTGGTAGCCCTTC
Rat TIMP2	CGCTGGACGTTGGAGGAAAG	CGTGTCCCAGGGCACAATAAA
Rat TIMP3	GGCCAAAGTGGTGGGAAAGA	AGGTGGTAGCGGTAATTGAGGC
Rat VEGF	CAATGATGAAGCCCTGGAGTG	GCTCATCTCTCCTATGTGCTGG
Rat NFKB	CAGATACCACTAAGACGCACCC	CTCCAGGTCTCGCTTCTTCACA
Rat SAA	GCCTCTTGATCCTGGGAGTTG	CCCGAGCATGGAAGTATTTGTC
Rat GAPDH	CTGGAGAAACCTGCCAAGTATG	GGTGGAAGAATGGGAGTTGCT

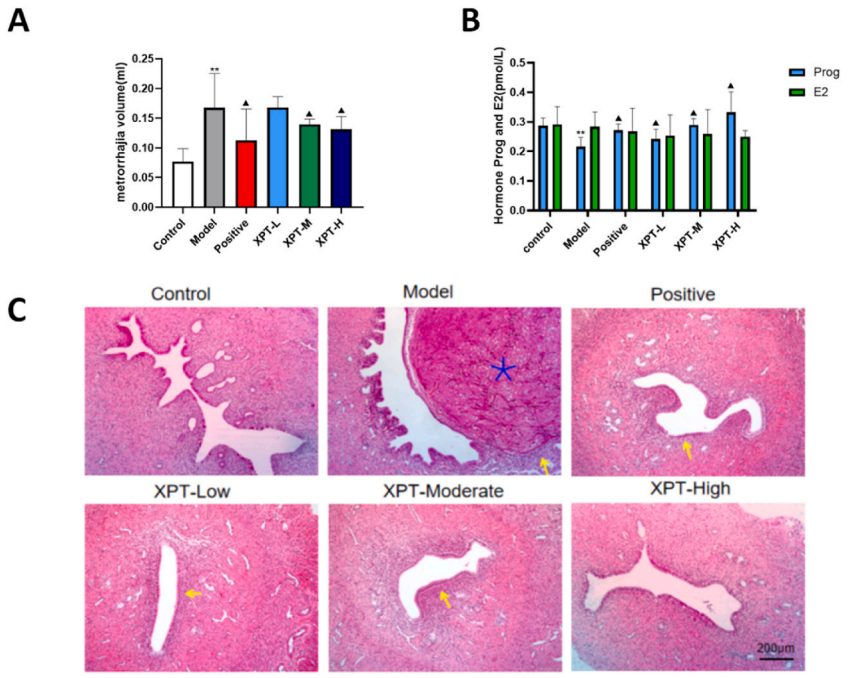

Fig. 2. Effects of XPT on uterine bleeding amount in rats (A). Effects of XPT on progesterone and estradiol in rats (B). Effects of XPT on endometrial histomorphology in rats (C). $^{**}P < 0.01$ as compared with the control group. $^{\triangle}P < 0.05$ as compared with the model group.

3.2. XPT alleviated pathological damage of endometrium

In the model group, H&E staining showed demonstrated that pregnancy sac was not completely discharged, and villous tissue resided under the endometrium, inflammatory cells infiltrated into the endometrium, and the number of blood vessels increased, but the number of endometrial glands decreased. Compared with the model group, there no embryo residue, more glands and blood vessels, fewer infiltrating inflammatory cells were observed in the endometrium of the low-dose, medium-dose, and positive control groups. Moreover, the morphology of embryos the high-dose group was close to that of the normal group (Fig. 2C).

3.3. Chemical components in XPT and their binding sites with VEGF

To investigate how XPT interact with VEGF, we performed high performance liquid chromatography (Fig. 3A). The main chemical components of XPT are Adenosine, Nicotinic acid, and Gallic acid (Fig. 3B–D). The binding modes of Adenosine, Nicotinic acid, and Gallic acid with VEGF are shown in Fig. 3E–G. The results showed that a lot of chemical compounds in XPT had binding sites with VEGF.

3.4. XPT promoted VEGF, p-ERK, inflammation indexes in vivo

According to the above results, we speculated that XPT may relieve AUB through repressing inflammatory responses. Therefore, we observed the effects of XPT on VEGF, ERK, *p*-ERK, inflammatory indexes in vivo. Western blot results indicated that XPT significantly decreased the expression of VEGF, *p*-ERK, MMP-2, MMP-9 and NF-κB in rats (Fig. 4A and B). qRT-PCR results showed that XPT significantly decreased the expression of VEGF, MMP-2, TIMP-1, TIPM2, TIMP3,NF-κB and SAA in rats (Fig. 5A–H). Immunohistochemistry showed that XPT significantly decreased the expression of VEGF, *p*-ERK, SAA, NF-κB, MMP-2, MMP-9, TIMP-1, TIPM2 and TIMP3 in rats (Fig. 6A–J). ELISA results showed that XPT significantly decreased the expression of TNF-α, IL-6 and IL-8 (Fig. 7A–C). These data suggested that XPT could downregulate inflammation indexes in vivo to relieve AUB. The schematic diagram of XPT in treating AUB via the VEGF-ERK1/2 pathway is shown in Fig. 8.

4. Disscussion

AUB caused by ovulatory dysfunction (AUB-O) occurs in about 50 % of all AUB cases [19]. In 2020, the AUB-O criteria for diagnosis and treatment incorporated five features in TCM, including “metrorrhagia”, “premenstruation”, “excessive menstruation”, “prolonged menstruation” and “menstrual bleeding” [20]. Drugs for ABU mainly include tranexamic acid, oral contraceptives, progesterone and estrogen and progesterone. However, hormone treatment takes a long time, and may be poorly complied by some patients [21]. In addition, juvenile and menopausal women are not suitable for hormone treatment. Therefore, a considerable number of patients are

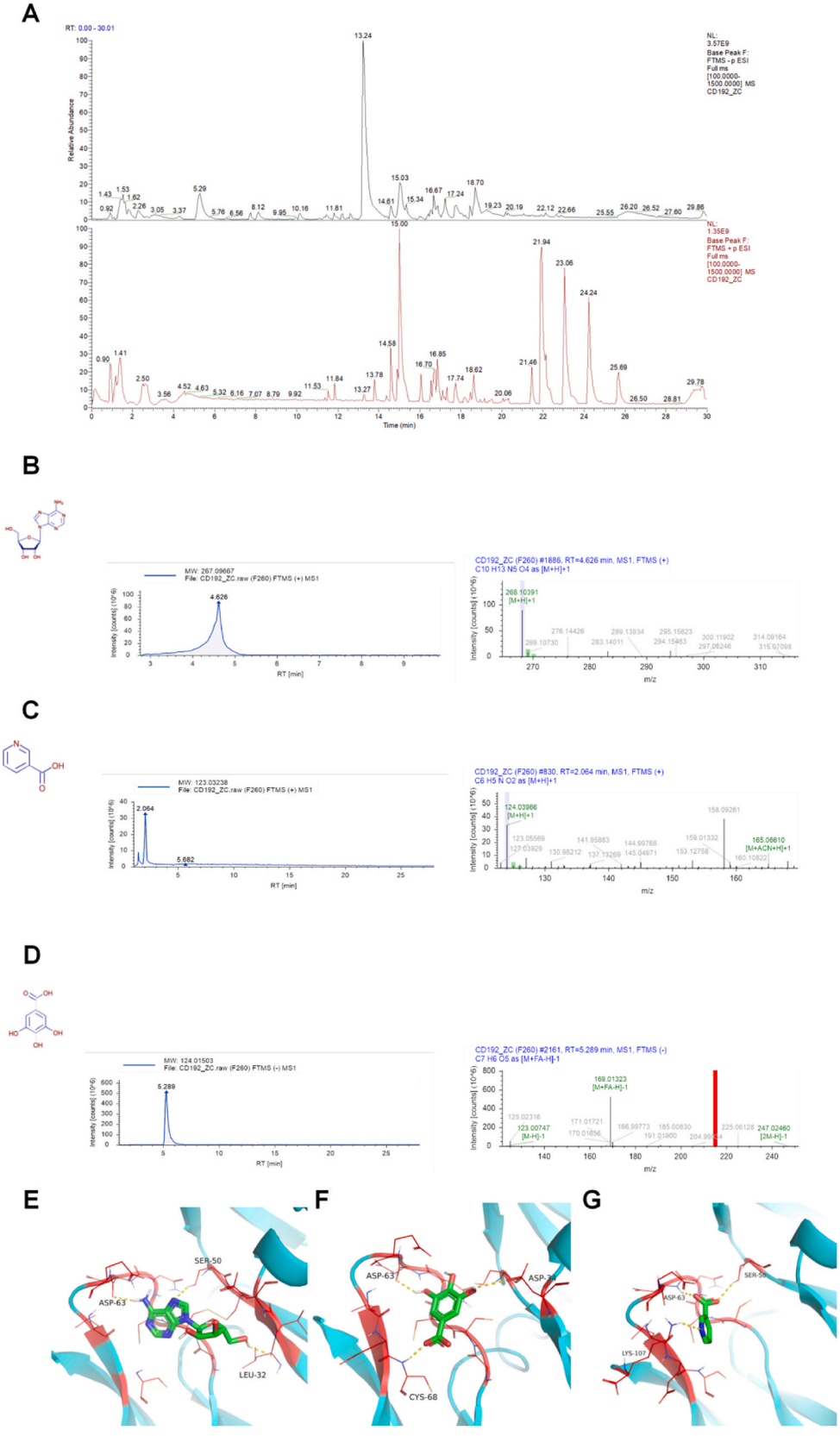

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Fig. 3. Chemical components in XPT and their binding sites with VEGF. A. Total ion chromatograms of XPT (green-negative mode, red-positive mode). (B–D) Chemical structures, chromatograms and MS/MS pictures of Adenosine (B), Nicotinic acid (C), and Gallic acid (D). (E–G) Possible binding sites of VEGF and Adenosine (E), Nicotinic acid (F), or Gallic acid (G) were predicted using computer.

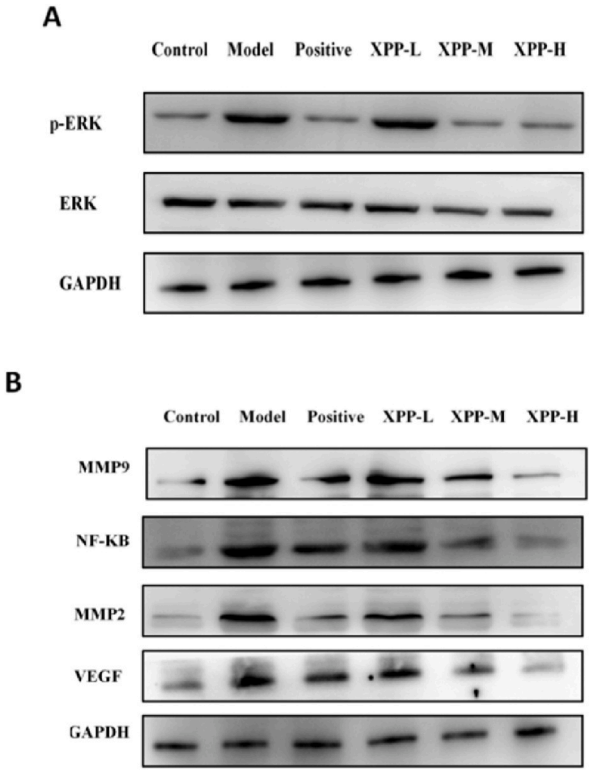

Fig. 4. Effects of XPT on protein levels of ERK, *p*-ERK (A), MMP9, NF-κB, MMP2, VEGF (B) in rats were examined by Western Blot.

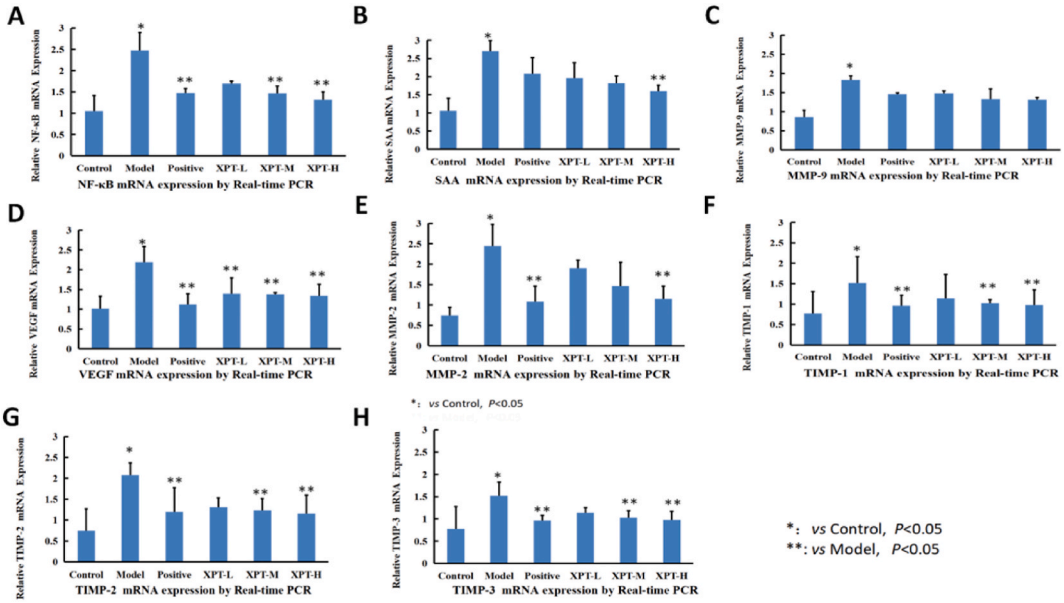

Fig. 5. Effects of XPT on mRNA levels of VEGF (A), NF-κB (B), SAA (C), MMP2 (D), MMP9 (E), TIMP-1 (F), TIMP-2 (G), TIMP-3 (H) in rats were examined by using qRT-PCR. *$P < 0.05$ as compared with the control group. **$P < 0.05$ as compared with the model group.

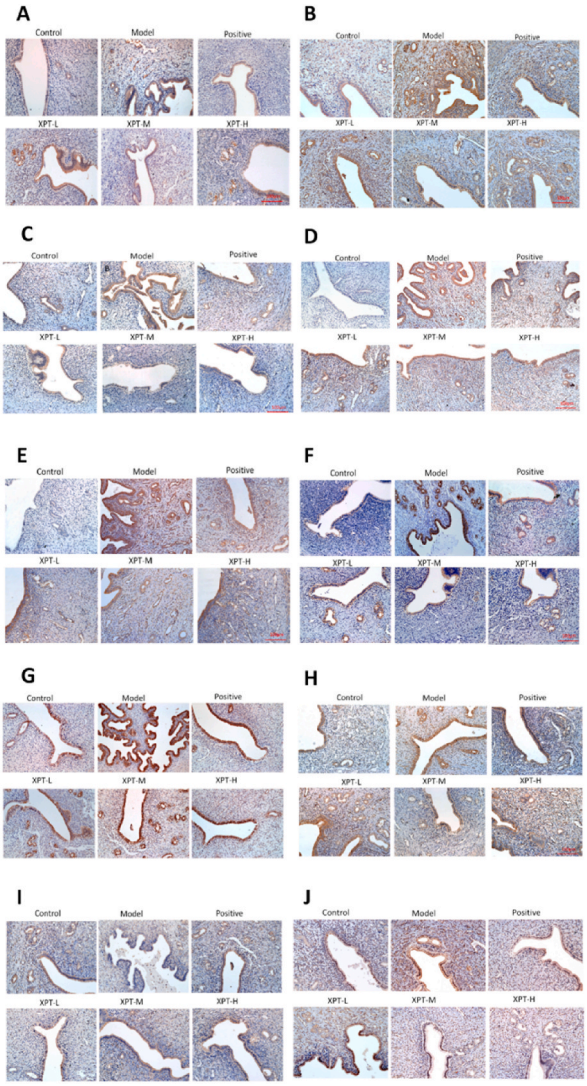

Fig. 6. Effects of XPT on protein levels of VEGF (A), NF-κB (B), SAA (C), MMP2 (D), MMP9 (E), TIMP-1 (F), TIMP-2 (G), TIMP-3 (H), ERK (I), *p*-ERK (J) in rats were examined by immunohistochemistry.

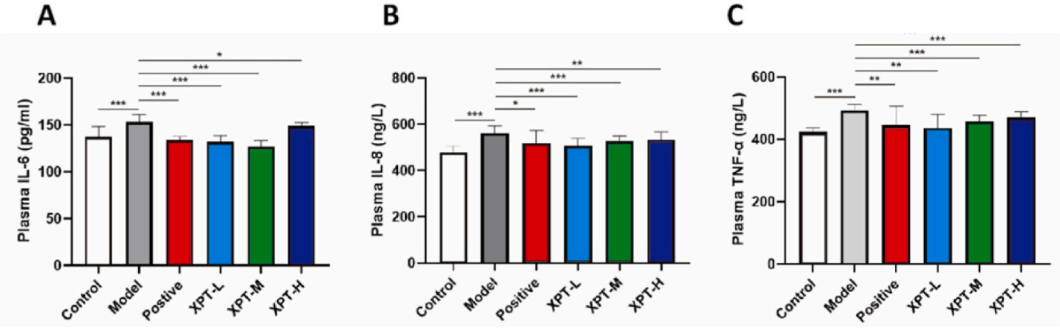

Fig. 7. Effects of XPT on the serum levels of TNF-α (A), IL-6 (B) and IL-8 (C) in rats were examined by using ELISA. *$P < 0.05$, $P < 0.01$, $P < 0.001$.

more prone to using TCM therapies, such as XPT [22–25], due to its evident efficacy, mild side effects, convenient use, etc.

In local angiogenesis, new microvessels generate to form a network. Upon local hypoxia and ischemia, inflammatory factors increase in the endometrium damaged or infected, and then the genes encoding VEGF are activated to overexpress VEGF. During the endometrial repair in rats with AUB, continuous uterine bleeding may undermine vascular regeneration and blood supply, resulting in

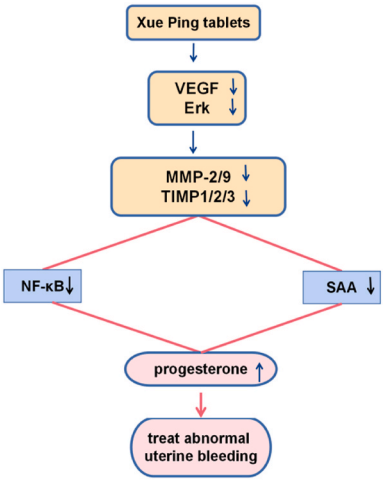

Fig. 8. Schematic diagram of VEGF-ERK1/2 pathway in the mechanism of XPT.

a persistent and high level of VEGF and finally a poor repair. In the present study, compared with that in the control group, the endometrium in the model group had a higher expression of VEGF, indicating that VEGF may participate in the occurrence and development of AUB. XPT can be used to stop the blood flow out of endometrium by removing blood stasis and facilitating hemostasis, thus promoting the repair of endometrium and reducing the expression of VEGF. Jiang et al. have found [26,27] that some TCM preparations can down-regulate the expression of VEGF in the serum and endometrium of patients with uterine cavity adhesion, the mechanism of which may involve tonifying the kidney and activating the blood circulation cycle.

MMPs are proteolytic enzymes that can degrade and reshape ECM. TIMPs are natural inhibitors of almost all MMPs [28–30]. MMP-9, one member of the MMP family, is a type of gelatinase. Its main function is to dissolve gelatin and type IV collagen that are mainly distributed in the stroma, adventitia, glandular epithelium and other parts of the endometrium. If MMP-9 excessively dissolves collagens and destroys vascular basement membranes, the stroma and vascular wall may degenerate, leading to AUB. As a specific inhibitor of MMP-9, TIMP-1 works with MMP-9 to maintain the integrity of endometrial ECM and basement membrane. In our present in vivo experiments, a rat model of AUB was constructed using mifepristone and misoprostol. Then, XPT were used to intervene for seven consecutive days. The results showed that XPT employ the VEGF-ERK1/2 pathway to repress inflammatory response in AUB. The inflammatory response in AUB engages multiple mechanisms and effectors. The present study verifies the specific function of the VEGF-ERK1/2 pathway in AUB. With the deepening of research, it may reveal more secrets about inflammatory response. Nevertheless, inflammatory inhibitors can exert therapeutic effects on AUB. Our experimental data demonstrated that XPT improved AUB via VEGF-ERK1/2 pathway. The schematic diagram of the therapeutic mechanism of XPT is shown in Fig. 8.

It has been found that inflammatory reactions run through the entire process of AUB and change with in external stimuli. Therefore, interventions into inflammation should be taken as long-term strategy. Meanwhile, more explorations are needed to standardize the dose of XPT. Our current study is preliminary. More depth researches should be performed to validate our findings.

5. Conclusions

The present study demonstrated the effects of XPT on AUB and the underlying mechanism related to VEGF-ERK1/2 pathway. XPT inhibited the expression of VEGF, ERK, MMP2/9 and TIMP1/2/3, to attenuate uterine inflammation and improve AUB. In total, our study provides the basis for the treatment of AUB with XPT.

Ethics approval

This experiment was approved by the Ethics Committee of Experimental Animal Research of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Animal Ethics No.: 2021 DW-39-02).

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Horizontal Subject of Nanjing University of Chinese Medicine (2021067).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Suqin Zhong: Writing – review & editing, Project administration, Formal analysis, Conceptualization. **Lichao Qian:** Writing – original draft, Data curation. **Yong Tan:** Writing – review & editing, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30079.

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