Life Sciences 240 (2020) 117085

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Tanshinone IIA contributes to the pathogenesis of endometriosis via renin angiotensin system by regulating the dorsal root ganglion axon sprouting

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ARTICLE INFO

Keywords:
Endometriosis
Tanshinone IIA
Renin angiotensin system
Dorsal root ganglion
Estradiol
Angiotensin II

ABSTRACT

Aims: Our study was designed to explore the function and mechanism of Tanshinone IIA in alleviating pain syndrome caused by endometriosis (EMs).

Main methods: Female Sprague-Dawley rats went through autotransplantation operation to establish EMs model. The rats were randomly divided into five groups: sham, model, positive, Tanshinone IIA (L) (3 mg/kg/d) and Tanshinone IIA (H) (12 mg/kg/d) group. Volume of ectopic endometrium was measured after 21 days of continuous administration. Serum estradiol (E2) was detected by enzyme linked immunosorbent assay (Elisa). The protein expression of angiotensinogen (AGT), renin (REN), angiotensin converting enzyme (ACE), angiotensin II (ANGII) and angiotensin II type 2 receptor (AT2) in the dorsal root ganglion (DRG) neurons were measured by immunohistochemistry and Western Blotting. The mRNA expression levels of AGT and ANGII were measured by Real-time polymerase chain reaction (PCR).

Key findings: Tissue measurements showed that tanshinone IIA significantly inhibited the growth of ectopic endometrium. Tanshinone IIA could improve the paw withdrawal threshold thus reducing the mechanical hyperalgesia of EMs rats. Moreover, Tanshinone IIA regulated the DRG renin angiotensin system (RAS) by reducing the protein expression of AGT, REN, ACE, ANGII and AT2 in DRG neurons. Furthermore, Real-time PCR results also showed that the mRNA expression levels of AGT and ANGII in the DRG neurons were decreased.

Significance: The Tanshinone IIA inhibitory effect on the EMs associated pain in EMs rats might occur through decreasing the expression of E2, ANGII and AT2, thus halting DRG sprouting and promoting hyperalgesia threshold.

1. Introduction

Endometriosis (EMs) is a gynecologic disease characterized by extra-uterine endometrial hyperplasia, which affects 6–10% women of childbearing age. The prevalence of EMs among infertile women reaches up to 30–50% [1]. EMs severely affects women's quality of life, impacting their careers, fertility, sexual and nonsexual relationships [2]. EMs is considered to be the major cause of pelvic pain, dysmenorrhea, dyspareunia, and infertility. So far, the exact etiology and pathophysiology of EMs are still unknown. Most scholars agree with Sampson's EMs implantation theory, which believes that EMs is caused by menstrual reflux [3].

Pain is the central clinical feature in EMs and often remains despite treatment of the disease. The mechanisms of pain in EMs include inflammation, pain perception, and changes in peripheral and central nervous system pain processing [4]. Although chronic pain may be caused by a variety of factors, increasing evidence suggests that peripheral axonal remodeling may play an important role. Previous studies have shown that E2 can promote axon regeneration and sprouting in many nerve tissues [5], including the dorsal root ganglion (DRG) [6]. In the early postpartum period, ERα and ERβ are expressed in the DRG neurons, which contribute to the survival and development of these nerves [7]. In addition, studies have shown that there is an intrinsic DRG renin angiotensin system (RAS) and that synthesis of angiotensin II (ANGII) is a necessary condition for E2 to induce the growth of sensory neurons [8]. Studies have shown that E2 promotes the sprouting of DRG pain receptor axons by upregulating AT2 receptor. Therefore, pharmacological regulation of RAS may provide a strategy for changing the course of some EMs pain syndromes.

Currently, hormonal medication and surgery are the standard therapeutic options for EMs. Surgery can eliminate ectopic lesions, restore normal pelvic anatomy, relieve pain and increase the chance of

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https://doi.org/10.1016/j.lfs.2019.117085
Received 15 July 2019; Received in revised form 15 November 2019; Accepted 15 November 2019
Available online 20 November 2019
0024-3205/

pregnancy. However, the recurrence rate of EMs patients five years after surgery is > 40% [9]. Hormone therapy is designed to reduce pain and delay relapses by inhibiting the production of estrogen in the ovaries, which can cause side effects, such as menopausal symptoms and osteoporosis [10]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used first-line treatment for endometriosis pain in women. However, till now, there is lack of high-quality evidence that NSAIDs are effective in managing pain caused by EMs. Therefore, new medications for EMs that can reduce the recurrence rate and relieve the associated pain with fewer side effects have become the current EMs research hotspot.

Tanshinone IIA is the highest content of diterpene quinone compound extracted from a traditional Chinese medicine Danshen (*Salvia miltiorrhiza* Bge.) [11]. Tanshinone IIA was initially used to treat cerebral ischemia reperfusion injury, acute cerebral infarction, coronary heart disease and other cardiovascular and cerebrovascular diseases [12–14]. Subsequent studies have also shown that it had good curative effect in other diseases, including cancer [15], liver diseases [16], Alzheimer's disease [17], polycystic ovary syndrome [18] and endometriosis [19]. So far, the molecular mechanism of tanshinone IIA therapeutic effects in EMs diseases remains unclear. Recent studies have shown that tanshinone IIA can inhibit the development of EMs lesions in mice, prevent epithelial mesenchymal transition and fibroblast to myofibroblast trans-differentiation, inhibit the expression of CA-125, IL-18 and TNF-alpha, induce ectopic endometrial stromal cells apoptosis [11,19,20]. However, the regulatory effects of tanshinone IIA on DRG and its role in EMs related pain remains to be determined. Therefore, this study aims to explore the therapeutic effect of Tanshinone IIA on EMs rats, and to explore the interaction between E2 and RAS mediated DRG axon sprouting. Ultimately, this study will provide a reference for its further study and clinical therapeutic use in EMs diseases.

2. Materials and methods

2.1. Animals

Thirty-six adult female Sprague-Dawley (SD) rats (weighing180–200 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Certificate of Quality No: SCXK 2016-0006). All animal protocols and procedures used in this study were reviewed and approved by the Animal Care Welfare Committee of the Capital Medical University (number AEEI-2018-031). All rats were allowed 1 week of acclimation to the environment and were fed ad libitum with standard rodent diet and water.

2.2. Chemicals and reagents

Sulfotanshinone sodium injection (contains 5 mg/mL of tanshinone IIA, molecular formula: $C_{19}H_{18}O_3$, molecular weight: 294.34, CAS No.:568-72-9) was purchased from Shanghai No.1 biochemical pharmaceutical Co., Ltd. (Shanghai, China). Medroxyprogesterone acetate tablets were purchased from Zhejiang Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). Estradiol valerate tablets were purchased from Bayer Vital GmbH (France). Estrogen elisa kits were purchased from Wuhan Huamei Biological Engineering Co., LTD (Wuhan, China). The polyclonal rabbit anti-AGT, anti-Renin, anti-ANGII, anti-ACE and anti-AT2 receptor were all purchased from Beijing Bioss biotechnology Co., LTD (Beijing, China).

2.3. Establishing the model of EMs in rats

Model preparation methods refer to the procedures described by Vernon and Wilson [21]. The rats (n = 30) were anesthetized by intraperitoneal administration of 40 mg/kg pentobarbital sodium. Their abdominal skin was sterilized with 75% alcohol, and then an incision was made in the abdomen to expose the uterus. After that, the left uterine was ligated, removed and immediately put into PBS. The endometrium was separated from the muscle layer and cut into 5 × 5 mm slices. The uterine segments were then transplanted to the right

Table 1
Quantitative polymerase chain reaction primer sequences.

Gene	Primer sequences (5′ → 3′)	Product size (bp)
GAPDH	F: CCTGGGCTACACTGAGGA R: TGAGGTCCACCACCCTGT	164
ANG II	F: CACTGCGGGTCTGGAGAAAT R: GACGGAACAACGAACTGTGC	295
AGT	F: AGGAGCAGCCCACAGAGT R: ACCTGAGTCCCGCTCGTA	104

ANG II, angiotensin; AGT, angiotensinogen; F, forward; R, reverse.

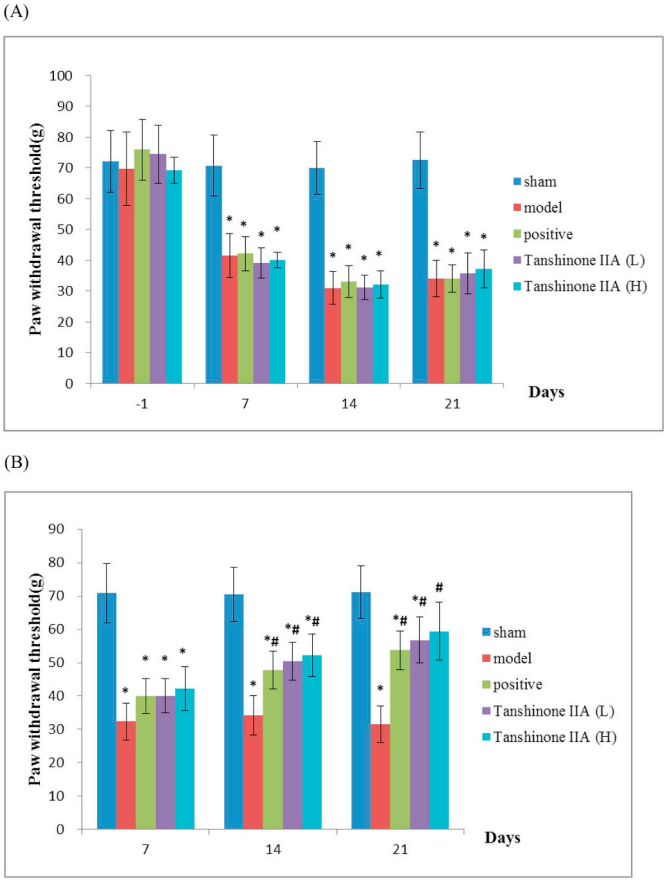


Fig. 1. Effect of Tanshinone IIA on the paw withdrawal threshold response in rats measured by mechanical hyperalgesia.
(A) Mechanical hyperalgesia was measured in rats 1 days before surgery (day −1) and on day7, 14, 21 after sham or surgery. (B) Mechanical hyperalgesia was measured in rats on day7, 14, 21after drug therapy. Data are presented as mean ± standard deviation. $^{*}P < 0.05$ vs sham group; $^{\#}P < 0.05$ vs model group.

Table 2
Volume of lesions before and after treatment.

Group	Pretreatment (mm^3)	Posttreatment (mm^3)
Model	69.43 ± 8.86	114.05 ± 11.68**
Positive	74.44 ± 11.65	49.53 ± 7.31**
Tanshinone IIA (L)	70.82 ± 9.71	57.74 ± 10.26*
Tanshinone IIA (H)	67.34 ± 8.75	34.33 ± 13.03**

$^{*}P < 0.05$, $^{**}P < 0.01$ compared with pretreatment.

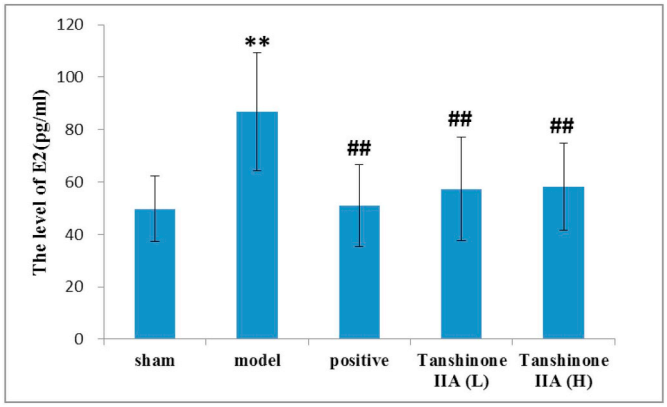


Fig. 2. Effect of Tanshinone IIA on the level of E2 in serum as assessed by the Elisa.
Data are presented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model group.

peritoneal surface near the great vessels, with the endometria surface attached to the wall. After the operation, all the rats were administered 0.02 mg estradiol valerate daily for 3 days. At the same time, another 6 rats were served as sham operated group.

After 21 days, the operation was performed again. We used a Vernier caliper to measure the three dimensions of endometrial grafts. The endometrial volume was calculated by formula: volume (mm^3) = 0.52 × (length × width × height) [22]. The successful EMs model standard is considered to be rats with endometrial volume larger than 25 mm^3 [23]. A total of 24 rats were qualified to be used in the following experiments. Rats in the sham operation group only underwent laparotomy, and no measurement was made at the second laparotomy.

2.4. Animal treatments

The 24 rats were randomly divided into model, positive, Tanshinone IIA low dose, and Tanshinone IIA high dose groups ($n = 6$ in each group). There was no significant difference in autograft volume among groups before medication treatment. Rats in the sham and model groups were given solvent (0.5% carboxymethyl cellulose sodium). Rats in the positive group were treated with 2 mg/kg/day medroxyprogesterone acetate by oral gavage refer to previous studies [24]. Rats in the Tanshinone IIA (L) and Tanshinone IIA (H) group were given 3 and 12 mg/kg/day of Tanshinone IIA by intraperitoneal injection, respectively.

The third laparotomy was performed 21 days after the medication treatment. Briefly, the volumes of ectopic endometrium were measured again with Vernier caliper. Blood was collected from abdominal aorta after anesthesia, and then serum was extracted and stored at −20 °C. After removing L1-L6 DRGs, the tissues were partially fixed in 4% neutral paraformaldehyde solution and partially preserved at −80 °C.

2.5. Measurement of mechanical hyperalgesia

To assess mechanical hyperalgesia, a digital force transducer electronic von Frey (Bioseb, France) was used 1 days before surgery (day −1), on day 7, 14, 21after sham or surgery, and on day 7, 14, 21 after drug therapy. Before each experiment, the rats were placed on a metal platform and allowed to adapt to the environment for > 15 min. The stimulus intensity was successively increased and/or decreased to determine the paw withdrawal threshold response. Take an average of 3 readings at 5 min intervals as the paw withdrawal threshold [25].

2.6. Enzyme linked immunosorbent assay (ELISA)

Blood was centrifuged at 3000 r/min for 10 min, after that the supernatant fluid was removed. Serum levels of E2 were determined by ELISA according to manufactures instructions (Huamei, China). The luminescent signal produced was measured at 450 nm using a microplate reader (Thermo Scientific, USA). The concentration of E2 was determined from the standard calibration curve and expressed as pg/mL serum. Each sample was tested in duplicate in two separate measurements. The minimum detectable dose of rat E2 is typically < 40 pg/mL. Three samples of known concentration were tested twenty times on one plate to assess the intra-assay precision (CV% < 15%). Three samples of known concentration were tested in twenty assays to assess the inter-assay precision (CV% < 15%).

2.7. Immunohistochemical staining

The DRGs were embedded in paraffinize and sliced into 4 μm thick sections. After deparaffinized and rehydrated, antigen retrieval was performed with 0.01 M sodium citrate buffer. Sections were incubated with 0.3% catalase for 20 min to inactivate endogenous peroxidase activity. After blocking the non-specific antibodies, the tissues were incubated overnight with primary antibody at 4 °C. The dilution ratio of anti-AGT, anti-Renin, anti-ANGII, anti-ACE and anti-AT2 was 1:400. After washing with PBS, the secondary antibody was sequentially incubated at 37 °C for 30 min. Subsequently, the sections were then rinsed again with PBS, 0.01% 3, 3-diaminobenzidine tetrahydrochloride (DAB) for staining, and then dehydrated with alcohol and xylene. On each section five fields were selected randomly using a BX53 microscope (Olympus). The optical density (OD) of positive cells was analyzed by the Image-pro plus 6.0 (IPP) software. The average integral OD (AIOD) was calculated as follows, AIOD = positive area × OD / total area.

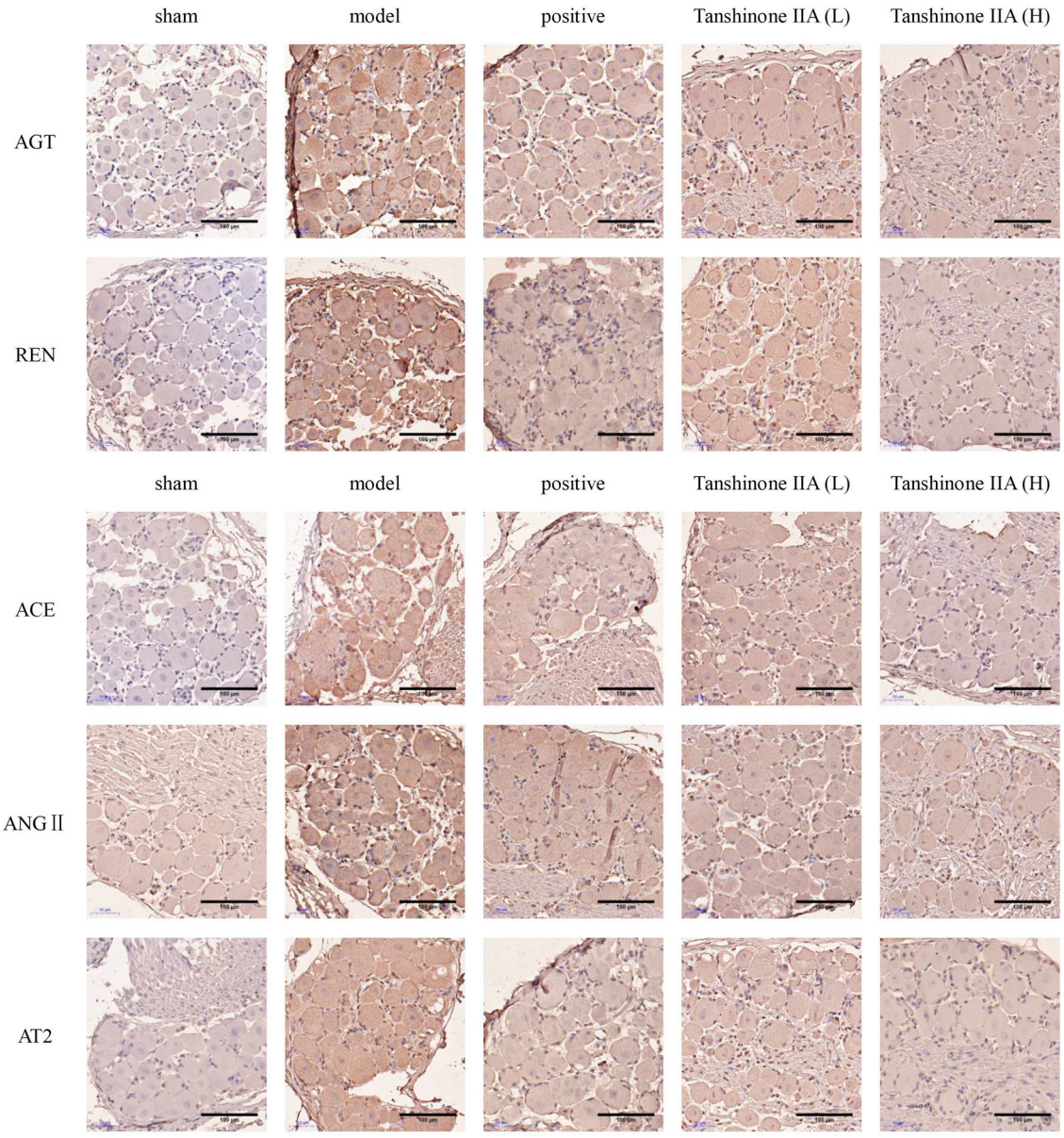
2.8. Western blotting analysis

Western blot analysis was performed by referring to the literature [26]. The total protein was extracted from DRG in each group. After protein quantitation using bicinchoninic acid (BCA) method, 80 μg proteins in each lane were separated by 10% SDS-PAGE gels. The proteins were transferred to polyvinylidene difluoride membranes. Then, the membranes were blocked with 5% skimmed milk for 1 h. The membranes were then incubated with ACE, Renin, ANGII and AGT polyclonal antibodies (diluted 1:1000 with 5% BSA), AT2 receptor polyclonal antibodies (diluted 1:500 with 5% BSA), β-actin monoclonal antibodies (diluted 1:5000 with 5% BSA) overnight at 4 °C. After washes with PBST for three times, the membranes were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (diluted 1:5000 with 5% BSA) for 1 h at 37 °C. The membranes were washed three times with PBST, and photographs were taken immediately after color development using a Gel Imaging System (Bio-Rad Laboratories, California, USA). The band intensities were analyzed using Image J software (v1.46, NIH, USA).

2.9. Real-time PCR

Total RNA from DRGs was isolated using TRIzol reagent (Invitrogen, USA). The quality of RNA was evaluated by measuring absorbance at 260 and 280 nm, and yield was determined by measuring absorbance at 260 nm. The cDNA was reversed transcribed according to standard protocols for PCR (30 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min, 4 °C for 5 min). Real-time PCR was performed using SYBR Green Master Mix (Invitrogen, USA) in an Applied Biosystems 7500 system (Applied Biosystems) following the manufacturer's instructions. The conditions were as follows: pre-denaturation at 95 °C for 2 min, denaturation at 95 °C for 15 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s,

(A)



(B)

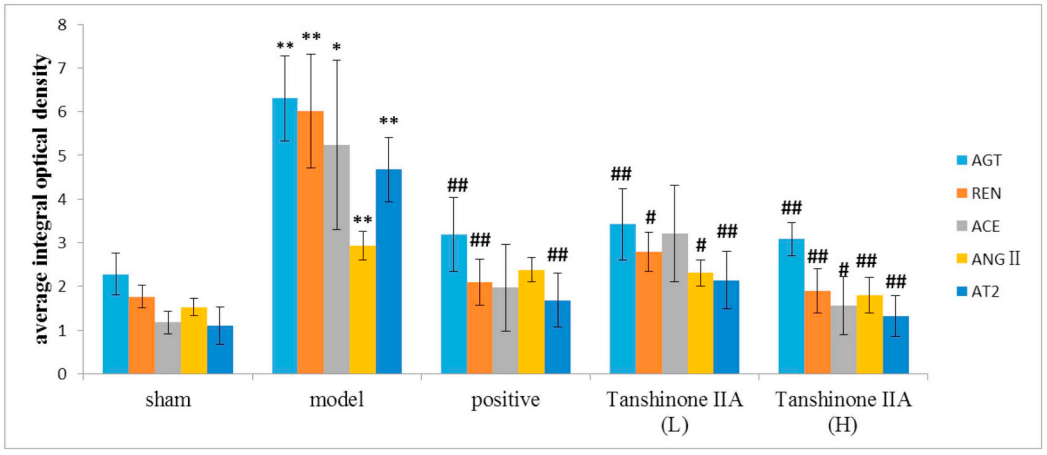


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Fig. 3. Effect of Tanshinone IIA on AGT,REN, ACE, ANGII and AT2 in the DRG measured by immunohistochemically (×200).
(A) Positive staining was visualized in brown color. (B) Quantification of AGT, REN, ACE, ANGII and AT2 expression. Scale bars:100 μm. Data are presented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model group. DRG, dorsal root ganglion. IOD, integrated optical density. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for a total of 35 cycles. GAPDH served as the internal control. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [27]. The primers used in real-time PCR are listed in Table 1.

2.10. Statistical analysis

Measurement data were shown as mean ± standard deviation (SD). The data analysis was performed using SPSS 20.0 (Chicago, USA). The comparisons among groups were made using one-way ANOVA for normally distributed data followed by Bonferroni test, and the Mann-Whitney rank sum test for nonparametric analysis. $P < 0.05$ were accepted as significant.

3. Results

3.1. Effect of Tanshinone IIA on mechanical hyperalgesia

There was no significant difference in the paw withdrawal threshold among groups 1 day before surgery. Compared with sham group, paw withdrawal threshold was significantly decreased in animals receiving endometrial autotransplantation in the model, positive, Tanshinone IIA (L) and Tanshinone IIA (H) groups on day 7, 14, 21 after surgery. After 2 or 3 weeks treatment with tanshinone IIA or medroxyprogesterone acetate, paw withdrawal threshold was significantly improved and mechanical hyperalgesia was relieved (Fig. 1).

3.2. Effect of Tanshinone IIA on lesion volume in rats

Three weeks after transplantation, the mean volumes of the lesions were comparable between the four operation groups (Table 2). After treatment, the volume of ectopic endometrial lesions decreased to different degrees, and the positive group, tanshinone IIA (L) group and tanshinone IIA (H) group all showed significant decreases compared with the model group. However, the mean volume of the lesions in the model group increased from 69.43 ± 8.86 mm^3 to 114.05 ± 11.68 mm^3.

3.3. Effect of Tanshinone IIA on estrogen (E2)

The results showed that the level of serum estrogen (E2) in the model group was higher than that in the sham group ($P < 0.01$). The levels of E2 were reduced significantly ($P < 0.01$) in the positive group and tanshinone IIA (L, H) groups compared with the model group. There was no statistically significant difference between the positive group and tanshinone IIA (L, H) group (Fig. 2).

3.4. Effect of Tanshinone IIA on the expression of AGT, REN, ACE, ANGII and AT2 in the DRGs measured by immunohistochemically

Compared with the sham group, the AGT, REN, ACE, ANGII and AT2 expression were significantly up regulated in the model group. Compared with the model group, Medroxyprogesterone acetate treatment significantly reduced the AGT, REN and AT2 expressions. Compared with the model group, the AIOD of AGT, REN, ANGII and AT2 in both Tanshinone IIA (L) and Tanshinone IIA (H) groups manifested a statistically significant decrease. In addition, we also found that the ACE level in DRGs after Tanshinone IIA (H) treatment was markedly reduced compared with that in the model group (Fig. 3).

3.5. Effect of Tanshinone IIA on the AGT, REN, ACE, ANGII and AT2 expression in the DRGs assessed by Western blotting

Studies have demonstrated that the expression of AGT, REN, ACE, ANGII and AT2 in the DRG in model group significantly increased than that in the sham group, which was consistent with the results of immunohistochemical. The relative expression levels of AGT, REN, ACE, ANGII and AT2 were significantly decreased in the positive and Tanshinone IIA (H) groups compared with the model group. Moreover, Tanshinone IIA (L) treatment significantly reduced the relative expression levels of REN, ACE and AT2 compared with the model group (Fig. 4).

3.6. Effect of Tanshinone IIA on gene expression of AGT and ANGII

We investigated mRNA levels of AGT and ANGII by real-time PCR. The mRNA levels of AGT and ANGII in the model group were significantly higher in comparison with the control group. Compared to the model group, treatment of medroxyprogesterone acetate or Tanshinone IIA (L, H) significantly reduced the gene expression levels of AGT and ANGII in the DRG (Fig. 5).

4. Discussion

EMs is a benign gynecological disease, but it has aggressiveness, invasive and recurrent malignant behaviors. Although many theories have been proposed to explain the pathophysiology of endometriosis, none is complete and the mechanism remains elusive. The pathophysiology of endometriosis is related to the changes of endometrial related steroids and alterations in ovarian steroidogenesis [28]. It has been reported that abnormally high E2 levels are closely related to the incidence of EMs. There are many ways to treat the symptoms of EMs, but treating the disease often requires repeated medical or surgical interventions. Surgical treatments include excision (using scissors or electricity) and ablative techniques (destroying the EMs with laser or electricity). The goal of excision surgical is to relieve symptoms while conserving reproductive function. Medical treatments for EMs include progestogens, oral contraceptives, gonadotrophin-releasing hormone (GnRH) agonists and testosterone derivatives [29]. Conventional medication is designed to suppress ovarian hormone production, but the effect is temporary and has side effects such as menopausal symptoms and osteoporosis. NSAIDs are the most commonly used first-line drugs in the treatment of EMs. NSAIDs may act on local cytokines within ectopic endometrium and may act as analgesics. But they do not remove or decrease deposits of ectopic endometrium and they may cause adverse effects such as nausea, vomiting, headache and drowsiness. In order to clarify the pathological of EMs and to establish a new treatment strategy for this disease, our research group is studying the mechanism of Tanshinone IIA reducing the volume of ectopic endometrium tissue and alleviating EMs related pain.

Although EMs is difficult to grow naturally in SD rats, its endometrium is sensitive to hormone stimulation. Moreover, the cycle pattern of rat endometrium was similar to that of women [30]. So, rats are relatively ideal experimental animals to study EMs. In this paper, rat model of EMs was established by autotransplanting of uterine horn slice near abdominal wall artery. After three weeks, the uterine transplants develop into cysts that resemble ectopic growths in women [31].

A combination of oral contraceptives (COCs) and progesterone has been used to treat pain associated with EMs in women. These drugs have been reported to significantly reduce dysmenorrhea, deep

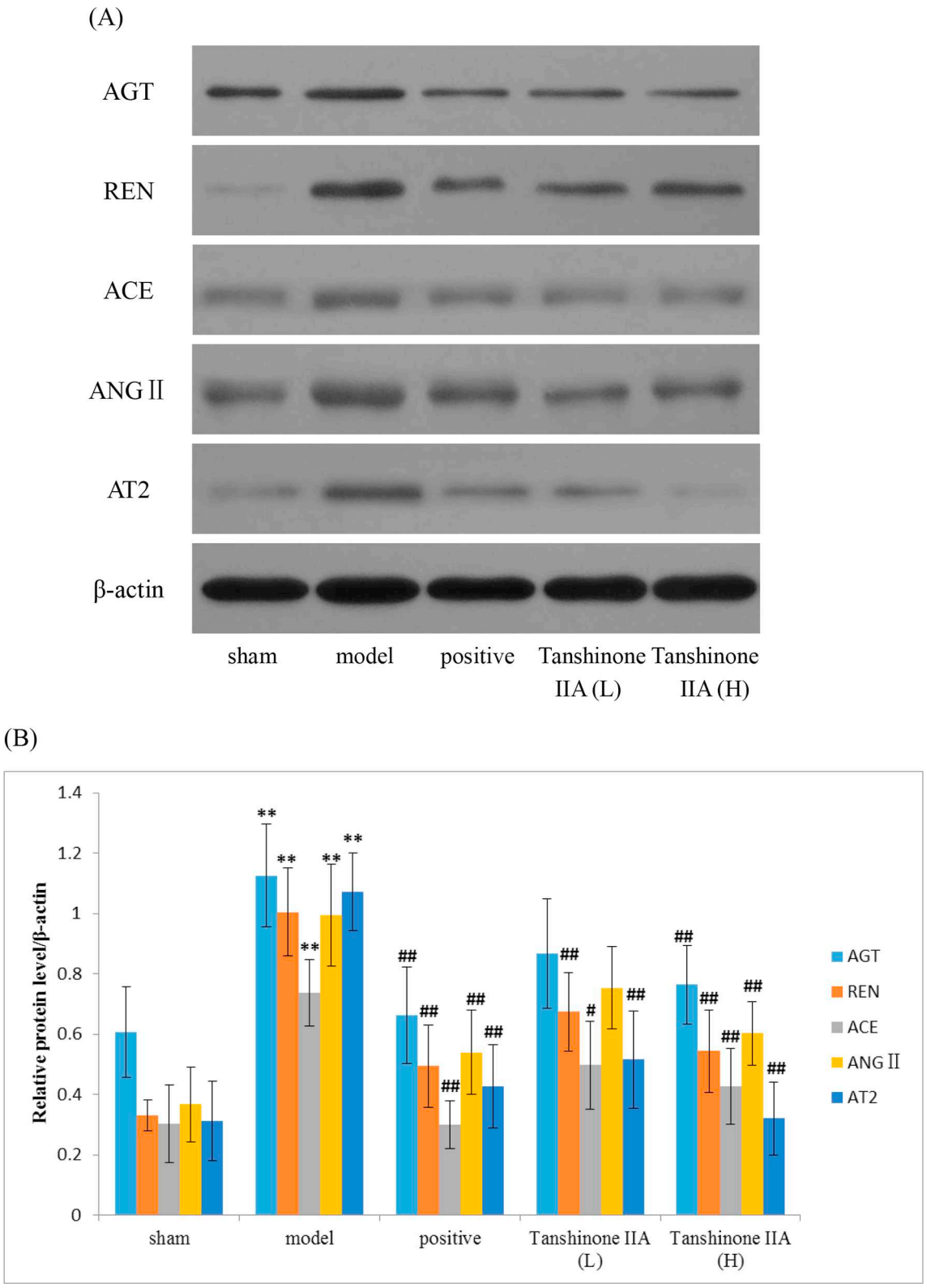

Fig. 4. Protein levels of AGT, REN, ACE, ANGII and AT2 in the DRG in rats.
(A) Representing Western blot analysis of AGT, REN, ACE, ANGII and AT2. (B) Quantification of AGT, REN, ACE, ANGII and AT2 expression. β-actin was used as a loading control. Data are presented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model group. DRG, dorsal root ganglion.

dyspareunia and non-menstrual pelvic pain in women with EMs [32]. Further studies have reported that COCs and progesterone significantly reduce the density of nerve fibers in eutopic endometrium in patients with EMs [33]. Medroxyprogesterone acetate is an effective standard drug for relieving pain associated with EMs and reversing the proliferation of EMs tissues, which was selected as the positive control drug in this study [34,35]. The results showed that medroxyprogesterone acetate significantly inhibited the growth of EMs, as follows

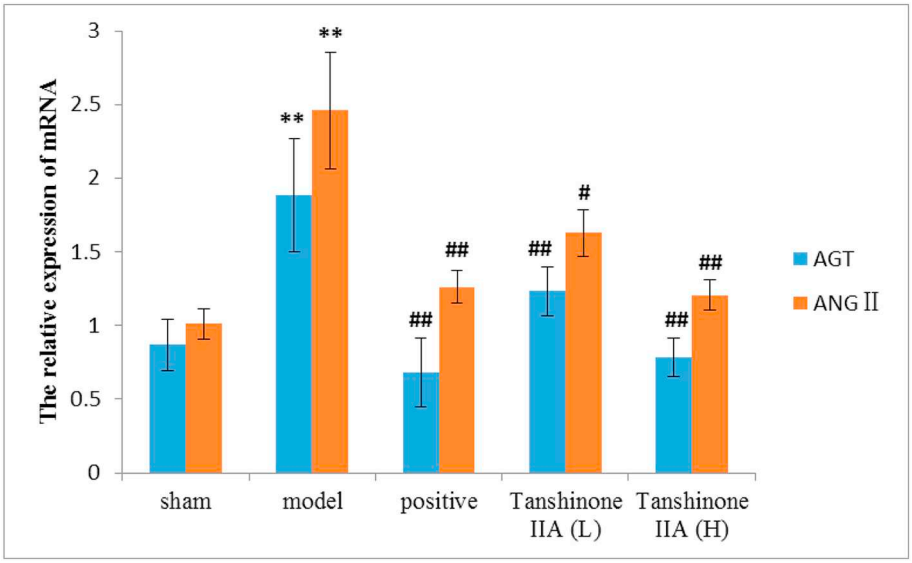

Fig. 5. Effect of Tanshinone IIA on gene expression levels of AGT and ANGII against reference gene in the DRG in rats.
Data are presented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model group. DRG, dorsal root ganglion.

49.53 ± 7.31 mm^3 post-treatment and 74.44 ± 11.65 mm^3 pre-treatment. The results of this study showed that the volume of EMs cyst in the tanshinone IIA group was significantly reduced after treatment compared with that before treatment, while the difference between the positive group and the Tanshinone IIA (L, H) group was not statistically significant. These results suggest that Tanshinone IIA can inhibit the growth of EMs tissues.

EMs is an estrogen dependent disorder, and E2 plays an important role in the growth of ectopic endometrium. The hormone regulation of ectopic EMs lesions may be less effective than that of EMs in situ, and the continuous local estrogen secretion may lead to greater cell proliferation, neuronutrient level and nerve fiber density, and reduced apoptosis, than that of patients with EMs in situ. Herein, we detected the serum concentration of E2 after treatment with Tanshinone IIA. We found that the serum levels of E2 obviously decreased in both 3 and 12 mg/kg Tanshinone IIA treated group compared to that in the model group. The present study suggested that tanshinone IIA inhibiting the proliferation of EMs tissues may be related to the reduction of serum E2 level. In addition, we also found that medroxyprogesterone acetate markedly reduced serum level of E2. And this result was consistent with previous report [36].

Since the early 1930s, the chemical constituents and biological activities of Danshen (*Salvia miltiorrhiza* Bge.) have been studied extensively. > 30 lipophilic diterpenes and 50 hydrophilic components were separated and identified from Danshen (*Salvia miltiorrhiza* Bge.) [37,38]. The main lipophilic components include tanshinone IIA, tanshinone IIB, tanshinone I, and cryptotanshinone, among which TIIA is the structural representative and most abundant derivative of tanshinone [39]. Numerous experimental studies have shown that tanshinone IIA has multiple biological activities, including but not limited to antiplatelet aggregation [40], antiinflammatory [41], antioxidant [42] and antitumor effects [43]. In recent years, its positive effects in EMs diseases have been widely studied. Tanshinone IIA could inhibit cell migration and invasion, induce cell apoptosis, and decrease cell viability in ectopic endometrial stromal cells by regulating the expression of 14-3-3 protein [20]. In addition, Tanshinone IIA could prevent fibrosis, the epithelial mesenchymal transition, fibroblast to myofibroblast transdifferentiation and smooth muscle metaplasia [19]. Moreover, Tanshinone IIA showed promises in treating EMs by increasing the levels of IL-13 in the peritoneal fluids and decreasing the serum levels of CA-125 and the levels of IL-18 and TNF-alpha in the peritoneal fluids [11]. According to the current knowledge, Tanshinone IIA is less toxic and is considered safe for clinical use. Tanshinone IIA has the anticancer effect, but it does not have cytotoxic against hepatocytes [44]. In addition, treatment with 100 μM TIIA for 48 and 72 h had no side effects on human amniotic mesenchymal stem cells (HAMCs) [45]. Moreover, the use of Tanshinone IIA during the last third of pregnancy did not cause any biochemical changes related to the liver and kidney function in the mother or fetus, which provided a new basis for guiding the use of Tanshinone IIA during pregnancy [46]. Therefore, it is necessary to understand the efficacy of tanshinone IIA and reveal its potential mechanism in the treatment of EMs, which can promote the development of new treatment strategies for EMs.

The main complaint of EMs patients is pain, which has a negative impact on quality of life. However, the pathogenesis of EMs related pain, especially chronic pain, is still unclear and further studies are needed. The pain sensor in DRG neuron is the first site of pain transmission, and it is a hot spot of pain research. The DRG afferent nerve not only innervates the secondary sensory neurons, but also innervates the laminae II interstitial neurons, stimulating or inhibiting the secondary sensory neurons and regulating pain signals [47,48]. The studies of animals and in humans have shown that pain sensitivity and somatosensory perception are affected by E2 [49,50]. In the early postpartum period, ER α and ER β are expressed in the DRG, and both of which are important for the development and survival of DRG [7]. Studies have shown that E2 promotes DRG axon sprouting through a renin angiotensin system (RAS) [8]. In brief, renin lysis angiotensinogen (AGT) to produce angiotensin I (ANGI), which is then converted by angiotensin converting enzyme (ACE) to the biologically active angiotensin II (ANGII). Recently, it has been reported that ANG II can promote axon elongation of DRG neurons in vitro through its ANG II type 2 (AT2) receptor [51]. In addition, E2 can increase the expression of AT2 mRNA in DRG neurons. So, E2 may aggravate some female related pain syndromes by promoting nerve excitation in peripheral tissues.

In the current study, tanshinone IIA can significantly decrease the serum level of E2 in rats and may prevent the growth of ectopic endometrial tissue. In addition, we demonstrated that tanshinone IIA could improve the paw withdrawal threshold thus reducing the mechanical hyperalgesia of EMs rats. Furthermore, it was found that tanshinone IIA can markedly inhibit protein expression of AGT, REN, ACE, ANGII and AT2 in the DRG neurons in rats. The mRNA expression of AGT and ANGII in the DRG in Tanshinone IIA (L) and Tanshinone IIA (H) groups were consistent with the results of protein expression. These

findings demonstrated that Tanshinone IIA may inhibit DRG RAS to regulate the DRG development and thus affects sensory function and pain sensitivity.

5. Conclusion

In conclusion, this finding suggests that the Tanshinone IIA inhibitory effect on the EMs associated pain in rats might occur through decreasing the expression of E2, ANGII and AT2, suppressing the intrinsic DRG RAS thus halting DRG sprouting and promoting hyperalgesia threshold. This may be the potential mechanism of Tanshinone IIA in EMs treatment. However, other molecular pathways involved in the pathogenesis of EMs remain to be explored.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (grant No. 81603649) and the Beijing Municipal Excellent Talents Foundation (grant No. 2016000020124G077).

Author contribution

Xin Gong designed the research; Zhen-zhen Chen and Xin Gong performed the research; Zhen-zhen Chen analyzed the data and wrote the paper; Xin Gong revised the manuscript.

Declaration of competing interest

The authors have no financial conflict of interests.

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