JTCM 中医杂志 Journal of Traditional Chinese Medicine

Online Submissions: http://www.journaltcm.com
info@journaltcm.com

J Tradit Chin Med 2015 April 15; 35(2): 184-191
ISSN 0255-2922

EXPERIMENTAL STUDY

Effect of Bushenwenyanghuayu decoction on nerve growth factor and bradykinin/bradykinin B_1 receptor in a endometriosis dysmenorrhea mouse model

Chen Jingwei, Du Huilan, Tong Ruixiao, Yang Hua, Ma Huirong

Chen Jingwei, Du Huilan, Ma Huirong, Gynecology and Obstetrics Staff Room of Integrative Medicine, Hebei University of Chinese Medicine, Shijiazhuang 050200, China
Tong Ruixiao, Department of Gynaecology, Chinese Medicine Hospital of Handan, Handan 056000, China
Yang Hua, Department of Public Courses, Hebei College of Science and Technology, Baoding 071000, China
Supported by Hebei University of Chinese Medicine Youth Funded Projects (Effects of Bushenwenyanghuayu Decoction on Algogenic Substances in a Mouse Model of Endometriosis Dysmenorrhea, No. QNZ2014002)
Correspondence to: Prof. Du Huilan, Gynecology and Obstetrics Staff Room of Integrative Medicine, Hebei University of Chinese Medicine, Shijiazhuang 050200 China. duhuilan@163.com
Telephone: +86-311-83932190
Accepted: June 15, 2014

Abstract

OBJECTIVE: To observe the effects of Bushenwenyanghuayu decoction (BD), a Traditional Chinese Medicine (TCM), on the serum concentration of nerve growth factor (NGF) and bradykinin (BK), and protein and mRNA levels of NGF and bradykinin B_1 receptor (BKB1R) in a mouse model of endometriosis dysmenorrhea.

METHODS: Seventy-five experimental female BALB/c mice were randomly divided into five groups, 15 mice each: sham, model, BD high dose (61.67 g/kg), BD low dose (15.42 g/kg), and gestrinone (0.4 mg/kg) groups. All the mice except for those in the sham group underwent auto-transplantation surgery and were gavaged estradiol valerate (0.5 mg/kg, daily for 12 days) after surgery. On the 12th day, 1 h after administration, writhing response was induced by intraperitoneal injection of oxytocin at 2 U/mouse. The writhing frequency and latency were recorded and the volume of the ectopic foci was measured. The concentration of serum NGF and BK was detected by enzyme-linked immunosorbent assay, the protein expression of NGF and BKB1R was tested by immunohistochemistry and western blotting, and NGF and BKB1R mRNAs were detected by real-time PCR.

RESULTS: Compared with the model group, the volume of the ectopic foci in the treatment groups was significantly lower ($P < 0.01$), the writhing frequency was decreased ($P < 0.05$), and the writhing latency was prolonged ($P < 0.01$). Compared with the sham group, serum NGF and BK levels in the model group were significantly increased ($P < 0.01$). There were positive correlations for writhing frequency among the NGF and BK groups ($P < 0.01$). The serum NGF and BK levels were significantly lower in the treatment groups than the model group ($P < 0.05$). The protein expression of NGF, BKB1R was significantly decreased in the treatment groups compared with the model group ($P < 0.01$). NGF and BKB1R mRNA expression was significantly decreased in the treatment groups compared with the model group ($P < 0.01$).

CONCLUSION: NGF and BK/BKB1R may play an important role in the development of endometriosis-associated dysmenorrhea, and BD was found to inhibit the development of endometriosis and relieve dysmenorrhea by influencing NGF and BK/BKB1R mRNA and protein levels.

Key words: Endometriosis; Dysmenorrhea; Nerve growth factor; Bradykinin; Bradykinin B_1 receptor; Bushenwenyanghuayu decoction

INTRODUCTION

Endometriosis is a gynecological disease affecting 10%-14% of reproductive-aged women, with symptoms such as dysmenorrhea, dyspareunia, and infertility,[1] of which dysmenorrhea is the most common and most specific symptom. However, the mechanisms by which endometriotic lesions produce pain and hyperalgesia are poorly understood. Numerous theories have been proposed to explain the cause of pain in endometriotic tissue, including production and release of prostaglandins;[2] inflammatory mediators such as kinins, histamine, interleukins, etc.;[3] and abnormal distribution of nerve fibers.[4] Recently, nerve growth factor (NGF) has been proposed as one of the key factors responsible not only for the promotion of nerve fiber growth but also for the onset and maintenance of pain in a variety of diseases.[5] Additionally, since NGF is a pain mediator, it can promote mast cells to release bradykinin (BK) and other inflammatory mediators.[6] The combination of bradykinin and its B_1 receptor (bradykinin B_1 receptor, BKB1R) are able to activate pain. Whether NGF, BK, and BKB1R help in improving endometriosis-associated dysmenorrhea remains to be seen. In our previous study,[7,8] we found that Bushenwenyanghuayu decoction (BD) had a significant clinical effect, especially in alleviating pain related to endometriosis. Based on previous studies,[9,10] a BALB/c mouse endometriosis model was established. In the present study writhing response was examined and the effect of BD on NGF and BK/BKB1R levels was investigated.

MATERIALS AND METHODS

Animals

A total of 75 female BALB/c mice, weighing 18-22 g and aged 6-8 weeks (License No. 1211067), were acquired from the Hebei Experimental Animal Center. Mice were maintained at constant temperature (20-23 ℃) and humidity (45%-60%) under a 12-h light/dark cycle, and all mice had free access to food and water. All animal studies were approved by the Institutional Animal Care and Use Committee of the Hebei University of Chinese Medicine. The mice were anesthetized with an injection of 5% chloral hydrate (6 mL/kg, supported by Shanghai Jin Sui biological technology Co., Ltd.,) into the abdominal cavity before operation and sacrifice to relieve the animal's suffering.

Bushenwenyanghuayu decoction

BD consists of Fuzi (*Radix Aconiti Lateralis Preparata*) 6 g, Rougui (*Cortex Cinnamomi Cassiae*) 10 g, Xiaohuixiang (*Fructus Foeniculi*) 10 g, Yanhusuo (*Rhizoma Corydalis Yanhusuo*) 15 g, Chuanxiong (*Rhizoma Chuanxiong*) 10 g, Chuanniuxi (*Radix Cyathulae*) 10 g, Chuanlianzi (*Fructus Toosendan*) 15 g, Dangshen (*Radix Codonopsis*) 15 g, Baizhu (*Rhizoma Atractylodis Macrocephalae*) 15 g, Moyao (*Myrrh*) 10 g, Jiuxiangchong (*Aspongopus*) 9 g, Gouji (*Rhizoma Cibotii*) 30 g, Sangjisheng (*Herba Taxilli Chinensis*) 30 g. These crude herbs were purchased from the Shijiazhuang Lerentang Drugstore. Following Traditional Chinese Medicine (TCM), the BD was made at a high dose (6.17 g/mL) and a low dose (1.54 g/mL) prepared by the Chinese Medicine Pharmacological Laboratory of Hebei University of Traditional Chinese Medicine. Gestrinone capsules made by Zizhu Pharmaceutical Limited Company (Beijing, China), were powdered and dissolved in deionized water at a concentration of 0.04 mg/mL. Estradiol valerate tablets made by DELPHARM Lille S.A.S. (Lys-Lez-Lannoy, France), were powdered and dissolved in deionized water at a concentration of 0.05 mg/mL. Gentamycin sulfate injection was purchased from Zhuofeng Pharmaceutical Limited Company (Zhengzhou, China).

Reagents

BK enzyme-linked immunosorbent assay (ELISA) kit was purchased from Enzo Life Sciences, Inc, (Farmingdale, NY, USA). NGF ELISA kit was purchased from Shanghai Senxiong Science and Technology Industrial Limited Company (Shanghai, China). Rabbit polyclonal antibody to BKB1R was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit monoclonal to NGF antibody was purchased from Epitomics (Burlingame, CA, USA). Rabbit polyclonal to β-actin antibody was purchased from Hangzhou Huaan Biology Technology Limited (Hangzhou, China). Reverse Transcription kit M-MLV and Fluorescent PCR Core Reagent Kits SYBR Green I were purchased from MBI (Vilnius, Lithuania). Trizol Reagent was purchased from Invitrogen (Carlsbad, CA, USA).

Grouping and treatment

Seventy-five mice total were randomly divided into five groups, 15 mice each, by the random number table method: sham group, model group, BD high dose group, BD low dose group, and gestrinone group. Before establishing the model, estradiol valerate at 0.5 mg/kg was administrated to all mice by gavage for 2 days to be in the estrus period. Except for the sham group, other mice underwent auto-transplantation surgery to establish endometriosis dysmenorrhea as described in the literature.[11,12] Mice were anesthetized by intraperitoneal injection with 5% chloral hydrate (6 mL/kg). Using aseptic technique, an abdominal incision was made to expose the uterus and a 1-1.5 cm segment of the left uterine horn was removed and placed in warm saline. The uterus was cut open longitudinally

and attached to the intimal surface to the right side of the abdominal wall and sutured diagonally with 4-0 absorbable thread. Sham surgeries were performed on the sham group using the same steps as the endometriosis surgeries, but no tissue was sutured to the abdominal wall. After confirming hemostasis, the incision was sutured closed in layers, and gentamycin sulfate injection (8 mg/mouse) was intraperitoneally injected for 3 days to prevent infection. From the first postoperative day, each group was administrated agents according to the following scheme: deionized water was administrated to mice in the model group and 6 h later estradiol valerate at 0.5 mg/kg; BD at 61.67 g/kg to mice in the high dose group and 6 h later estradiol valerate at 0.5 mg/kg; BD at 15.42 g/kg to mice in the low dose group and 6 h later estradiol valerate at 0.5 mg/kg; gestrinone at 0.4 mg/kg to mice in the gestrinone group twice a week, and other time water instead, 6 h later estradiol valerate at 0.5 mg/kg; deionized water was orally administered to mice in the sham group. Mice in the five groups were all administrated agents by gavage for 12 days at an intragastric volume of 1 mL/100 g. At the 12th day after 1 h of administration of estradiol valerate, oxytocin at 2U/mouse was injected intraperitoneally to all mice except for those in the sham group to induce writhing response. Normal saline at 0.2 mL/mouse was injected to mice in the sham group.

Specimen collection

Thirty minutes after observing the writhing response, the mice were anesthetized by intraperitoneal injection of 5% chloral hydrate (6 mL/kg) and decapitated. Then the blood was collected and centrifuged at 1800 ×*g* for 15 min to separate the serum, and then the serum samples were stored at - 80 ℃ for ELISA testing. The volume of ectopic foci (length × width × height, unit mm^3) was measured. Some of the samples including the uterus and ectopic foci were fixed in 4% paraformaldehyde and embedded in paraffin. The residual samples were immediately placed in liquid nitrogen, and stored at - 80 ℃ before use.

ELISA for measuring serum NGF and BK

Both NGF and BK were determined using ELISA assay according to the kit instructions.

Immunohistochemistry for detection of NGF and BKB1R

Immunohistochemistry streptavidin-perosidase method was used for detection of NGF and BKB1R. Cells positive for NGF and BKB1R appeared with pale brown stained cytoplasm. The positively-stained cells were counted in 10 random high-power fields of each sample under light microscope. HMIAS-2000 pathology picture analysis system (Qianping Audiovisual Company, Wuhan, China) was used to view the tissues and staining was measured by optical density (OD) of the positive signal.

Western blotting for NGF and BKB1R

Whole protein lysates were processed as described previously.[13] After protein content was determined by the Bradford method, proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in tris buffer saline. The membrane was then incubated at 4 ℃ overnight with primary antibodies. After washing, the membranes were incubated with a secondary conjugated antibody (anti-rabbit IgG antibody 1∶10000) for 1 h at room temperature. The protein bands were scanned, and analyzed on an Odyssey scanner (LI-COR, Lincoln, NE, USA). Data are presented as a ratio of NGF or BKB1R and β-actin, respectively.

Real-time PCR for measuring NGF and BKB1R mRNA

Total RNA was extracted from the tissue specimens using Trizol Reagent and reverse transcription of RNA to cDNA was performed using a reverse transcriptase kit according to the manufacturer's protocol. The synthetic oligonucleotide primer sequences for NGF, BKB1R, and GAPDH were as follows: NGF 5′-TCATCATCCCATCCCATCT-3′ and 5′-TTCACCTCTCCCAACACCA-3′ , BKB1R 5′-GCTGCCAACATTTATCATCTCC-3′ and 5′-GCCCAAGACAAACACCAGA-3′ , GAPDH 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-GCTTCACCACCTTCTTGATGTC-3′ . Real-time PCR was performed using SYBR green I according to the manufacturer's instructions. PCR was performed at 42 ℃ for 50 min, 95 ℃ for 5 min, followed by 40 cycles at 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s. A comparative cycle of threshold fluorescence (Ct) method was used and the relative transcript amount of the target gene was normalized to that of GAPDH using the $2^{-\Delta\Delta Ct}$ method based on a previous study.[14] The final results of the real-time PCR were expressed as the ratio of mRNA of control.

Statistical analysis

Data are presented as mean ± standard deviation ($\bar{x} \pm s$). Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons were based on analysis of one-way analysis of variance followed by post-hoc least significant differece and Student-Newman-Keuls test. Correlation between two variables was evaluated by linear correlation analysis with Pearson correlation coefficient, and differences were considered statistically significant at $P < 0.05$.

RESULTS

Comparison of writhing response among groups

Except for the sham group, the mice in the other groups all displayed the writhing response. Compared with the model group, the writhing latency was pro-

longed and the writhing frequency was reduced in the BD high dose and low dose groups and the gestrinone group. The differences were statistically significant ($P < 0.01$ or $P < 0.05$), but there were no significant differences among treatment groups ($P > 0.05$) (Table 1).

Table 1 Comparison of writhing response ($\bar{x} \pm s$)

Group	*n*	Writhing latency (s)	Writhing frequency (times)
Sham	15	-	-
Model	15	32±10	22±10
BD high	13	88±25[a]	13±5[b]
BD low	15	91±32[a]	10±5[a]
Gestrinone	14	89±24[a]	7±5[a]

Notes: the sham group was administered deionized water; the model group was treated with estradiol valerate combined with oxytocin. The BD high dose group, BD low dose group, and gestrinone group were intragastrically administered the BD high dose decoction at 61.67 g/kg, BD low dose decoction at 15.42 g/kg, and gestrinone solution at 0.4 mg/kg, respectively for 12 days. BD: Bushenwenyang decoction. Comparison with the model group, [a]$P < 0.01$, [b]$P < 0.05$.

Comparison of ectopic foci volume among groups

Except for the sham group, the ectopic foci were well developed in the other groups. Compared with the model group, the volume of the ectopic foci in the BD high-dose and low-dose groups and the gestrinone group became significantly smaller ($P < 0.01$), but there were no significant differences among treatment groups ($P > 0.05$) (Table 2).

Table 2 Comparison of ectopic foci volume ($\bar{x} \pm s$)

Group	*n*	Volume (mm^3)
Sham	15	-
Model	15	43±11
BD high	13	23±9[a]
BD low	15	20±8[a]
Gestrinone	14	28±10[a]

Notes: the sham group was administered deionized water, the model group was treated with estradiol valerate combined with oxytocin. The BD high dose group, BD low dose group, and gestrinone group were intragastrically administered the BD high dose decoction at 61.67 g/kg, BD low dose decoction at 15.42 g/kg, and gestrinone solution at 0.4 mg/kg, respectively for 12 days. BD: Bushenwenyang decoction. Comparison with the model group, [a]$P < 0.01$.

Comparison of serum NGF and BK

The level of NGF and BK in the model group was significantly higher than that of the sham group ($P < 0.01$), whereas those in the treatment groups were significantly lower than that of the model group ($P < 0.05$, $P < 0.01$), and there were no significant differences among the treatment groups ($P > 0.05$) (Table 3).

Correlation analysis for NGF, BK, and writhing response

The results suggested that there were positive correlations between NGF and BK, and they both were positively correlated with writhing frequency ($P < 0.01$), but there was no correlation with writhing latency ($P > 0.05$) (Table 4).

Table 3 Serum level of NGF and BK ($\bar{x} \pm s$)

Group	*n*	bradykinin (ng/mL)	NGF (pg/mL)
Sham	15	3.4±1.0[a]	38.8±11.2[a]
Model	15	7.3±0.7[b]	73.4±14.2[b]
BD high	13	4.4±1.1[a]	48.4±17.8[c]
BD low	15	4.5±1.9[a]	52.6±16.5[c]
Gestrinone	14	4.6±1.4[a]	49.7±17.9[c]

Notes: the sham group was administered deionized water. The model group was treated with estradiol valerate combined with oxytocin. The BD high dose group, BD low dose group, and gestrinone group were intragastrically administered the BD high dose decoction at 61.67 g/kg, BD low dose decoction at 15.42 g/kg, and gestrinone solution at 0.4 mg/kg, respectively for 12 days. BD: Bushenwenyang decoction; BK: bradykinin; NGF: nerve growth factor. Comparison with the model group, [a]$P < 0.01$, [c]$P < 0.05$; comparison with the sham group, [b]$P < 0.01$.

Comparison of NGF and BKB1R protein expression by immunohistochemistry

The NGF and BKB1R proteins were mainly expressed in the cytoplasm of cells in the endometrial stroma or presented as brown granule staining in glands. The expressions of NGF and BKB1R in the uteri of the model-group mice were significantly higher than that in the sham mice ($P < 0.01$), and those in treatment groups were significantly lower than that of the model group not only in the uterus but also in the ectopic foci ($P < 0.01$), but there was no significant difference among the treatment groups ($P > 0.05$) (Figures 1, 2).

Comparison of NGF and BKB1R protein expression by Western blot

Western blot analysis showed that NGF and BKB1R protein expression in the model group increased significantly compared with the sham group ($P < 0.01$), and protein expression in the treatment groups decreased significantly compared with that of the model group ($P < 0.01$). There was no significant difference among treatment groups ($P > 0.05$) (Figure 3)

Comparison of mRNA for NGF and BKB1R by real-time PCR

mRNA for NGF in the uterus increased significantly in the model group compared with the sham group ($P < 0.01$), and decreased significantly in the treatment groups compared with the model group (all $P < 0.01$), and there were significant differences between BD high dose and gestrinone groups ($P < 0.01$). The ectopic foci level of NGF mRNA in the treatment groups decreased significantly compared with the model group (all $P < 0.01$), and the differences between BD high-dose group and gestrinone, and the BD low-dose group and gestrinone were statistically significant ($P < 0.01$, $P < 0.05$).

BKB1R mRNA in the uterus increased significantly in the model group compared with the sham group ($P <$

0.01), and decreased significantly in the treatment groups compared with the model group (all $P < 0.01$). The differences between the BD high-dose group and gestrinone, and the BD low-dose group and gestrinone were statistically significant ($P < 0.01$, $P < 0.05$). The level of BKB1R mRNA in ectopic foci in the treatment groups decreased significantly compared with the model group (all $P < 0.01$), and the difference between the BD high-dose and gestrinone groups was statistically significant ($P < 0.01$) (Table 5).

DISCUSSION

In gynecological concepts of TCM, the nature of endometriosis is blood stasis, and the existence of blood stasis blocks Chong Ren and Bao channels, followed by dysmenorrhea, which slowly leads to kidney deficiency. Therefore, we consider the basic pathogenesis of the disease as kidney deficiency and blood stasis,[15,16] and BD as a therapy for it. In this study, we investigated the association among endometriosis-associated dysmenorrhea, NGF, and BK/BKB1R. We measured the concentration of NGF and BK in mouse serum, and found that there were positive correlations between the concentrations of NGF and BK and writhing frequency, indicating that NGF and BK participate in the generation or persistence of pain due to endometriosis. The higher the content of NGF and BK, the greater was the degree of dysmenorrhea. The present study verified that the expression of NGF and BKB1R in the model group was stronger than the sham group both at the protein level or the mRNA level. On the one hand, NGF can increase the number of sensory neurons and is selectively trophic for small fiber sensory neurons and sympathetic ganglion neurons that participate in mediating pain sensation.[5] On the other hand, as a type of pain substance, NGF directly participates in the generation of pain sensation, and there is a combined effect of NGF and BK in the occurrence of endometriosis-associated dysmenorrhea. The combination of BK and its over-expressed receptor BKB1R can cause the release of Ca^{2+}, activation of phosphoric acid protein kinase C, phosphorylation of transient receptor potential vanilloid receptor complexes, and can strengthen the entry of ions and reduce the activation threshold of algesiroreceptor, causing different types of pain.[17] This process promotes the release of prostaglandins and directly causes pain and hyperpathia.[18] Thus, NGF and BK/BKB1R may play pivotal roles in the occurrence and development of dysmenorrhea with endometriosis.

The results showed significant alterations in serum NGF and BK after BD treatment. The expression of NGF and BKB1R proteins decreased, and NGF and BKB1R mRNA levels were decreased. On the one hand, BD can inhibit nerve fiber regeneration by reducing NGF level, so as to block the transmission of pain signals. On the other hand, it is able to slow down the release of pain substances by decreasing BK concentration, and thus alleviating dysmenorrhea. Inhibiting the expression of NGF and BK/BKB1R has the possible effect of reducing the incidence of inflammatory pain by blocking the inflammatory response.

Table 4 Correlation of nerve growth factor and bradykinin with writhing response

Factor 1	Factor 2	*r* value	Sig
Nerve growth factor	Writhing frequency	0.574	0.008
Nerve growth factor	Writhing latency	- 0.335	0.149
Bradykinin	Writhing frequency	0.712	0.000
Bradykinin	Writhing latency	- 0.307	0.188
Nerve growth factor	BK	0.569	0.009

Notes: *r*: correlation coefficient values; Sig: significance; BK: bradykinin.

Table 5 Comparison of nerve growth factor and bradykinin B_1 receptor mRNAs ($\bar{x} \pm s$)

Group	*n*	Nerve growth factor mRNA		Bradykinin B_1 receptor mRNA	
		Uterus	Ectopic foci	Uterus	Ectopic foci
Sham	15	1.01±0.12[a]	-	0.90±0.09[ac]	-
Model	15	3.27±0.52[b]	3.85±0.20	2.84±0.63[bc]	4.02±0.63[c]
BD high	13	1.24±0.14[ac]	1.32±0.32[ac]	1.31±0.20[ac]	1.17±0.19[ac]
BD low	15	2.17±0.33[ab]	1.70±0.45[ad]	1.38±0.29[ad]	1.62±0.28[a]
Gestrinone	14	2.24±0.20[ab]	2.50±0.47[a]	2.06±0.20[ab]	2.08±0.20[a]

Notes: the sham group was administered deionized water. The model group was treated with estradiol valerate combined with oxytocin. The BD high dose group, BD low dose group, and gestrinone group were intragastrically administered the BD high dose decoction, BD low dose decoction, and gestrinone solution, respectively. BD: Bushenwenyang decoction. Comparison with the model group, [a]$P < 0.01$; comparison with the sham group, [b]$P < 0.01$; comparison with the gestrinone group, [c]$P < 0.01$, [d]$P < 0.05$.

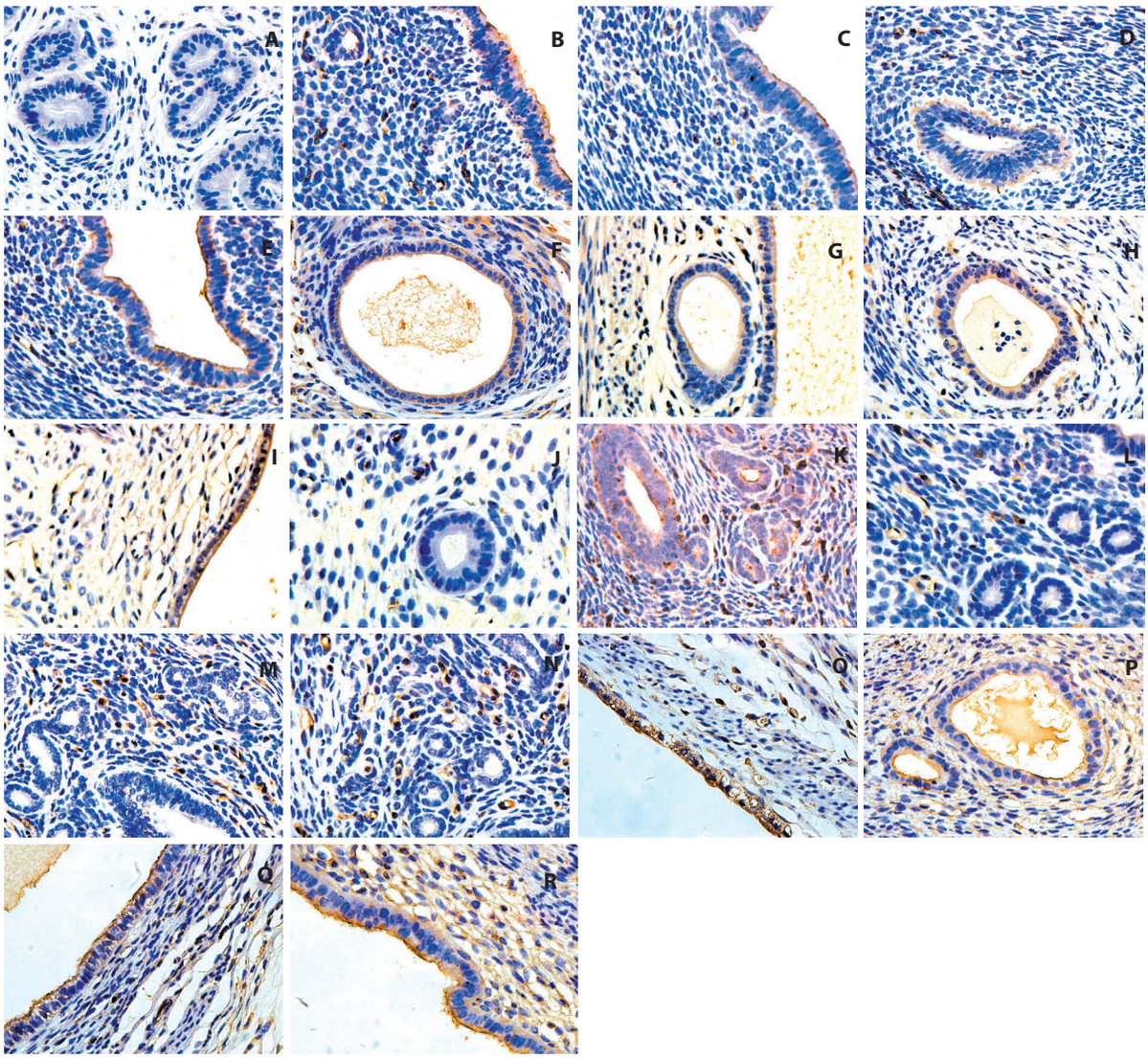

Figure 1 Expression of NGF / BKB1R in the uterus and ectopic foci of different groups (streptavidin-perosidase, ×400)
A: the expression of NGF in the uterus of the sham group; B: the expression of NGF in the uterus of the model group; C: the expression of NGF in the uterus of the high dose group; D: the expression of NGF in the uterus of the low dose group; E: the expression of NGF in the uterus of the gestrinone group; F: the expression of NGF in the ectopic foci of the model group; G: the expression of NGF in the ectopic foci of the high dose group; H: the expression of NGF in the ectopic foci of the low dose group; I: the expression of NGF in the ectopic foci of the gestrinone group; J: the expression of BKB1R in the uterus of the sham group; K: the expression of BKB1R in the uterus of the model group; L: the expression of BKB1R in the uterus of the high dose group; M: the expression of BKB1R in the uterus of the low dose group; N: the expression of BKB1R in the uterus of the gestrinone group; O: the expression of BKB1R in ectopic foci of the model group; P: the expression of BKB1R in ectopic foci of the high dose group; Q: the expression of BKB1R in ectopic foci of the low dose group; R: the expression of BKB1R in ectopic foci of the gestrinone group. NGF: nerve growth factor; BKB1R: bradykinin B_1 receptor.

In the present study, BD had a similar effect to gestrinone, and the dose-dependent efficacy of BD on improving the state of dysmenorrhea or decreasing NGF and BK/BKB1R level was not observed.

In conclusion, we found that BD had beneficial effects in shrinking the volume of ectopic foci, lengthening the writhing latency and reducing writhing frequency, indicating that the decoction from TCM has a protective effect on dysmenorrhea with endometriosis. The mechanisms may be associated with the reduction in the level of serum NGF and BK, and by the reduction of protein and mRNA levels of NGF and BKB1R. However, due to the limitation of small sample size and short study duration, the underlying mechanisms need further investigation, which might provide more scientific validation for the clinical application of BD.

REFERENCES

1 **Burns KA**, Rodriguez KF, Hewitt SC, Janardhan KS, Young SL, Korach KS. Role of estrogen receptor signaling required for endometriosis-like lesion establishment in a mouse model. Endocrinology 2012; 153(8): 3960-3971.

2 **Wu MH**, Shoji Y, Chuang PC, Tsai SJ. Endometriosis: disease pathophysiology and the role of prostaglandins. Expert Rev Mol Med 2007; 9(2): 1-20.

3 **Wu MY**, Ho HN. The role of cytokines in endometriosis. Am J Reprod Immunol 2003; 49(5): 285-296.

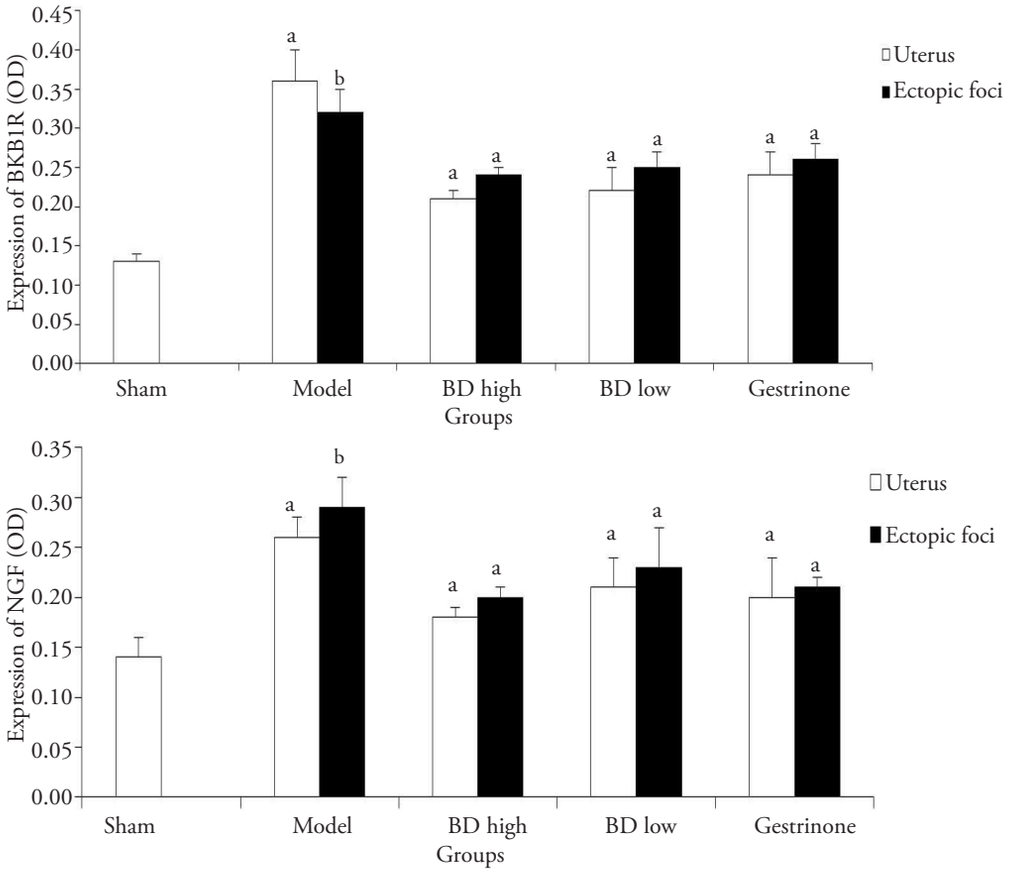

Figure 2 Comparison of NGF and BKB1R by immunohistochemistry

A: comparison of NGF; B: comparison of BKB1R. The sham group was administered deionized water. The model group was treated with estradiol valerate combined with oxytocin. The BD high dose group, BD low dose group, and gestrinone group were intragastrically administered the BD high dose decoction, BD low dose decoction, and gestrinone solution, respectively. BD: Bushenwenyang decoction; NGF: nerve growth factor; BKB1R: bradykinin B_1 receptor. Comparison with the model group, $^aP < 0.01$; comparison with the sham group, $^bP < 0.01$. BD: Bushenwenyang decoction; NGF: nerve growth factor; BKB1R: bradykinin B_1 receptor.

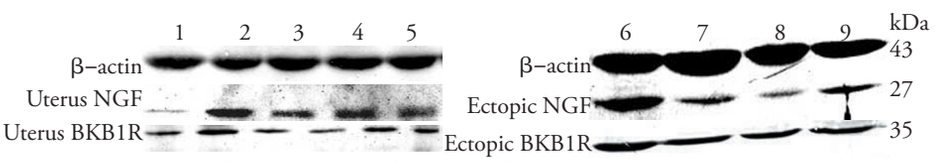

Figure 3 Expressions of NGF and BKB1R by Western blot

1: sham group; 2 and 6: model group; 3 and 7: BD (Bushenwenyanghuayu decoction) high dose group; 4 and 8: BD low dose group; 5 and 9: gestrinone group. the sham group was administered deionized water. The model group was treated with estradiol valerate combined with oxytocin. The BD high dose group, BD low dose group, and gestrinone group were intragastrically administered the BD high dose decoction, BD low dose decoction, and gestrinone solution, respectively. NGF: nerve growth factor; BKB1R: bradykinin B_1 receptor.

4 **Tokushige N**, Markham R, Russell P, Fraser IS. High density of small nerve fibres in the functional layer of the endometrium in women with endometriosis. Hum Reprod 2006; 21(3): 782-787.

5 **Apfel SC**. Neurotrophic factors and pain. Clin J Pain 2000; 16(2 Suppl): S7-S11.

6 **Austin PJ**, Moalem-Taylor G. The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. J Neuroimmunol 2010; 229(1-2): 26-50.

7 **Chen JW**, Du HL, Yang J, Bai FL, Cui JX. The clinical study of Bushenwenyanghuayu Method in treating endometriosis. Xian Dai Zhong Xi Yi Jie He Za Zhi 2009; 18 (25): 3053-3054.

8 **Bian WH**, Du HL, Chen HJ, Jia YB. The Clinical Study of Bushenwenyanghuayu method on dysmenorrhea patients with endometriosis. Lin Chuang Yi Yao Shi Jian Za Zhi 2008; 1(6): 370-373.

9 **Chen JW**, Tong RX, Sun XH, Fan LJ, Du HL. Establishment of a mouse model of primary dysmenorrhea model with progynova and oxytocin. Zhong Guo Shi Yan Dong Wu Xue Bao 2013; 21(5): 78-81.

10 **Chen JW**, Tong RX, Li QX, Tian QH, Du HL. Effects of Bushenwenyanghuayu decoction on dysmenorrhea in endometriosis model. Zhong Yao Yao Li Yu Lin Chuang 2013; 29(5): 113-116.

11 **Vernon MW**, Wilson EA. Studies on the surgical induction of endometriosis in the rat. Fertil Steril 1985; 44(5): 684-694.

12 **Chen JW**, Tong RX, Fan LJ, Sun XH, Ma HR, Du HL. Establishment of endometriosis dysmenorrhea model in BALB/c mice. Zhong Guo Shi Yan Dong Wu Xue Bao

2014; 22(1): 83-86.

13 **Ji C**, Aisa HA, Yang N, et al. Gossypium herbaceam extracts inhibited NF-kappa B activation to attenuate spatial memory impairment and hippocampal neurodegeneration induced by amyloid-beta in rats. J Alzheimers Dis 2008; 14(3): 271-283.

14 **Pfaffl MW**. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29 (9): e45.

15 **Zhou H**, Qi C .Effect of reinforcing kidney and activating blood therapy on apoptosis of rat models with endometriosis. Zhong Yi Za Zhi 2009; 50(2):165-168.

16 **Zhang M**, Cao BL. Influence of Fufang Ezhu San on MMP-2 and VEGF expressions of ectopic endometrial tissues of endometriosis rats. Zhong Yi Za Zhi 2011; 52(14): 1225-1228.

17 **Kohno T**, Wang H, Amaya F, et al. Bradykinin enhances AMPA and NMDA receptor activity in spinal cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity. J Neurosci 2008; 28(17): 4533-4540.

18 **Fuchs D**, Birklein F, Reeh PW, Sauer SK. Sensitized peripheral nociception in experimental diabetes of the rat. Pain 2010; 151(2): 496-505.