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Research

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TJCYR ameliorates embryo implantation dysfunction through PI3K/Akt/eNOS signalling to improve endometrial receptivity

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Key words Tiao Jing Cu Yun Recipe; endometrial receptivity; embryo implantation dysfunction; biomarker; angiogenesis; PI3K/Akt/eNOS

Abstract:

Backgroud: In our clinical practice, we found that Tiao Jing Cu Yun Recipe (TJCYR), which was composed of Dangshen, Danshen, Danggui, Huangqi, Shudihuang, Bajitian and Yinyanghuo, had obviously enhanced the rate of pregnancy in the women with infertility. Although the application effects are desirable and satisfactory, the therapeutic mechanism of TJCYR remains poorly understood. In this study, we evaluated the effect of TJCYR on embryo implantation dysfunction (EID)-induced damage of endometrial receptivity in mice and investigated the mechanisms underlying the effect.

Methods: Pregnant mice were randomly divided into six groups: Control, EID only, Progesterone (Prog)+EID, TJCYR-low-dose+EID, TJCYR-medium-dose+EID, TJCYR-high-dose+EID. Mifepristone was injected to make EID model. On the eighth day of pregnancy, the mice were sacrificed and the number of uterus-implanted blastocysts was counted. On the fourth day of pregnancy, the serum was to analyze the level of hormone by radioimmunoassay, the uterus was to analyze morphology by H&E and SEM, the combination of immunofluorescence and western blot were to identify the related proteins.

Results: Compared with the EID group, the mice treated with high-dose TJCYR had a greater number of implanted sites, so we choose that dose of TJCYR as treatment in the following study. The mice treatment with

TJCYR could significantly enhance the level of P_4, and inhibite the decrease in the expression of PR that induced by EID. Compared with the EID only, the SEM showed that a marked increase in the number of well-developed pinopodes in the TJCYR treatment group. Except morphological marker, several molecules in relation to pinopodes that could be used as biomarkers. TJCYR abrogated the EID-induced weakened in those biomarkers. Additionally, the vascular density and VEGF were decreased in the EID group, it appeared severe tissue hypoxia, while TJCYR reversed that change. Compared with the control, the p-Akt and p-eNOS were decreased in EID group, accompanied with decline of NO. While TJCYR promoted the activation of Akt and eNOS, to improve the poor microvascular environment of endometrium.

Conclusion: TJCYR has therapeutic potential against poor endometrial receptivity via activation of the PI3K/AKT/eNOS signaling pathway.

Background

The incidence of infertility has been one of the health problems worldwide in women[1], which increase the economic, physical, and emotional burden on approximately 9% of couples worldwide[2]. Although access to assisted reproductive technology (ART) is abundant in developed countries and has overcome the majority of infertility causes, success rates have stagnated to around 30%[3]. The successful implantation of embryo is a dynamic process that occurs as a result of interplay among the quality of embryo and the status of endometrium. In the recent years, advances in ART allowed for the selection of high quality embryos, but poor reproductive outcomes remained high. It has been confirmed that 2/3 of in vitro fertilization and embryo transfer (IVF-ET) implantation failures were considered to be due to uterus with decreased endometrial receptivity[4]. Endometrial receptivity refers to the finite window in each menstrual cycle in which the endometrium is sufficiently prepared for an implanting blastocyst, therfore improving endometrial receptivity is the key point to rise the pregnancy rate in women with infertility[5-8].

After a long period of research, there are some markers to assess endometrial receptivity, including morphological and biochemical changes. Pinopodes are ultrastructural changes on the apical surface of the luminal epithelium of the uterus. There are also some biochemical

changes, such as Integrins, leukaemia inhibitory factor, osteopontin and so on, which are synchronous with pinopodes.

Tiao Jing Cu Yun Recipe (TJCYR), which consists of Dangshen, Danshen, Danggui, Huangqi, Shudihuang, Bajitian, Yinyanghuo, is a traditional Chinese medicine that has been widely used as an infertility treatment. Lin et al. found that TJCYR could improve kidney deficiency syndrome of anovulatory infertility patients, regulate the follicular development, and elevate the pregnancy rate. Its actions might be associated with regulating their sex hormones, expressions of ovary local factors such as INHB, ACTA, and FS[9]. Huang et al. found that TJCYR can significantly improve the ovulation rate and the reproductive function of endocrine axis in sterile rats caused by androgen[10]. Yu et al. reported that *Epimedium brevicornu* Maxim. and *Morinda officinalis* How. improved endometrial receptivity in ovulation stimulation (OS) and EID mice through significant improvements in the spatial and temporal expression of pinopodes, accompanied with significantly increase the number of embryonic implantation sites[11]. Tao et al. reported that Bushen Huoxue Formula, which is composed of TJCYR, can improve the ACT-INH-FS system in patients with polycystic ovary syndrome of kidney deficiency and blood stasis pattern[12]. In our clinic, we found that TJCYR had a good effect on pregnancy, but the pharmacological mechanism of its action is still under investigation. The aim of this study was to document

the beneficial effect of TJCYR on EID-induced damage of endometrial receptivity in the mice and to investigate the mechanism of action. From this point of view, we have strived to find novel candidates from traditional medicinal herbs for improving endometrial receptivity.

Materials and Methods

Preparation of TJCYR

TJCYR is composed of Dangshen, Danshen, Danggui, Huangqi, Shudihuang, Bajitian, and Yinyanghuo in a dry weight ratio of 20:20:20:20:15:12:12. All herbs were supplied by the Shanghai Kangqiao Chinese Medicine Tablet Co., Ltd (Shanghai, China) (Table 1). The decoction pieces mixture was added with water of 4 times volume, boiled 2 times, concentrated using a rotary evaporator, and obtained the equivalent crude content of 2.0g/ml. The dosage of TJCYR represents the dry weight of the raw herbs used to produce decoction. 10 times of the normal dosage for adult human was defined as the dosage of TJCYR for mice. Thus, mice in TJCYR group were given 10g/kg/day of TJCYR.

Table1. Tiao Jing Cu Yun Recipe (TJCYR) components

Chinese term	Generic name	Scientific name	Product lot
Dangshen	*Codonopsis pilosula (*Franch.*)* Nannf.	Codonopsis radix	171011
Danshen	*Salvia miltiorrhiza*	Salviae miltiorrhizae	170701

	Bge.	radix et rhizoma	
Danggui		Angelicae sinensis	170606
	Angelica sinensis	radix	
	(Oliv.) Diels		
Huangqi	*Astragalus*	Astragali radix	160829
	membranaceus		
	(Fisch.) Bunge.		
Shudihuang	*Rehmannia glutinosa*	Rehmanniae radix	170420
	*(*Gaert.*)* Libosch.ex	praeparata	
	Fisch.et Mey.		
Bajitian	*Morinda officinalis*	Morindae officinalis	171219
	How.	radix	
Yinyanghuo	*Epimedium brevicornu*	Epimedii folium	170723
	Maxim.		

Fingerprinting high-performance liquid chromatography (HPLC) analysis

The phytochemical property of TJCYR was examined by HPLC analysis, comparing it to known compounds that included Calycosin-7-glucoside, Acteoside, Salvianolic acid B, Icariin, Tanshinone IIA. The multi-components of the TJCYR were performed on the 1200 series HPLC device (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler (G1329B), thermostatted column compartment (G1316A), quaternary pump (G1311A), photodiode array detector (G1315D), and degasser (G1322A). HPLC was performed on an Apollo

C18 (4.6×250mm;partical size, 5μm; GRACE, Columbia, Maryland, USA) with a mobile phase of acetonitrile (A) - 0.1% (v/v) phosphoric acid (B) for gradient elution (0–70 min, 1-40% A; 70-90 min, 40-80% A). The analysis was operated at a flow rate of 1 mL/min, a column temperature of 30 ℃ and a detection wavelength of 260nm. The injection volume was 10μL. The standard solutions and sample were filtered with a 0.45μm membrane filter before subjecting them to HPLC analysis.

Experimental animals

All experimental protocols were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China). Adult female and male Kunming mice (weighing 25-28g) were purchased from Beijing Vital River Laboratory Animal Technology Co, Ltd. (Beijing, China). The female mice were virginal and the male mice had been proven to be fertile. The mice were housed in cages separately with controlled temperature and humidity, 12h light-dark periods, and free access to water and a standard diet. Prior to the experiments, all mice were given a period of about 2 days for acclimatization. The two estrous cycles were observed by using vaginal smears in female mice before the treatment.

Treatments and Establishment of EID

The female mice were mixed with male mice in ratio of 2:1 mating from 19:00 to 07:00 and then checked for the presence of a vaginal plug at

08:00 the next day. Females presenting vaginal plugs (classified as day 1 of pregnancy, Pd1) were randomly divided into six experimental groups (n=20 for each group), including the Control, EID only, Progesterone (Prog) +EID, TJCYR-low-dose+EID, TJCYR-medium-dose+EID and TJCYR-high-dose+EID. In the Control and the EID only group, the mice were given intragastric administration of physiological saline solution once daily for 4 days, the treatment group were given Prog and TJCYR respectively. Mifepriston (0.1mg/mouse) was subcutaneously injected at Pd4 in the morning, to establish the EID model. Mice were sacrificed on Pd4 and Pd8, respectively. The uteri were harvested for the evaluation of endometrial receptivity and the potential mechanism (Figure1A).

Determination of implantation sites number

In one set of our experiment, the gestational mice were sacrificed on Pd8. On this time, the implantation sites in the uterus were captured easily. And the number of implantation sites was counted.

Radioimmunoassay for the test of E_2 and P_4

Serum sex-steroid levels were determined from the serum of blood collected on Pd4. Anesthesia was administered using 0.4% sodium pentobarbital. Blood samples were collected from the abdominal aortic under anesthesia. The serum was separated by centrifugation at 3000×*g* (4℃) for 10 min. Serum was stored at -20℃. The level of E_2 and P_4 were

detected using radioimmunoassay, which followed the instructions of the Estradiol Radioimmunoassay Kit (Beijing north institute of biotechnology, Beijing, China) and Iodine[^{125}I] Progesterone Radioimmunoassay Kit (Beijing north institute of biotechnology, Beijing, China) respectively.

Histologic analysis using hematoxylin and eosin (H&E) staining Tissue samples were fixed in 4% paraformaldehyde overnight at 4℃, rinsed, and transferred to PBS, followed by paraffin embedding, then serially sectioned at a thickness of 5 μm for histologic analysis. Sections were stained with hematoxylin for 15 min and washed with dripping water. The sections were then stained with eosin for 3 min and washed with dripping water. The slides were mounted with Permount TM Mounting Medium (Sinopharm Chemical Reagent Co.,Ltd, Shanghai, China) and then covered with coverslips. Finally, the slides were investigated using an optical microscope (Stemi DV4, Carl Zeiss, Oberkochen, Germany).

Scanning electron microscopy (SEM)

The uteri were excised and then cut open along the longitudinal axis. The endometrial tissue was rinsed in saline solution for 1min, fixed in 1.25% (w/v) glutaraldehyde solution and 1% osmic acid at 4℃ for 2h respectively. After several washes in buffer, the samples were dehydrated through graded concentrations of ethanol and subsequently dried in a critical-point drier with carbon dioxide, then mounted onto the specimen

holder and coated with gold palladium. Finally, all specimens were observed under the scanning electron microscope (SU8010, Hitachi Instruments, Tokyo, Japan).

Immunofluorescence staining

For immunofluorescence assay, the samples were frozen in OCT in liquid nitrogen and were serially frozen-sectioned at 8 μm thick. Nonspecific protein were blocked with 5% fetal bovine serum for 1h at room temperature (RT). Then sections were incubated with primary antibodies or fluorescence probe overnight at 4℃. Such as Estrogen Receptor alpha antibody (ERα, 1:200, Abcam, Cambridge, CB, UK), Estrogen Receptor beta1 antibody (ERβ1, 1:500, Abcam, Cambridge, CB, UK), Progesterone Receptor antibody (PR, 1:200, Abcam, Cambridge, CB, UK), Integrin alpha V antibody (Integrin αV, 1:200, Abcam, Cambridge, CB, UK), Integrin beta 3 antibody (Integrin β3, 1:100, Abcam, Cambridge, CB, UK), Osteopontin antibody (OPN, 1:500, Abcam, Cambridge, CB, UK), LIF antibody (LIF, Abcam, Cambridge, CB, UK), Tomato Lectin (Lycopersion Esculentum Lectin, LEL, Vector Laboratories, Inc., Burlingame, CA, USA), FITC-MAb1 antibody (HP2 Hypoxyprobe^{TM}-1 Plus Kit, Hypoxyprobe, Inc., Burlington, MA, USA). The next day, the sections were incubated with the Alexa Fluor 488 nm (green, CST, Danvers, MA, USA) or Alexa Fluor 555 nm (red, CST,

Danvers, MA, USA) for 1h at RT in dark, then stained with DAPI (blue, Sigma, St.Louis, MO, USA). After rinsed three times with PBST, the slides were mounted with Antifade Polyvinylpyrrolidone Mounting Medium (Beyotime, Shanghai, China) and then covered with coverslips. Finally, images were obtained at 488 nm or 555 nm excitation by a Carl Zeiss LSM800 confocal microscope (Carl Zeiss Microscope GmbH, Jena, Germany).

Quantification of NO content

Nitric Oxide (NO) concentration in uteri tissue was measured with the NO assay kit (Beyotime, Shanghai, China) following the user manual. The quantitative determination of nitrite levels represents NO production. The level of NO was shown in μM/μg protein.

Western blotting analysis

Uteri tissue protein lysates were separated by 10% SDS-PAGE and then transferred to PVDF (0.45μm, EMD Millipore, Billerica, MA, USA). The membranes were probed overnight at 4℃ with primary antibodies against ERα (1:1000, Abcam, Cambridge, CB, UK), ERβ1 (1:1000, Abcam, Cambridge, CB, UK), PR (1:1000, Abcam, Cambridge, CB, UK), Integrin αV (1:1000,Abcam, Cambridge, CB, UK), Integrin β3 (1:1000, Abcam, Cambridge, CB, UK), OPN (1:500, Abcam, Cambridge, CB, UK), LIF (1:1000, Abcam, Cambridge, CB, UK), VEGF (1:1000,

Proteintech, Rosemont, IL, USA), Akt (1:1000, CST, Danvers, MA, USA), p-Akt (1:1000, CST, Danvers, MA, USA), eNOS (1:1000,Abcam, Cambridge, CB, UK), p-eNOS (1:1000, Proteintech, Rosemont, IL, USA), GAPDH (1:3000, Proteintech, Rosemont, IL, USA), or β-tubulin (1:1000, CST, Danvers, MA, USA) . The next day, the membranes were washed and incubated with the appropriate secondary antibody for 1h at RT. After the final wash, signals were detected using the FluorChem E imaging system (ProteinSimple, San Francisco, CA, USA) and a chemiluminescence detection kit (EMD Millipore). Protein band densities were quantified by using an image analysis system (Alpha View SA, ProteinSimple, San Francisco, CA, USA) and expressed as ratios to GAPDH or β-tubulin.

Statistical analysis

Data were analyzed using SPSS21.0, and results were presented as means ±SEM. Data from the experimental groups were compared by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis, and differences were considered significant at $P<0.05$.

Results

The quality evaluation of TJCYR by HPLC.

HPLC chromatograms of TJCYR crude sample and the standard substance were seen in Figure 1B. Under this separation condition, the index components of herbs can be separated and quantified. It well

guaranteed the quality and pharmacodynamics of TJCYR.

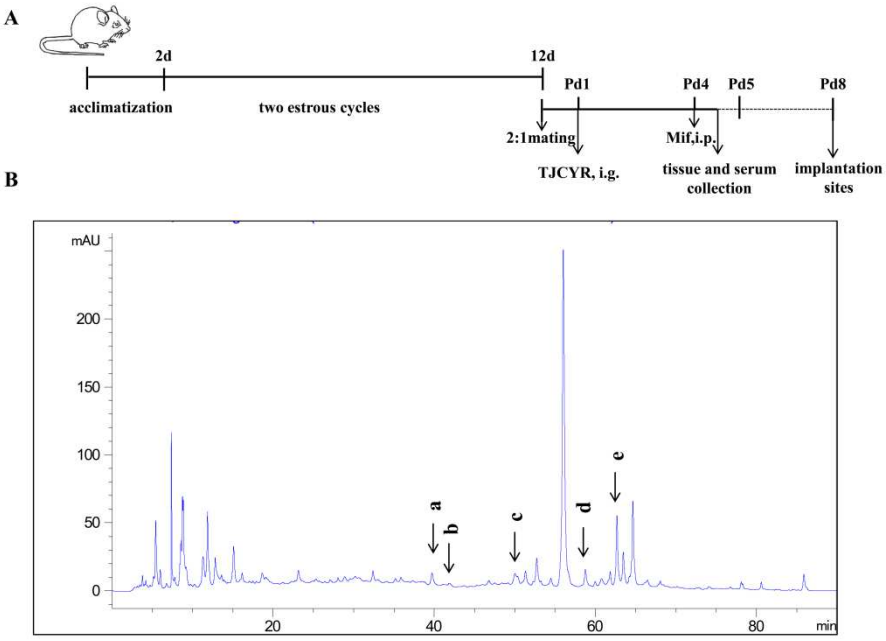

Figure 1. Study scheme and the fingerprinting of TJCYR. (A) Schematic diagram of experimental protocol; (B) Characteristic fingerprint of TJCYR was analyzed by HPLC. Pd, day of pregnancy; EID, embryo implantation dysfunction; TJCYR, Tiao Jing Cu Yun Recipe; HPLC, High Performance Liquid Chromatography; a, Calycosin-7-glucoside (standard substance for *Astragalus membranaceus* (Fisch.) Bunge); b, Acteoside (standard substance for *Rehmannia glutinosa (*Gaert.*)* Libosch.ex Fisch.et Mey.); c, Salvianolic acid B (standard substance for *Salvia miltiorrhiza* Bge.) ; d, Icariin (standard substance for *Epimedium brevicornu* Maxim.); e, Tanshinone IIA (standard substance for *Salvia miltiorrhiza* Bge.).

TJCYR was able to protect blastocysts implantation

Numbers of blastocyst sites were recorded on Pd8. As our results indicated (Figure 2), the mean number of implanted embryos was markedly lower in EID group than in the control group (P<0.05), which suggested successful model. The medium and high-dose of TJCYR treatment increased the implantation numbers compared with the EID only. By comparison, the high-dose of TJCYR dramatically enhances blastocysts implantation, especially, so we choose that dose of TJCYR as treatment in the following study.

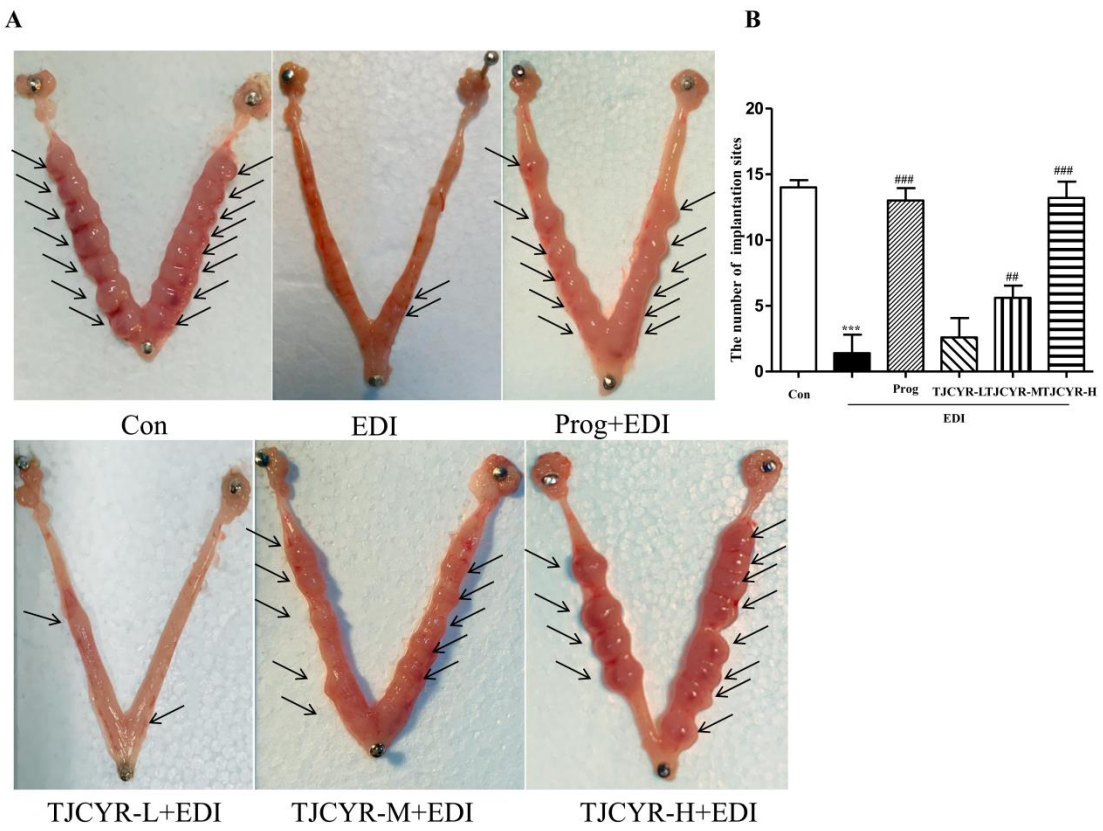

Figure 2. ***TJCYR incresed the number of implantation sites.*** (A) A representative photograph showing the number of implantation sites (the arrow pointed) at Pd8; (B) Quantification of implantation sites (n=8).

Results are expressed as mean ± SEM. $^{***}P<0.001$versus Control; $^{\#\#}P<0.05$, $^{\#\#\#}P<0.01$ versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe; TJCYR-H, the high-dose TJCYR; TJCYR-M, the medium-dose TJCYR; TJCYR-L, the low-dose TJCYR.

TJCYR improved EID-induced endometrium morphological changes. As shown in the Figure 3A, H&E staining was used to evaluate the pathological changes of endometrium. We discovered that there were little loose endometrial stroma, followed with insufficient glands and vessels in EID only group, which were opposite in mice treated with TJCYR. To further morphologically assess the TJCYR caused changes on EID, we checked the effect of TJCYR on the pinopodes by SEM. As shown in the Figure 3B, in the control group, the sample revealed that the majority of well-developed pinopodes were evenly distributed over the endometrial epithelial surfaces, while EID-induced surfaces only sparsely presented a part of well-developed pinopodes and a few developing pinopodes . However, the restrained expression of pinopodes in the EID group was significantly improved following treatment with TJCYR. The results suggest that TJCYR can improved the morphology and vasculature of endometrium of EID model to a certain extent, which is beneficial to embryo implantation.

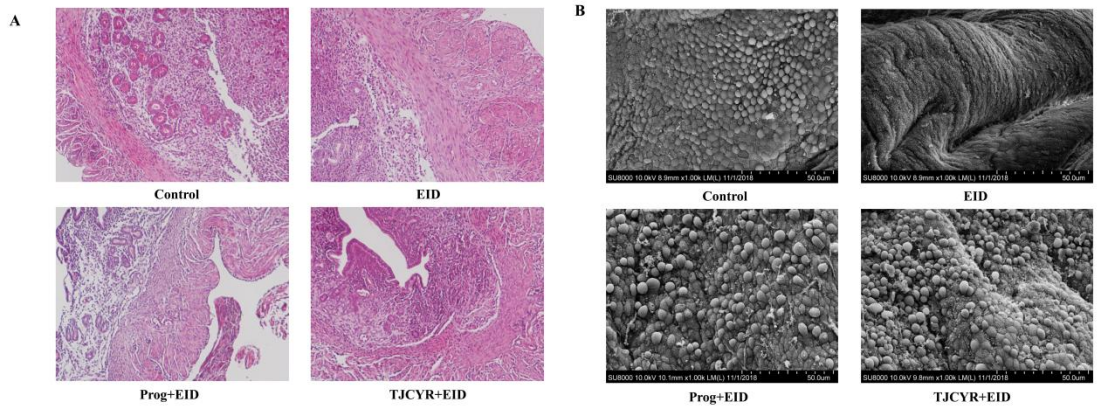

Figure 3. Effect of TJCYR on EID-induced changes in endometrial morphology. (A) H&E showing pathological changes in endometrium (×200); (B) SEM showing ultrastructure changes in pinopodes.

The effect of TJCYR on hormones and hormone receptors.

The receptive mouse uterus displays stromal proliferation and epithelial differentiation as the indicator of proper arrangement for blastocyst implantation, which are governed by ovarian hormones, such as: 17β-estradiol (E_2) and progesterone (P_4). And the primary mediators of these E_2 and P_4 -induced events are their receptors- progesterone receptor (PR) and estrogen receptor (ER). In our results, we found that in the EID only group, the level of P_4 was obviously lower than those in the Control group, and the expression of PR lowered induced by EID . However, the treatment with TJCYR could reverse those changes (Figure 4) .

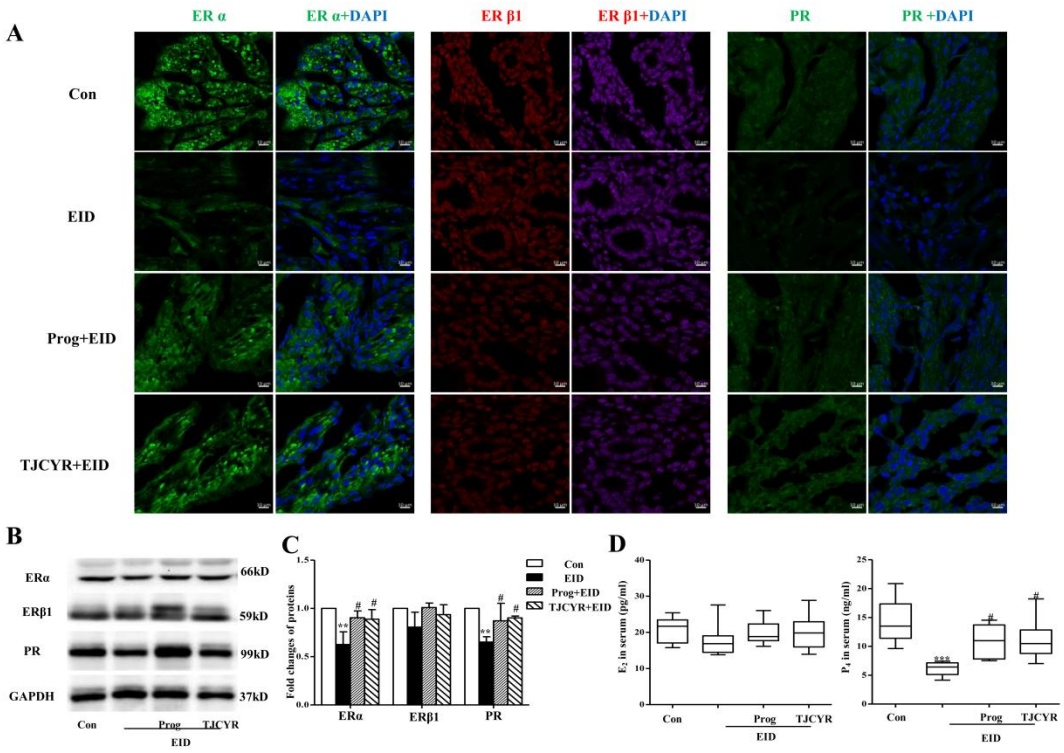

Figure 4. Effect of TJCYR on EID-induced changes in the hormones and receptors. (A) Representative confocal fluorescent images of the uterine sample with ERα (Green), ERβ1 (Red) and PR (Green), respectively. (B) Protein levels of ERα, ERβ1 and PR in uterine tissue were determined by Western blotting (n=3); (C) Quantification of protein levels. (D) The level of E_2 and P_4 in serum were tested by radioimmunoassay. Results are expressed as mean ± SEM. $^{***}P<0.001$, $^{**}P<0.01$versus Control; $^{\#}P<0.05$ versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe; E_2, 17β-estradiol; P_4, progesterone; ERα, Estrogen Receptor alpha; ERβ1, Estrogen Receptor beta1; PR, Progesterone Receptor.

TJCYR enhanced the related proteins as the markers of endometrial

receptivity.

There are several molecular markers related to the endometrial receptivity, including Integrin αV, Integrin β3, LIF, and OPN. As shown in the Figure 5, we found that the expression of Integrin αV, Integrin β3, LIF, and OPN significantly decreased in EID only group by immunofluorescence and western blot analysis. TJCYR had been found to attenuate the EDI-induced damage through increasing the expression of Integrin αV, Integrin β3, LIF, and OPN. These results were consisted with the changes of pinopodes shown in Figure 3B .

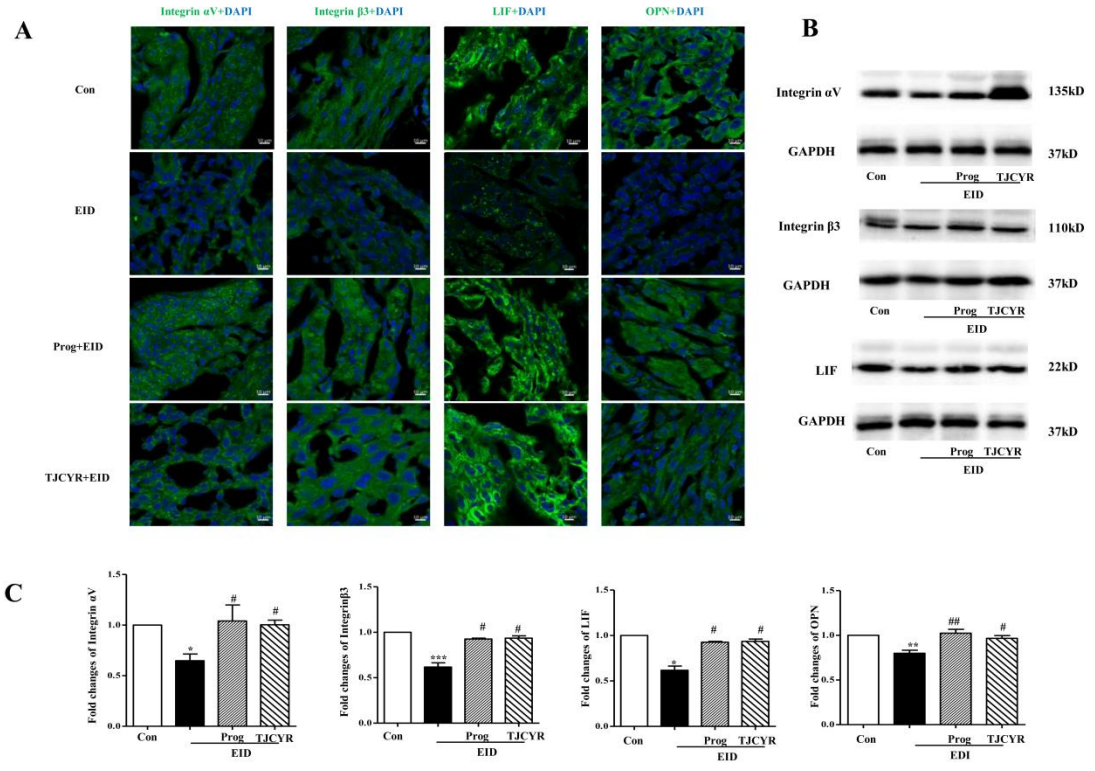

Figure 5. Effect of TJCYR on the biomarkers of endometrial receptivity. (A, D, G and J) Representative confocal fluorescent images of the uterine sample with Integrin αV, Integrin β3, LIF and OPN,

respectively. (B, E, G and K) Protein levels of Integrin αV, Integrin β3, LIF and OPN in uterine tissue were determined by Western blotting (n=3); (C, F, I and L) Quantification of protein levels. Results are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$versus Control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe .

Upregulation of angiogenesis and alleviation of endometrium hypoxia probably contribute to the improvement of endometrial receptivity by TJCYR.

Angiogenesis is one of important biological events which could be involved in implantation. Tomato Lectin is recognized as the most sensitive vessel marker, the fluorescence intensity could reflect the vascular density. Compared with the control group, we discovered that in the EID only group, the vascular density decreased significantly, which was prevented by TJCYR (Figure 6A). Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis, which has beneficial effects on endometrial receptivity and plays a key role in the embryonic development of mice. As shown in Figure 6B and C, the treatment of TJCYR obviously inhibited the decrease of VEGF in mice that subjected to EID, which is consistent with the immunofluorescence analysis (Figure 6A) that showed increased density of vascular.

Hypoxyprobe-1 is the hypoxia marker associated monoclonal and

polyclonal antibodies that bind to pimonidazole adducts in hypoxia tissue. As shown in the Figure 6D, the fluorescence intensity was increased significantly in mice that subjected to EID only, which suggested serious hypoxia in tissue, while, TJCYR could ameliorat hypoxia

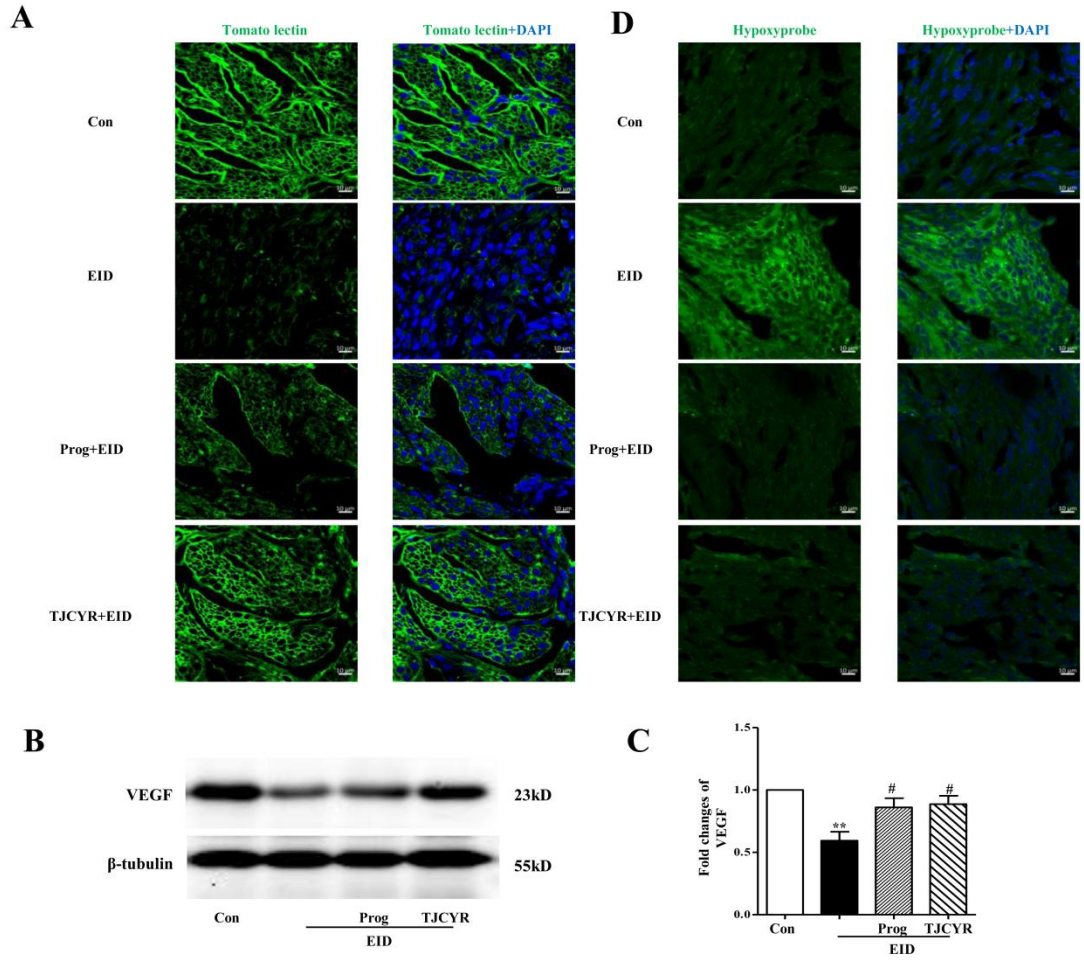

Figure 6. Effect of TJCYR on angiogenesis and hypoxia. (A) Representative confocal fluorescent images of the uterine sample with Tomato Lectin (Green) and DAPI (Blue); (B) Protein levels of VEGF in uterine tissue were determined by Western blotting (n=3); (C) Quantification of protein levels. Results are expressed as mean ± SEM; (D) Representative confocal fluorescent images of the uterine sample

with Hypoxyprobe (Green) and DAPI (Blue). $^{*}P<0.05$, $^{**}P<0.01$ versus Control; $^{\#}P<0.05$ versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe.

TJCYR improved angiogenesis through PI3K/Akt/eNOS signaling pathway

The pathway of PI3K/Akt/eNOS is the downstream of VEGF, which participated in angiogenesis. Here, we found that the expressions of p-Akt and p-eNOS were all reduced in the EID only group compared to the control group, accompanied with the decline of NO. While, all of these decreased expressions were restored by the treatment of TJCYR(Figure 7). These data suggested that TJCYR stimulated PI3K/Akt/eNOS signaling pathway, which was deactivated by EID.

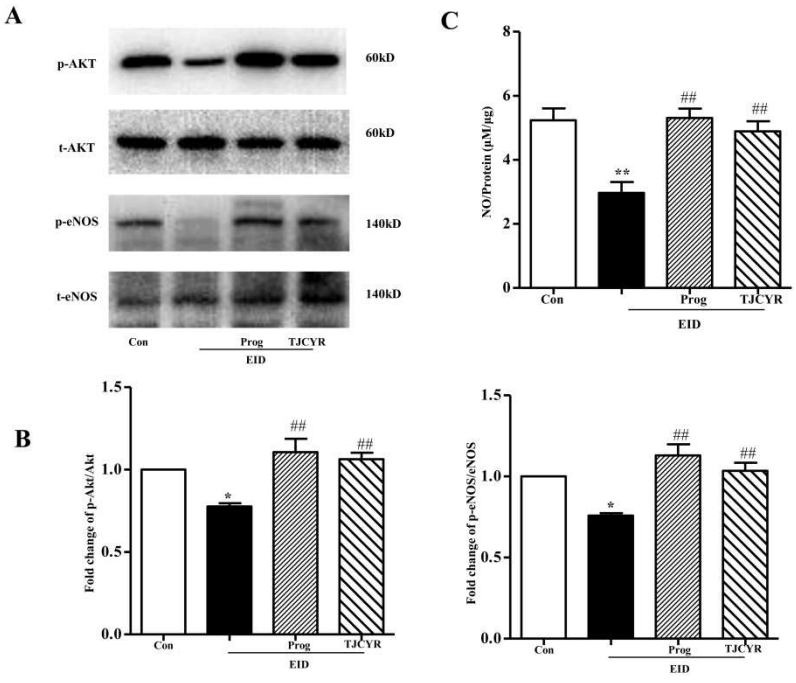

Figure 7. Effect of TJCYR on PI3K/Akt/eNOS signaling pathway. (A) Protein levels of PI3K/Akt/eNOS signaling pathway in uterine tissue

were determined by Western blotting (n=3); (B) Quantification of protein levels. Results are expressed as mean ± SEM; (C) NO production was showed by NO/protein (n=10). Results are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ versus Control; $^{\#\#}P<0.01$ versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe; NO, nitric oxide.

Discussion

For successful embryo implantation to be achieved, it is a highly orchestrated process that involves blastocyst-uterine interactions. While blastocyst quality has been extensively studied, equally as important is the preparation of the receptive endometrium, or more precisely, it is endometrial receptivity. By definition, endometrial receptivity is "that period of endometrial maturation during which the trophectoderm of the blastocyst can attach to the endometrial epithelial cells and subsequently proceed to invade the endometrial stroma and vasculature"[13].Therefore, improving endometrial receptivity is the key point to rise the pregnancy rate.

In mice, the implantation window extends from the morning of Pd4 to the end of Pd5 [14]. The use of mifepristone for both planned and emergency contraception has been widely accepted [15]. And mifepristone played a role in inhibiting development and maturation of endometrium, hence affecting embryo implantation in mice, as well as for terminatin of

pregnancy[16]. In our study, we used the EID mice model by treatment with mifepristone, and collected samples between 21:00 and 22:00 on Pd4 (as show in Figure 1A).

The endometrium is a unique tissue which undergoes dramatic and rapid changes throughout the menstrual cycle. It progresses through specific stages in response to ovarian hormones. The receptive mouse uterus displays stromal proliferation and epithelial differentiation as an indicator of proper arrangement for blastocyst implantation [17], which is governed by ovarian hormones. In another word, increased production of ovarian hormones E_2 and P_4 provides the uterus with a capacity of blastocyst attachment pre-decidualization. Circulating P_4 levels rises on Pd3 due to its production from newly-formed corpus luteum. Elevated level of P_4 takes over E_2 and becomes a dominant ovarian hormone on Pd4, increased P_4 promoted stromal proliferation and inhibited E_2-induced epithelial proliferation. E_2 and P_4 exerted their respective functions through ER and PR. We found that, compared with EID only, TJCYR could obviously enhance the level of P_4 and the corresponding receptor-PR , while the influence of E_2 and ER were little. We thought the difference of influence on E_2 and P_4 was due to EID model. In our study, mifepristone was prepared for EID model, which has a strong affinity with PR and competitively inhibits the effect of P_4. So the regulation of P_4 maybe obvious after treatment with TJCYR.

The pinopodes are membrane protrusions on the apical surface of luminal epithelium. The pinopodes play the key role in the initial stage of implantation by promoting attachment of the embryo, because they prevent the cilia from sweeping off the blastocyst as well as promoting withdrawal of uterine fluid and closure of the uterine cavity[5,18]. The pinopodes have been considered as the characteristic morphologic markers to assess endometrial receptivity and to locate the implantation window[16,19]. In our study, the specimens were examined by scanning electron microscopy (SEM) for the detection of pinopodes. The result revealed that TJCYR could enhance the number of fully developed pinopodes, accompany with reduced distribution of cilia, which may provide nutrients for embryo and enable its attachment to the uterine endometrium.

The pregnancy is a versatile and dynamic process for the implanting embryo and the endometrial epithelium, which requires a series of rather complicated and synchronous morphological and biochemical changes. Appearance of pinopodes is consistent with the expression of several molecular markers of endometrial receptivity, such as Integrin, leukaemia inhibitory factor (LIF), and osteopontin (OPN). It is meaningful to analyze pinopodes and the molecular biomarkers together, so our further studies exploring the effect of TJCYR on the expression of Integrin, LIF, and OPN. Integrins are cell surface receptors that are involved in

cell-to-cell and extracellular matrix adhesion. Some Integrins increase during the implantation window. There are many isoforms of integrins in mammals, only three isoforms α1β1, α4β1 and αVβ3 are found to be particularly involved in implantation, and αVβ3 is seemingly playing more conspicuous roles than others, especially [20]. The Integrin αVβ3 is a potential receptor for blastocyst attachment and is localized on pinopodes [18]. Some studies revealed that the blockade of the Integrin αVβ3 or lack of Integrin β3 may be related to unexplained infertility [21,22] . The role of Integrins in the implantation process had been reached extensively. Several studies had identified an association between Integrin expression and female fertility, which suggested the potential use of Integrins as markers of uterine receptivity[23,24]. LIF is a member of the interleukin-6 family of cytokines, which plays a critical role in implantation and pinopodes release secretory vesicles that contain LIF in uterine lumen to enable trophoblast invasion and also affect on immune tolerance during implantation [18,25]. Some studies [26,27]showed that the deletion or mutation of LIF reduced implantation failure. Other studies[28,29] reported that endometrial LIF and receptor were higher around the time of implantation in fertile women compared with unexplained infertile women. Other finding [30] also showed that LIF may maintain the proper development of the endometrium and implantation receptivity by regulating downstream target genes. OPN is one of the co-factors involved in cell adhesion and

invasion during the implantation process, and it is an acidic member of the small integrin- binding ligand family of proteins[31], had been shown to be maximally expressed in the epithelial layer in human, mouse and rabbit uterine. OPN was an important constituent of the uterus during pregnancy[31-33]. In this study, the EID group showed histopathological lesions characteristic of pinopodes, as well as low expression of Integrin αV, Integrin β3, LIF, and OPN. While the expression of these biochemical markers were significantly increased in mice treated with TJCYR. These data strongly promised a repairment of TJCYR in endometrial receptivity. During the window of implantation, a rich vascular network is necessary to supply nutrients and oxygen. Proper endometrial vascular development and maintenance are crucial for successful pregnancy. Vascular endothelial growth factor (VEGF) has a predominant role in successful implantation and maintenance of pregnancy by increasing vascular permeability or forming vascular network. The expression of VEGF and its receptor were confirmed in early pregnancy. It was reported that VEGF knock-out mice do not produce viable offspring[34]. The expression of VEGF increased significantly during implantation windows, which suggested that VEGF promotes angiogenesis and the establishment of capillary network, further improved endometrial receptivity and promoted embryo implantation. Otherwise, deficiency of VEGF may induce the reduction of angiogenesis on implantation site, then lead to miscarry[35].

In this study, we found that there was a poor vascular network in the mice treated with EID only, accompany with serious hypoxia. Here we found that treatment with TJCYR significantly inhibited decline in density of vessel and expression of VEGF in EID only group, and improved endometrial blood circulation, further prevented the hypoxia. All the data showed that VEGF regulated angiogenesis and built endometrial microenvironment for embryo implantation. As we all know, VEGF is an angiogenesis accelerator, which could activate the PI3K/Akt/eNOS pathway. Here, we found that the expressions of p-Akt and p-eNOS were all reduced in the EID mice compared to the control group, and all of these decreased expressions were restored by the treatment of TJCYR. And the activated eNOS could produce nitric oxide (NO). NO, as an endogenous vasodilator, could increase the vascular dilatation and permeability at the implantation site, further to provide safeguard for embryo implantation and pregnancy maintenance.

Conclusion

TJCYR can activate the PI3K/AKT/eNOS signaling pathway, promote angiogenesis, and increase the endometrial receptivity, which were beneficial to the implantation of embryos.

Abbreviations

TJCYR: Tiao Jing Cu Yun Recipe; EID: embryo implantation dysfunction; Prog: Progesterone; Pd: day of pregnancy; HPLC: high-performance liquid chromatography; HE: Hematoxylin-eosin; SEM: scanning electron microscopy; ERα: Estrogen Receptor alpha; ERβ1: Estrogen Receptor beta1; PR: Progesterone Receptor; Integrin αV: Integrin alpha V; Integrin β3: Integrin beta 3; LIF: leukaemia inhibitory factor; OPN: osteopontin; VEGF: Vascular endothelial growth factor; p-Akt: Phospho-Akt; p-eNOS: Phospho- eNOS ; NO: nitric oxide.

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Not applicable.

Authors' contributions

LD conceived and designed the experiments. LX, Y-QX, Y-PY and Z-JJ performed the experiments. H-LH and PZ analyzed the data. PZ wrote the paper. LD provided revision. Hong-li Huang and Lei Xia are equal contributors.

All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Consent for publication

The manuscript is approved by all authors for publication.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

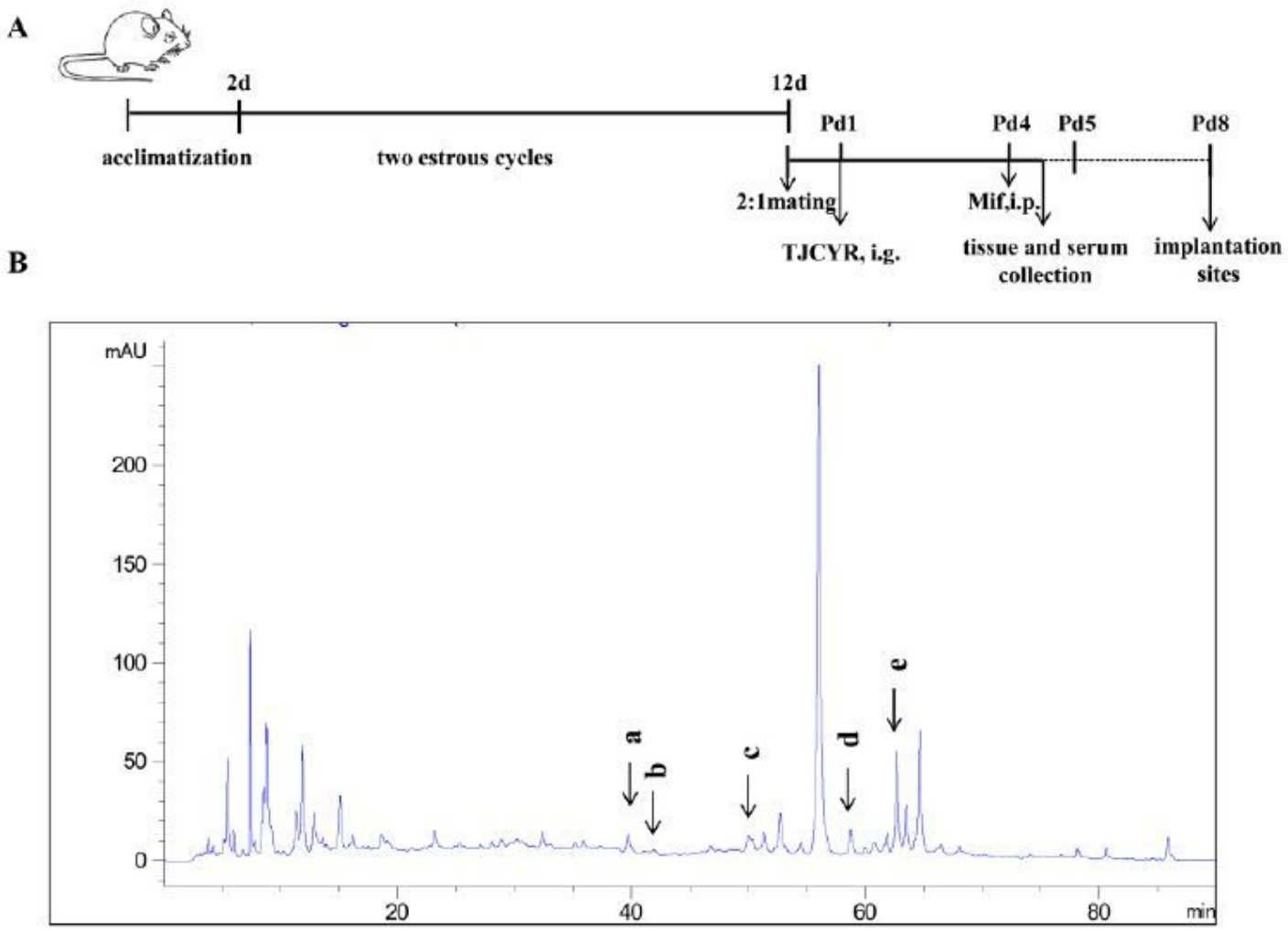

Figure 1

Study scheme and the fingerprinting of TJCYR. (A) Schematic diagram of experimental protocol; (B) Characteristic fingerprint of TJCYR was analyzed by HPLC. Pd, day of pregnancy; EID, embryo implantation dysfunction; TJCYR, Tiao Jing Cu Yun Recipe; HPLC, High Performance Liquid Chromatography; a, Calycosin-7-glucoside (standard substance for Astragalus membranaceus (Fisch.) Bunge); b, Acteoside (standard substance for Rehmannia glutinosa (Gaert.) Libosch.ex Fisch.et Mey.); c, Salvianolic acid B (standard substance for Salvia miltiorrhiza Bge.) ; d, Icariin (standard substance for Epimedium brevicornu Maxim.); e, Tanshinone IIA (standard substance for Salvia miltiorrhiza Bge.).

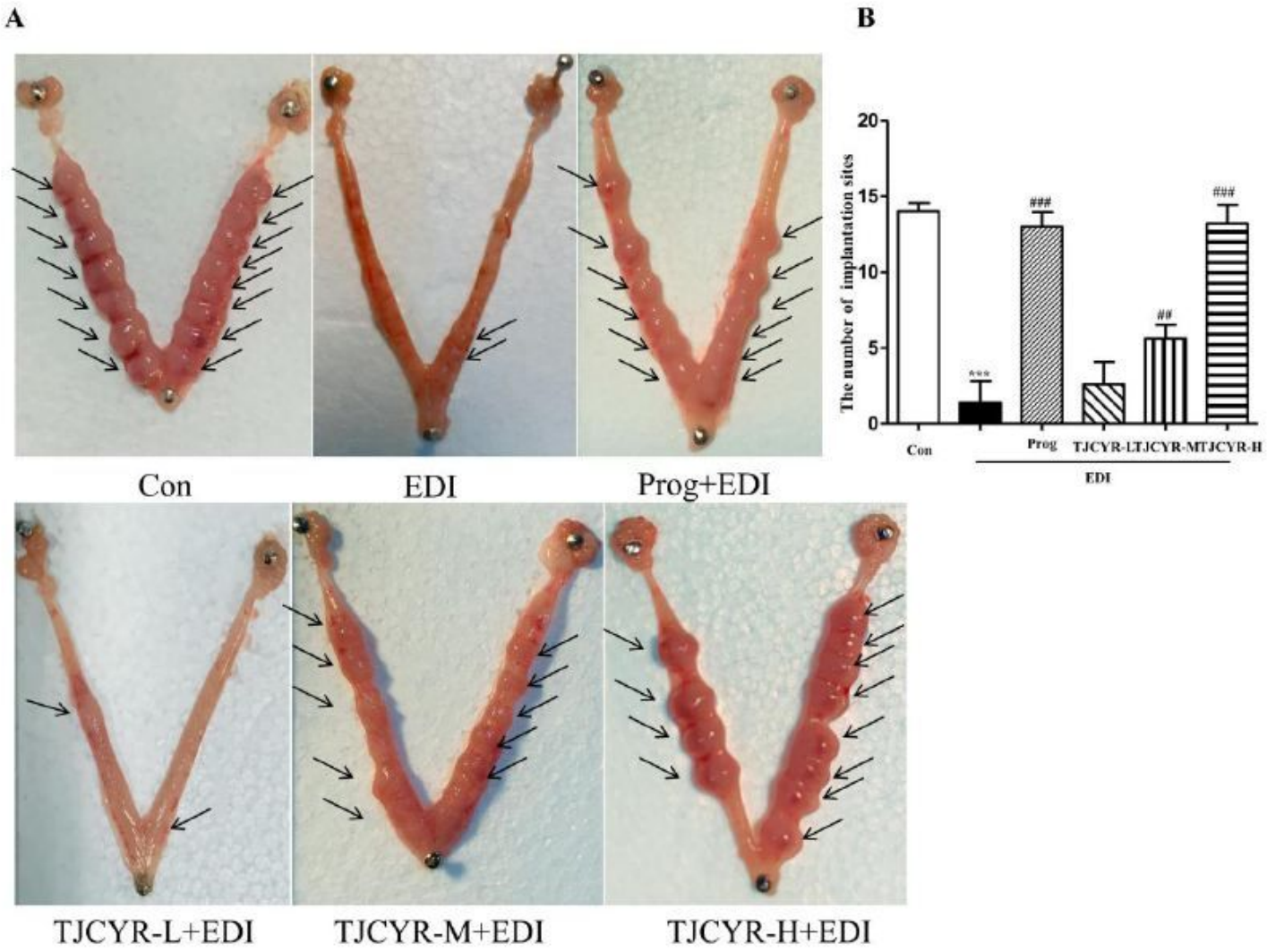

Figure 2

TJCYR incresed the number of implantation sites. (A) A representative photograph showing the number of implantation sites (the arrow pointed) at Pd8; (B) Quantification of implantation sites (n=8). Results are expressed as mean ± SEM. ***P<0.001versus Control; ##P<0.05, ###P<0.01 versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe; TJCYR-H, the high-dose TJCYR; TJCYR-M, the medium-dose TJCYR; TJCYR-L, the low-dose TJCYR.

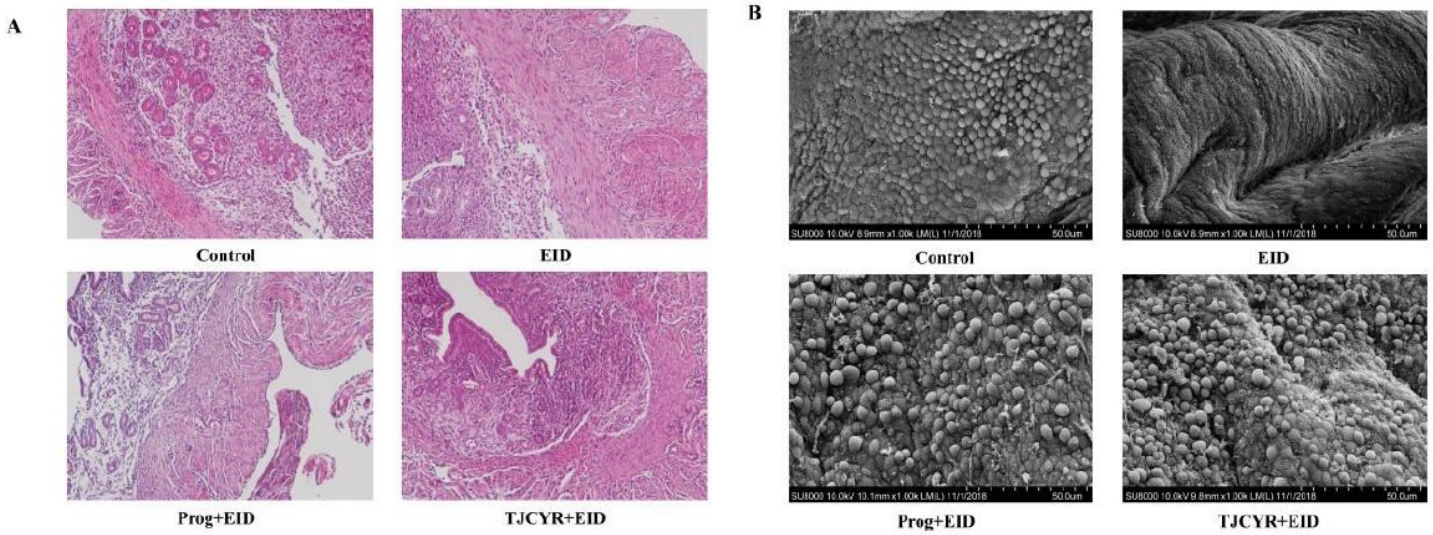

Figure 3

Effect of TJCYR on EID-induced changes in endometrial morphology. (A) H&E showing pathological changes in endometrium (×200); (B) SEM showing ultrastructure changes in pinopodes.

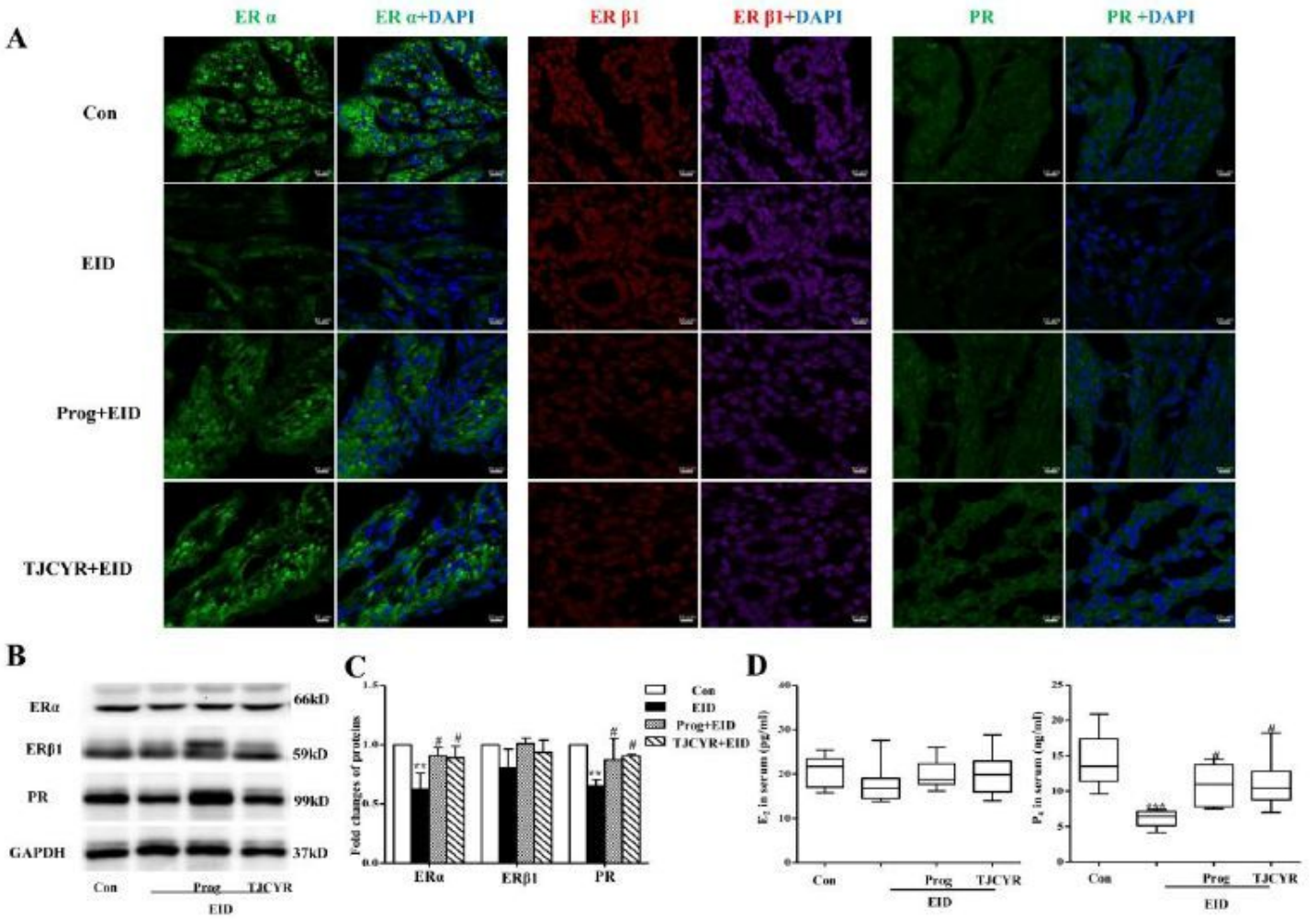

Figure 4

Effect of TJCYR on EID-induced changes in the hormones and receptors. (A) Representative confocal fluorescent images of the uterine sample with ERα (Green), ERβ1 (Red) and PR (Green), respectively. (B) Protein levels of ERα, ERβ1 and PR in uterine tissue were determined by Western blotting (n=3); (C) Quantification of protein levels. (D) The level of E2 and P4 in serum were tested by radioimmunoassay. Results are expressed as mean ± SEM. ***P<0.001, **P<0.01versus Control; #P<0.05 versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe; E2, 17β-estradiol; P4, progesterone; ERα, Estrogen Receptor alpha; ERβ1, Estrogen Receptor beta1; PR, Progesterone Receptor.

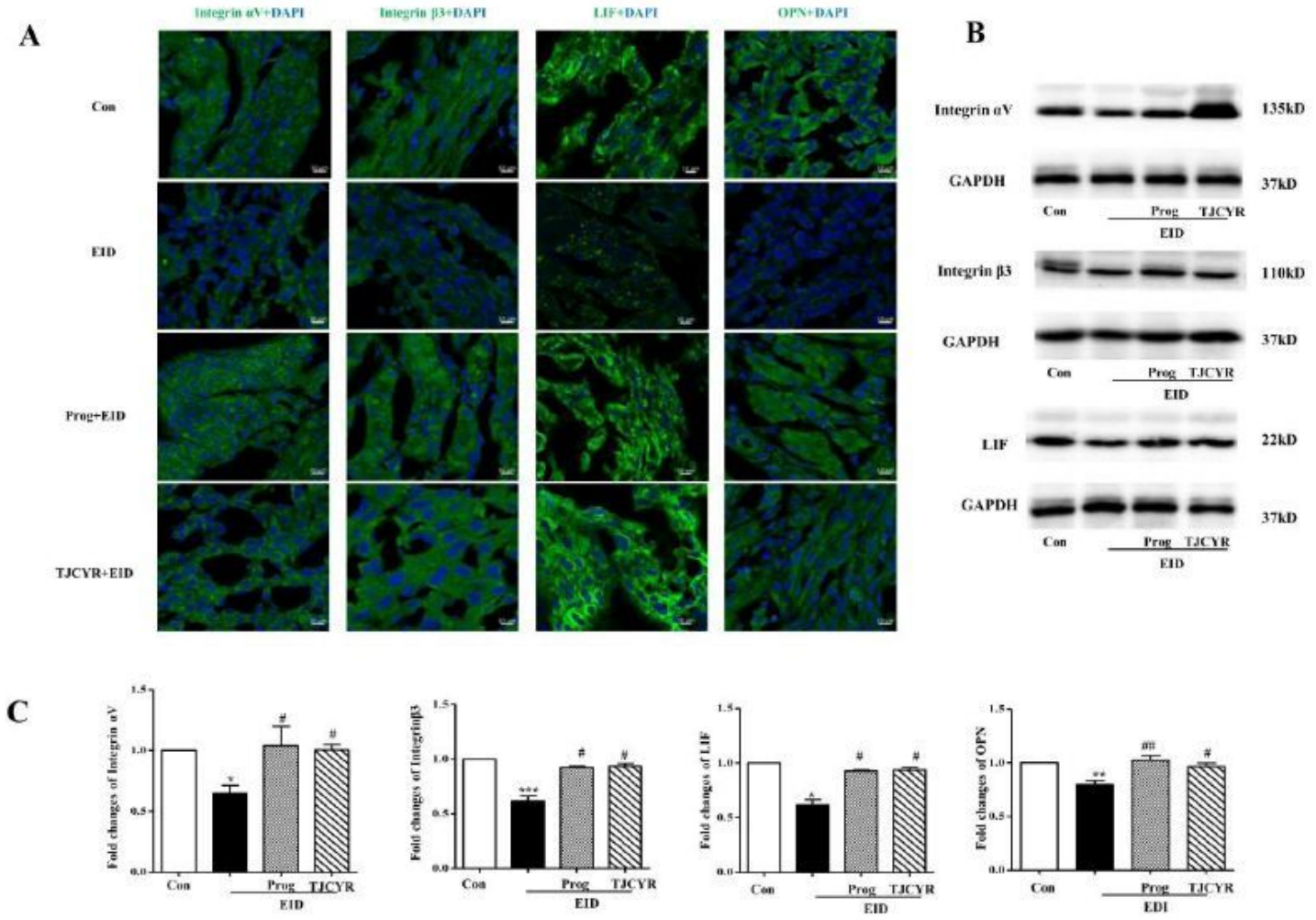

Figure 5

Effect of TJCYR on the biomarkers of endometrial receptivity. (A, D, G and J) Representative confocal fluorescent images of the uterine sample with Integrin αV, Integrin β3, LIF and OPN, respectively. (B, E, G and K) Protein levels of Integrin αV, Integrin β3, LIF and OPN in uterine tissue were determined by Western blotting (n=3); (C, F, I and L) Quantification of protein levels. Results are expressed as mean ± SEM.

*P<0.05, **P<0.01, ***P<0.001versus Control; #P<0.05, ##P<0.01 versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe .

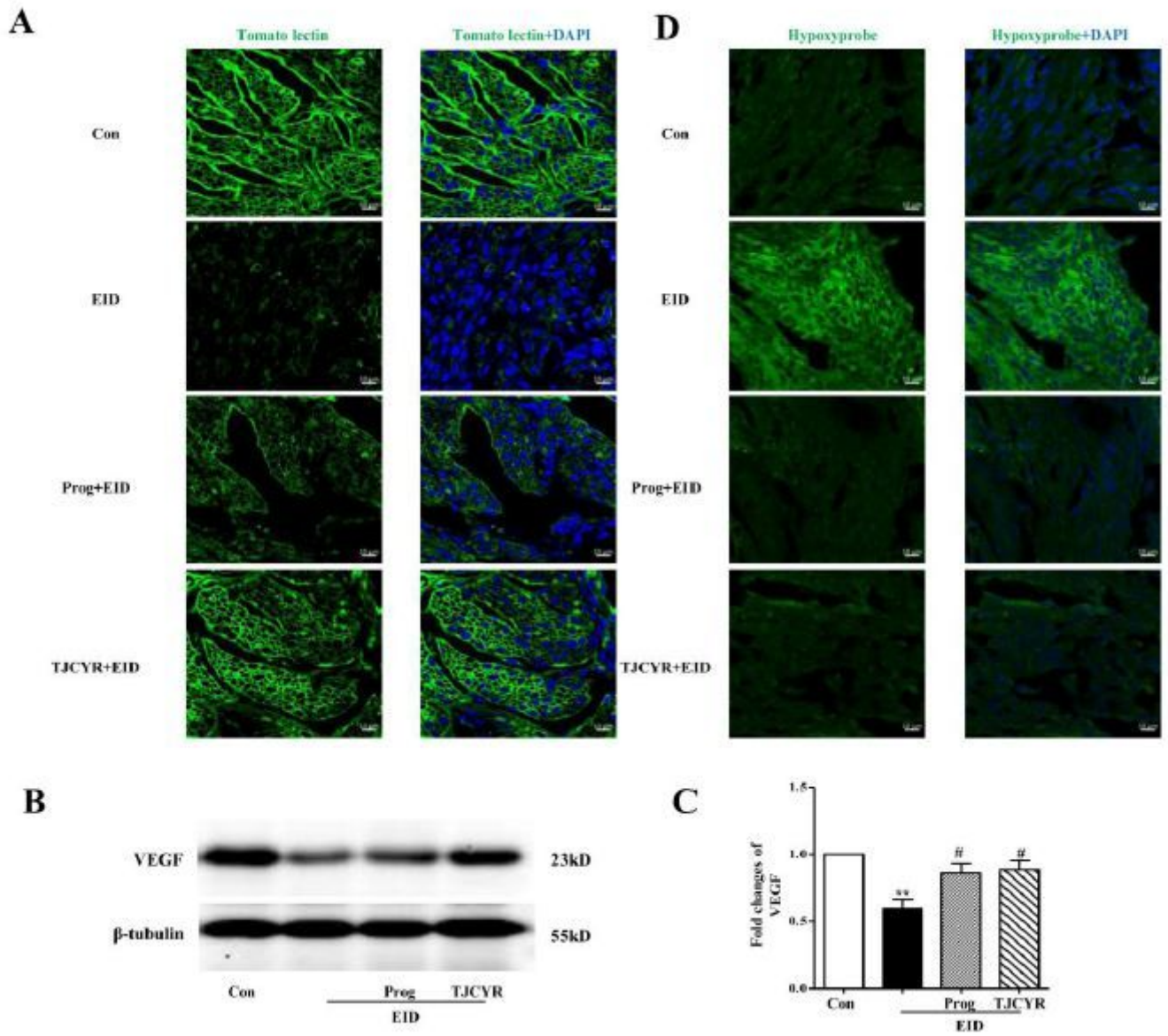

Figure 6

Effect of TJCYR on angiogenesis and hypoxia. (A) Representative confocal fluorescent images of the uterine sample with Tomato Lectin (Green) and DAPI (Blue); (B) Protein levels of VEGF in uterine tissue were determined by Western blotting (n=3); (C) Quantification of protein levels. Results are expressed as mean ± SEM; (D) Representative confocal fluorescent images of the uterine sample with Hypoxyprobe (Green) and DAPI (Blue). *P<0.05, **P<0.01 versus Control; #P<0.05 versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe.

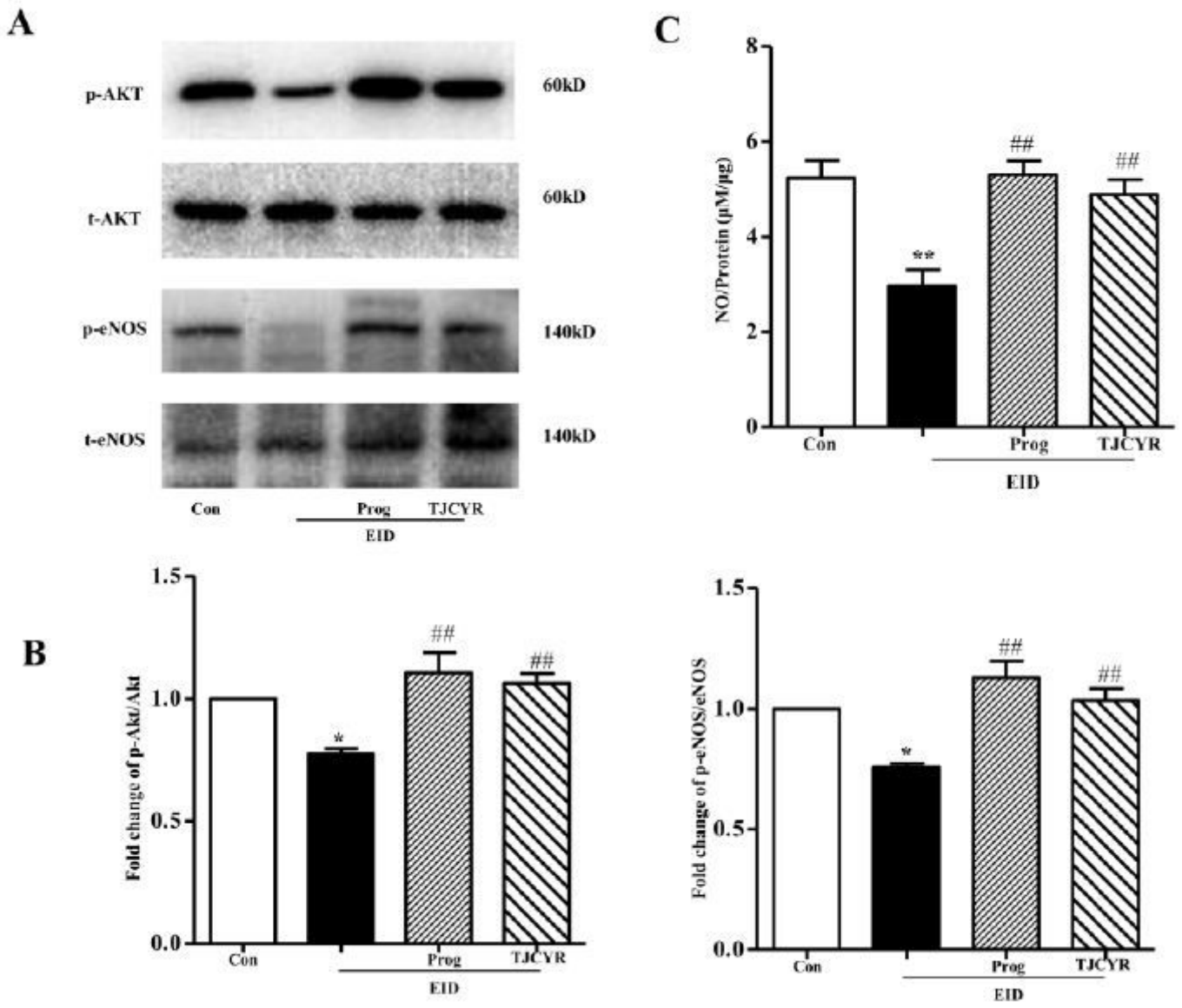

Figure 7

Effect of TJCYR on PI3K/Akt/eNOS signaling pathway. (A) Protein levels of PI3K/Akt/eNOS signaling pathway in uterine tissue were determined by Western blotting (n=3); (B) Quantification of protein levels. Results are expressed as mean ± SEM; (C) NO production was showed by NO/protein (n=10). Results are expressed as mean ± SEM. *P<0.05, **P<0.01 versus Control; ##P<0.01 versus EID only. EID, embryo implantation dysfunction; Prog, Progesterone; TJCYR, Tiao Jing Cu Yun Recipe; NO, nitric oxide.