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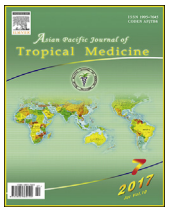

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Effect of Taoren Quyu Decoction on human endometrial cells and its anti-endometriosis activity in rats

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ABSTRACT

Objective: To study the effect of Taoren Quyu Decoction (TQD) on endometrial cells in patients with endometriosis (EMs) and EMs in rats.

Methods: A total of 60 female Wistar rats were randomly divided into 4 groups, namely, normal group, model group, positive group and TQD group, each group having 15 rats. Except the normal group, EMs model was established in the other three groups by transplanting the rat autologous endometrium. After 4 weeks of intragastric administration, blood, eutopic and ectopic endometrial tissues of rats in each group were collected to detect the serum levels of estrogen (E2), cancer antigen 125 (CA125), endometrial antibody (EMAb), and expressions of microvessel density (MVD), vascular endothelial growth factor (VEGF) and angiopoietin (Ang-2). The volume of endometriosis cyst was determined simultaneously. For the *in vitro* culture of human endometrial cells, 4 groups, namely, normal group, model group, positive group and TQD group were used. The positive group and TQD group were treated with danazol and TQD respectively. Then 24 h after the treatment, the expressions of survivin and tumor suppressor gene (p53) of each group were detected.

Results: The volumes of the endometriosis cysts in the positive group and the TQD group were significantly reduced compared with the model group ($P < 0.05$). The serum levels of E2, CA125 and EMAb, and the expressions of MVD, VEGF and Ang-2 in the model group were significantly increased compared with the normal group ($P < 0.05$); while they were all significantly reduced in the positive group and TQD group ($P < 0.05$). Compared with the normal group, the expression of survivin in the model group was significantly up-regulated ($P < 0.05$), and expression of p53 was significantly reduced ($P < 0.05$); compared with the model group, the expressions of survivin in the positive and TQD groups were significantly decreased ($P < 0.05$), and expression of p53 was significantly up-regulated ($P < 0.05$). The difference between positive group and TQD group was not statistically significant ($P > 0.05$).

Conclusions: TQD has a significant anti-EMs effect, and its mechanism of action may be related to anti-angiogenesis and promoting apoptosis of ectopic endometrial cell.

1. Introduction

Endometriosis (EMs) is a common gynecological disease that has higher incidence in women of childbearing age. The main clinical symptoms of this disease include dysmenorrhea, irregular menstruation, discomfort during sexual intercourse, chronic pelvic pain and infertility *etc.* [1]. Although EMs is benign lesion on pathology, it still has malignant biological behaviors of infiltration, metastasis and recurrence. Therefore, to search for a reliable and effective drug for the treatment of EMs is of great significance. EMs in Chinese medicine can be classified into the category of 'dysmenorrhea', 'abdominal mass', 'infertility', *etc.* In recent years, Chinese medicine has a satisfactory effect in the clinical treatment of EMs. The treatment of EMs with Chinese medicine has characteristics of multiple regimens, multiple approaches and comprehensiveness and the advantages of definite therapeutic effect and less side effects [2,3]. In this study, we used the Chinese medicine decoction Taoren Quyu Decoction to interfere with rat EMs

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model and human endometrial cells and investigated the mechanism of action of Taoren Quyu Decoction on EMs by evaluating the level or expression of related parameters in serum, tissue and cells.

2. Materials and methods

2.1. Experimental animals and cell line

A total of 60 female healthy Wistar rats of clean grade, weighing 200–220 g, were purchased from Guangdong Medical Laboratory Animal Center and fed adaptively for one week. The animal experiments in this study were approved by the Animal Care and Use Committee of Jinan University (Ref. No. 20131007) and all experimental procedures were in line with the Animal Welfare Act and the National Institutes of Health (NIH) Guidelines for the Management and Use of Laboratory Animals. The human endometriotic epithelial cell line (11Z) and human mesothelial cells (MeT-5A) used in this study were supplied by Nanjing Haeckel Biotechnology Co., Ltd.

2.2. Drugs and reagents

(1) The main drugs: Danazol purchased from Jiangsu Lianhuan Pharmaceutical Co. Ltd.; Taoren Quyu Decoction: 10 g Taoren, 10 g Danshen, 5 g Dang Gui and 5 g Yi Mu Cao together were decocted with 10-fold water for 40 min, and the decoction was repeated twice; then the two decoction solutions were combined, filtered and concentrated to 100 mL to obtain the Taoren Quyu Decoction with concentration of 300 mg/mL (crude drug/final volume). All crude drugs were purchased from Bozhou, Anhui, and identified by Professor Lin Hui, School of Pharmacy, Jinan University (voucher specimen No. TQD 20161005). (2) The main reagents: DMEM/F12 culture medium (100 U/mL penicillin G + 100 g/mL streptomycin) (Article No. 11039021), fetal bovine serum (FBS) (Article No. 10099141), Medium 199 (100 U/mL penicillin G + 100 g/mL streptomycin) (Article No. 31100035) were Gibco products (USA); thiazolyl blue (MTT) and dimethyl sulfoxide (DMSO) (cell culture level) were products of Sigma (USA); estradiol (E2), cancer antigen 125 (CA125), endometrial antibody (EMAb) detection kits (ELISA) were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd.; Rabbit anti-mouse vascular endothelial growth factor (VEGF) polyclonal antibody, rabbit anti-mouse angiopoietin (Ang-2) polyclonal antibody, rabbit anti-human survivin (Survivin) polyclonal antibody and rabbit anti-human tumor suppressor gene (p53) polyclonal antibodies were the products of Abcam (USA).

2.3. Main instruments

Ordinary optical microscope (Olympus, Japan), inverted phase contrast microscope (Olympus, Japan), low temperature high-speed centrifuge (Eppendorf, Germany), Gel Doc XR + gel imaging analysis system (Bio-Rad, USA).

2.4. Experimental methods

2.4.1. Animal experiments

(1) Animal grouping: 60 rats were randomly divided into 4 groups; normal group, model group, positive group and TQD group, each group having 15 rats. (2) Preparation of animal model: except the normal group, EMs model was established in the other three groups of rats during estrus by transplanting the rat autologous endometrium: after anesthesia with chloral hydrate, the hair on the abdomen of rats was removed. After routine disinfection, abdominal wall and peritoneum of rats were incised to find the uterus, and uterine artery was ligatured, then uterine horns (about 1–1.5 cm) was resected and placed in culture dish containing normal saline to wash the blood on the surface of tissue. The adventitia of collected uterus tissues was peeled off and the intima section of about 3 mm × 3 mm was collected and sewn in the right side of the abdominal wall with its superficial epithelium toward the abdominal cavity. The abdominal cavity and site of intima transplant were rinsed with normal saline and the abdominal wall sutured. The rats were intramuscularly injected with antibiotics every day after surgery for 5 consecutive days (3 weeks after surgery, the rats were laparotomized for the second time during estrus to observe the growth of ectopic endometrium. The presence of macroscopic nodule-like transplant lesion and sac-like transparent vesicles with angiogenesis on the ectopic endometrium indicate the success in establishing EMs model. The EMs model was successfully established in all the 60 rats in this study). Rats in normal group were subjected to sham operation; except endometrial transplantation, the rest of the operative procedures were the same. (3) Administration: one week after surgery, Danazol suspension (20 mg/kg/d) and TQD (400 mg/kg/d) were intragastrically administered to positive group and TQD group respectively, and the rats in normal group and model group were intragastrically administered with normal saline (5 mL/kg/d). The 4 groups were intragastrically administered for 4 consecutive weeks. (4) Sample collection: the rats were fasted one day prior to sample collection and sacrificed by cervical dislocation. Blood was collected from abdominal aorta to prepare serum, and the endometrium transplant lesion was collected in the mean time. (5) Parameters detection: serum levels of E2, CA125 and EMAb were measured by ELISA; the volume of endometriosis cyst was determined; the expressions of microvessel density (MVD), VEGF and Ang-2 in tissues were detected by immunohistochemical method.

2.4.2. Cell experiment

(1) Cell culture: the human endometriotic epithelial cell line and MeT-5A cells were cultured using DMEM/F12 medium, and the cell viability was determined by MTT assay. (2) Grouping and treatments: the second generation of human endometriotic epithelial cells with better growth were selected and divided into model group, positive group and TQD group. The positive group and TQD group were interfered with danazol solution (dissolved in PBS) and TQD (30 μg/mL) respectively; MeT-5A cells were regarded as normal group, and the normal group and model group were routinely cultured. (3) Extraction and detection of gene and protein: after 24-h culture, the total RNA and total protein of the cells in the four groups were extracted respectively. The expressions of survivin and p53 were detected by PCR and Western blot respectively.

2.5. Interpretation of immunohistochemical results

MVD: the clear stained blood vessels in tissues were scanned under low-magnification, and at the area with the maximum

quantity of microvessels, the number of blood capillary and small veins was counted under high-magnification; claybank positive endothelial cells or endothelial cell clusters which distinctly separated from the adjacent blood vessels and tissue cells can be marked as a microvessel. The mean of the three counts was regarded as the final MVD. Expressions of VEGF and Ang-2: the presence of claybank particles in tissue cell cytoplasm indicated positive staining. The sum of staining score and the score for proportion of positive stained cell count in total cell count ≥3 points was regarded as positive expression, and the expression rate of VEGF and Ang-2 was calculated. The scoring criteria for staining strength: negative 0 point, weak 1 point, moderate 2 points, and strong 3 points. The score for proportion of positive stained cell count in total cell count: 0 proportion 0 point, 1%–25% 1 point, 26%–50% 2 points, 51%–75% 3 points, and >75% 4 points.

2.6. Statistical analysis

The obtained data were analyzed using SPSS 17.0 software. Measurement data were expressed as mean ± SD, and the comparison between groups was performed using *t*-test; enumeration data were expressed as *n* (%), and comparison was done using χ^2 test; $P < 0.05$ indicates the statistically significant difference.

3. Results

3.1. Comparison of volume of endometriosis cyst in each group

Compared with the model group [(48.60 ± 15.11) mm^3], the volumes of the endometriosis cysts were significantly reduce in the positive group [(17.96 ± 3.34) mm^3, $P < 0.05$, $t = -7.669$] and the TQD group [(20.64 ± 6.05) mm^3, $P < 0.001$, $t = -6.653$]. The difference in volumes of the endometriosis cysts between positive group and the TQD group was non-significant ($P > 0.05$).

3.2. Comparison of serum levels of E2, CA125 and EMAb in each group

Compared with the normal group, the serum levels of E2 (pg/mL), CA125 (U/mL) and EMAb (ng/L) in the model group were significantly increased ($t = 15.785$, 12.361 and 8.651 respectively; all $P < 0.001$); compared with the model group, the serum levels of E2, CA125 and EMAb were significantly lower in the positive group ($t = -12.483$, −7.126 and −3.009 respectively; all $P < 0.05$) and TQD group ($t = -9.387$, −4.973 and −2.359 respectively; all $P < 0.05$); the difference in the serum parameters between positive group and the TQD group was non-significant ($P > 0.05$) (Table 1).

Table 2
Comparison of survivin, p53 gene and protein expression in cells of each group ($n = 15$, mean ± SD).

Group	mRNA		Protein	
	Survivin	p53	Survivin	p53
Normal	0.35 ± 0.08	0.87 ± 0.13	0.55 ± 0.10	1.58 ± 0.18
Model	1.12 ± 0.14**	0.21 ± 0.05**	1.26 ± 0.15**	1.06 ± 0.20**
Positive	0.63 ± 0.05##	0.70 ± 0.14##	0.75 ± 0.13##	1.33 ± 0.25#
TQD	0.67 ± 0.07##	0.61 ± 0.12##	0.82 ± 0.15##	1.27 ± 0.31#

Compared with normal group, **$P < 0.001$; compared with model group, #$P < 0.05$ and ##$P < 0.001$.

3.3. Comparison of MVD, VEGF and Ang-2 expressions in the tissues of each group

Compared with the normal group, the expressions of MVD, VEGF and Ang-2 were significantly increased in the model group ($t = 12.423$ for MVD; $\chi^2 = 16.205$ and 8.688 for VEGF and Ang-2 respectively; all $P < 0.05$); compared with the model group, the expressions of MVD, VEGF and Ang-2 were significantly lower in the positive ($t = -5.936$ for MVD; $\chi^2 = 8.688$ for VEGF and Ang-2; all $P < 0.05$) and TQD group ($t = -3.653$ for MVD; $\chi^2 = 6.806$ and 8.688 respectively for VEGF and Ang-2; all $P < 0.05$); the difference in positive expression rates of protein in the tissues between positive group and TQD group was non-significant ($P > 0.05$) (Table 1).

3.4. Comparison of survivin, p53 gene and protein expression in cells of each group

Compared with the normal group, the expression of survivin was significantly up-regulated in the model group ($t = 18.495$ and 15.253 respectively for mRNA and protein; each $P < 0.001$), and expression of p53 was significantly reduced ($t = -18.352$ and −7.485 respectively for mRNA and protein; each $P < 0.001$); compared with the model group, the expressions of survivin were significantly decreased in the positive ($t = -12.766$ and −9.951 respectively for mRNA and protein; each $P < 0.001$) and TQD group ($t = -11.135$ and −8.033 respectively for mRNA and protein; each $P < 0.001$), and expression of p53 was significantly up-regulated in the positive ($t = 12.766$ and 3.266 respectively for mRNA and protein; each $P < 0.05$) and TOD group ($t = 11.917$ and 2.205 respectively for mRNA and protein; each $P < 0.05$). The difference in survivin, p53 gene and protein expression levels between positive group and TQD group was not statistically significant ($P > 0.05$) (Table 2).

Table 1
Comparison of serum levels of E2, CA125 and EMAb, and the expressions of MVD, VEGF and Ang-2 in each group ($n = 15$, mean ± SD).

Group	E2	CA125	EMAb	MVD	VEGF [*n* (%)]	Ang-2 [*n* (%)]
Normal	23.28 ± 4.15	3.25 ± 1.13	0.20 ± 0.04	5.02 ± 1.85	1 (6.67)	2 (13.33)
Model	57.93 ± 7.42**	16.67 ± 4.05**	0.42 ± 0.09**	20.33 ± 4.40**	13 (86.67)**	11 (73.33)*
Positive	31.06 ± 3.80##	7.15 ± 3.22##	0.34 ± 0.05#	11.78 ± 3.43##	4 (26.67)#	2 (13.33)#
TQD	34.51 ± 6.19##	9.01 ± 4.38##	0.36 ± 0.04#	14.60 ± 4.19##	5 (33.33)#	2 (13.33)#

Compared with normal group, *$P < 0.05$ and **$P < 0.001$; compared with model group, #$P < 0.05$ and ##$P < 0.001$.

4. Discussion

EMs refers to the growth of endometrial tissue in other parts outside the uterine cavity mucous membrane. The incidence of EMs in women of childbearing age is 10%–20%, of which 30%–70% cases are accompanied with pelvic pain and infertility, seriously affecting women's reproductive health and quality of life [4,5]. At present, Western medicine therapy mainly employs surgery and hormone-based drug to treat EMs, but it is still difficult to achieve a radical treatment, and adverse reactions were remarkable with EMs tending to relapse. In recent years, Chinese medicine treatment for EMs has made some progress. Most clinical studies and basic experiments have confirmed the exact efficacy and safety of Chinese medicine on EMs [6–8]. Chinese medicine believes that EMs is caused by accumulation of blood, which is outside the vessels, in the pelvic cavity; according to its pathogenesis, EMs can be divided into qi stagnation and blood stasis syndrome type, renal deficiency and blood stasis syndrome type, cold coagulation blood stasis syndrome type, and blood stasis and heat accumulation syndrome type etc. The basic principles for treatment is to promote blood circulation to remove blood stasis, and the methods include promoting the circulation of qi and dispersing blood stasis, invigorating the kidney and eliminating blood stasis, clearing away heat and resolving blood stasis, warming meridians and removing blood stasis, and activating blood circulation and eliminating blood circulation *etc*. TQD is an effective traditional Chinese medicine prescription for the treatment of EMs, and has been applied clinically for many years and achieved a certain therapeutic effect. The prescription uses Taoren as the principal agent, supplemented with Danshen, Dang Gui and Yi Mu Cao and such crude drugs; this prescription has effects of promoting qi and blood circulation, removing blood stasis and lumps, and regulating menstruation and relieving pain. In this study, TQD was applied to rats EMs model and human endometrial cells, respectively, to explore the medicinal value of TQD from different aspects.

The results of this study revealed that the volume of endometriosis cyst in TQD group was significantly reduced compared to model group, and the difference was non-significant between positive group and the TQD group. This finding indicates that TQD has an inhibitory effect on the growth of EMs. Laboratory tests showed that the serum levels of CA125 and EMAb in TQD group were significantly lower than those in model group, and were not significantly different from those in normal group and positive group, indicating that TQD has remarkable effect on EMs.

It has been reported that angiogenesis plays an important role in the incidence and development of EMs; over angiogenesis may be the basis of the pathogenesis of EMs [9,10]. Most studies suggest that traditional Chinese medicine can inhibit neovascularization of ectopic tissue, thus controlling the progress of EMs [11–14]. In the present study, TQD can significantly reduce the serum level of E2 in rats. EMs is an estrogen-dependent disease; high levels of E2 can directly inhibit NK cell activity, leading to growth of ectopic endometrial tissues. Therefore, lowering E2 levels can reduce the proliferation speed of ectopic endometrial tissue and reduce neovascularization [15–17]. The angiogenesis mechanism of EMs involves a number of related signal transduction pathways, and is regulated by a variety of factors, mainly VEGF and Ang-2 [18,19]. The results of the present study revealed that the expression of VEGF and Ang-2 in EMs model rats treated with TQD was significantly decreased and MVD was significantly reduced, suggesting that the mechanism of action of TQD on EMs may be achieved by down-regulating VEGF and Ang-2 expression. VEGF is known to be one of the key factors in promoting angiogenesis, and can directly act on the vascular endothelial cells to promote endothelial cell proliferation, regulate blood vessels and increase vascular permeability [20,21]. Ang is a kind of endothelial cell-specific pro-angiogenic factor that regulates angiogenesis, blood vessel degeneration and stability. Ang-2 is most closely related to VEGF, and concurrence of both with up-regulated expression can synergistically promote angiogenesis [22,23]. In this study, the increased expression of VEGF and Ang-2 in the transplanted lesion tissue of EMs model rats could confirm this statement. Therefore, lowering VEGF and Ang-2 expressions, inhibiting and blocking angiogenesis may be an effective approach to treat EMs.

To further explore the mechanism of TQD on EMs, the present study also evaluated the effect of TQD on apoptosis of human endometrial cell. Cell proliferation and apoptosis is another important pathogenesis of EMs. Apoptosis is an important factor in maintaining the stability of endometrial cells, and over cell proliferation and reduced apoptosis promote the development of EMs [24,25]. At present, animal experiments and clinical studies evidenced that traditional Chinese medicine can achieve the treatment of EMs by inducing apoptosis [26,27]. The finding of the present study indicated that compared with the model group, the expression of survivin in the TQD group was significantly decreased, and expression of p53 was significantly up-regulated, which were not significantly different from those in positive group. Survivin is a novel antiapoptotic factor that promotes cell proliferation, inhibits apoptosis and participates in angiogenesis [28]. p53 is currently one of the most studied tumor suppressor genes, whose main function is regulating cell cycle and apoptosis [29]. The expressions of survivin and p53 in ectopic endometrial cells are reverse, and the increase in survivin expression and decrease in p53 expression indicate the reduction of apoptosis level, which would be beneficial to the development of EMs [30,31]. This finding confirms that TQD has the effect in promoting endometrial cell apoptosis and provides evidence for TQD treatment of EMs.

In conclusion, TQD has significant anti-EMs effect on rat model of EMs and human endometrial cells; its mechanism may be related to anti-angiogenesis and promotion of ectopic endometrial cell apoptosis. The present study focused on the angiogenesis mechanism and the apoptosis mechanism to evaluate the effect of TQD on EMs, but TQD may also act on EMs via other mechanisms, which need to be further investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

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