

—Original—

An *in vivo* mouse model of primary dysmenorrhea

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Abstract: Primary dysmenorrhea (PD) is a common gynecological disorder. Hitherto, animal models which recapitulate clinical features of PD have not been fully established. We aimed to examine whether a pain model in mice could mimic the clinic features of PD. After pretreated with estradiol benzoate (1 mg/kg/day) intraperitoneally (i.p.) for 3 consecutive days, non-pregnant female Imprinting Control Region mice (6–8 weeks old) was injected with 0.4 U of oxytocin to induce the stretching or writhing response which was recorded for a time period of 30 min. During the writhing period, the uterine artery blood flow alterations were examined by Doppler ultrasound detection. After writhing test, the uterine morphological changes were observed by hematoxylin and eosin (H&E) staining histopathology. In addition, enzyme-linked immunosorbent assay kit was used to measure the levels of prostaglandins F_{2α}/prostaglandins E₂ (PGF_{2α}/PGE₂) and TXB₂ (a metabolite of TXA₂)/6-keto-PGF_{1α} (a metabolite of PGI₂) in the uterine tissue homogenates and plasma, respectively. Western blot analyses were performed to determine the expressions of oxytocin receptor (OTR), beta2-adrenergic receptor (beta2-AR), and cyclooxygenase-2 (COX-2) in uterine, which are responsible for the uterine contraction. The writhing response only occurred in the estrogen pretreated female mice. The area of uterine myometrium significantly decreased along with the increased thickness in the oxytocin-induced estrogen pretreated mice model. The uterine artery blood flow velocity dropped, while the pulsatility index and resistance index slightly increased after the injection of oxytocin. The PGF_{2α}/PGE₂ level significantly increased and the plasma TXB₂/6-keto-PGF_{1α} level significantly enhanced. Compared with the control group, the uterine histopathology demonstrated moderate to severe edema of endometrium lamina propria. In consistent with the uterine morphological changes, a significant reduction of beta2-AR and a significant increase of OTR and COX-2 in the uterine tissue were observed. The writhing response was caused by the abnormal contraction of uterus. The uterine spasm and ischemia changes of oxytocin-induced estrogen pretreated female mice model were similar to the pathology of human PD. We reported an *in vivo* mice model, which can be used to study PD and for clinical therapeutic evaluations.

Keywords: *in vivo* animal model, primary dysmenorrhea, uterine pathological change, uterine artery blood flow, uterine contraction

Introduction

Primary dysmenorrhea (PD) is a common gynecological disorder, affecting 40–50% adolescent females

of reproductive age [10]. This disease is characterized by intense acute abdominal pain during the first or second day of the menstrual flow yet without visible pelvic lesions [24]. These manifestations can be accompanied

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by symptoms such as low backache, nausea, vomiting and diarrhea, which often lead to recurrent absence of school or work [13]. Previous etiological studies have demonstrated the close relationship between PD and abnormal increased prostanoid secretion [7, 9]. The abnormal prostanoid levels induce frequent or dysrhythmic uterine contractions, which can reduce the uterine blood flow and be regarded as the main factors leading to menstrual pain [4, 10].

Although the abnormal levels of prostanoids have been proposed to be a major contributor to the pain experience during PD, the pathological consequence still remains to be elucidated [2]. Internationally, most of the studies regarding PD were mainly relied on the *in vitro* experiments, which were confined to investigate the local effects on the uterus, while the comprehensive effects were impossible to be elucidated. In China, an *in vivo* mice model managed with oxytocin is recognized as a pharmacodynamic experiment mice model and is frequently used in PD study [15]. Since the intense acute abdominal pain is the main clinical symptoms of PD, the writhing response was considered as the major indicator and the writhing times were used to evaluate the model and the effects of the test drugs. The comparative studies of this *in vivo* animal model mimicking the clinical features of PD are scarce. In this study, we examined the pathological features of oxytocin-induced estrogen pretreated female mice. We demonstrated that this pain model in mice presented the increased level of PGF_{2 α} /PGE₂, and increased thickness along with the decreased area of uterine myometrium, as well as the uterine artery blood flow velocity drop, which were similar to the pathological features of human PD. The abnormal contraction and ischemia of uterus were also confirmed by the histopathological and biochemical measurements.

Material and Methods

Animals

Nonpregnant Female and Male Imprinting Control Region (ICR) mice weighing 18–22 g (6–8 weeks old) were obtained from the Experimental Animal Center of Yangzhou University. All animals were housed in a temperature, humidity and light controlled vivarium, with food and water *ad libitum*. Animal use protocol was approved by the Animal Ethics Committee of the School of Chinese Material Medica, China Pharmaceutical University.

Chemicals and reagents

Estradiol benzoate injection was obtained from Tianjin Jinyao Amino Acid Pharmaceutical Co., Ltd. (Tianjin, China). Oxytocin was purchased from Nanjing Xinbai Pharmaceutical Co., Ltd. (Nanjing, China). Prostaglandin F_{2 α} (PGF_{2 α}), prostaglandin E₂ (PGE₂), 6-keto-prostaglandin F_{1 α} (6-keto PGF_{1 α} , a metabolite of PGI₂) and thromboxane B₂ (TXB₂, a metabolite of TXA₂) test kit were purchased from Nanjing Jiancheng Bioengineering Institute Co., Ltd. (Nanjing, China).

Animal treatment and PD symptom recordings

Female and male mice were both pretreated with estradiol benzoate (1 mg/kg/day) intraperitoneally for 3 consecutive days. Female mice with saline exposure intraperitoneally were used as control group. On the fourth day, mice were intraperitoneally injected with 0.4 U of oxytocin. Next, the writhing responses, which mainly consisted of abdominal wall contractions, pelvic rotation, and followed by hind limb stretches, were observed and recorded for 30 min. After the writhing test, the animals were sacrificed by cervical dislocation. The areas of uterine myometrium were calculated by Quantity One software, version 4.6.2 (Bio-Rad, USA), and then the uterine tissues were collected for pathological and biochemical analysis.

Uterine artery blood flow analysis

Female mice were pretreated with estradiol benzoate (1 mg/kg/day) intraperitoneally for 3 consecutive days. On the fourth day, uterine artery blood flow of mice were monitored by Doppler ultrasound detection (Ultrasound biomicroscope, Vevo2100TM, VisualSonics, Canada) after the injection of oxytocin under 4% chloral hydrate anesthesia (10 ml/kg, intraperitoneally).

Histological tests

After fixing the uterine tissues in 10% buffered formalin, the organs were dehydrated with a graded ethanol series and embedded in paraffin. The uterine tissues were then tailored into 5- μ m-thick sections. The morphological evaluation of tissues stained with hematoxylin and eosin (H&E) was performed with a light microscope (OLYMPUS DX45, Japan).

Western blot analysis

Uterine tissue samples were prepared by homogenization in modified RIPA buffer (1 \times PBS, 1% Igepal CA-

630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 30 μ l/ml aprotinin, 100 mM sodium orthovanadate), and then maintained in constant agitation for 1.5 h at 4°C and then centrifuged twice at 12,000 g for 5 min at 4°C. The supernatants were collected and the protein concentration was determined by the BCA. Forty micrograms of total protein was applied to each well of a 10% SDS polyacrylamide gel and electrophoresed for 2 h at 120 V along with a set of protein markers. The proteins were then transferred onto PVDF membranes (Millipore Corp, Bedford, MA, USA) at 200 mA for 1.5 h at room temperature using a transfer buffer (25 mmol/l Tris base, 192 mmol/l glycine and 20% methanol). The blots were blocked for 1 h at room temperature with blocking buffer (in 10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20). The blocking buffer was decanted and blots were incubated with primary antibody: anti-oxytocin receptor (anti-OTR, 1:200, sc-8102; SantaCruz Biotechnology, Santa Cruz, CA, USA), anti-beta2-adrenergic receptor (anti-beta2-AR, 1:200, sc-9042-; SantaCruz Biotechnology, Santa Cruz, CA, USA), anti-cyclooxygenase-2 (anti-COX-2, 1:1,000, ab62331; Abcam, USA). As an internal control, blots were incubated with an anti-Glyceraldehyde 3-phosphate dehydrogenase antibody (anti-GAPDH, 1:5,000, Shanghai Kangcheng Bioengineering Institute Co., Ltd., Shanghai, China). After washing three times with TBS buffer (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20), the membrane was incubated with either a goat-anti-mouse or goat-anti-rabbit peroxidase-conjugated secondary antibody depends on the source of primary antibody. The blots were finally detected with ECL reagent and visualized by Quantity One software 4.6.2 (Bio-Rad, USA). Each experiment was repeated three times in order to have reproducible results. The value of band intensity for the tested protein was normalized to the intensity of GAPDH.

PGF_{2 α} , PGE₂ and TXA₂, PGI₂ measurement

After retro-orbital collection of the blood, the mice were sacrificed by cervical dislocation and the uterine tissues were then dissected for further investigation. The uterine tissues were homogenized in 9 volumes of normal saline. Following centrifugation at 3,000 rpm for 15 min at 4°C, the supernatant was used to measure the levels of PGF_{2 α} , PGE₂, 6-keto PGF_{1 α} (a metabolite of PGI₂) and TXB₂ (a metabolite of TXA₂) by enzyme-linked immunosorbent assay (ELISA) in accordance with

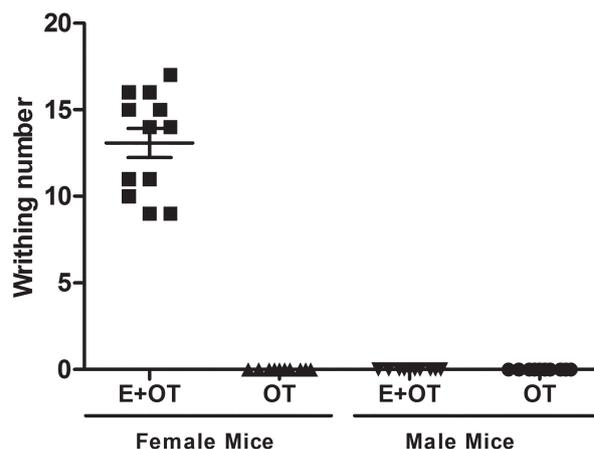


Fig. 1. Oxytocin-induced writhing response in different sex and whether estrogen pretreatment (n=12). “E+OT” group, the females and male mice were respectively pretreated with estrogen (E) for 3 consecutive days and each mouse was injected with 0.4 U of oxytocin (OT) on the fourth day; “OT” group, the females and male mice were respectively pretreated with saline for 3 consecutive days and each mouse was injected with 0.4 U of oxytocin on the fourth day. The number of writhes was counted for 30 min after oxytocin administration.

manufactures instructions (Nanjing Jiancheng Bioengineering Institute Co., Ltd., Nanjing, Jiangsu, China).

Statistical analysis

All data are presented as means \pm SEM. Statistical differences between paired groups were calculated using Student’s *t*-test. The $P < 0.05$ was considered statistically significant. Data were represented graphically using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

Results

Writhing Responses in estrogen pretreated mice

Oxytocin-induced writhing response only occurred during about 5–30 min after injection of oxytocin in the females pretreated with estrogen while male mice did not experience any writhing reactions (Fig. 1). The response mainly consisted of abdominal wall contractions, pelvic rotation, and followed by hind limb stretches.

Area of uterine myometrium in oxytocin-induced writhing mice

As shown in Fig. 2, oxytocin induced a significant decrease on the area of the uterine myometrium along

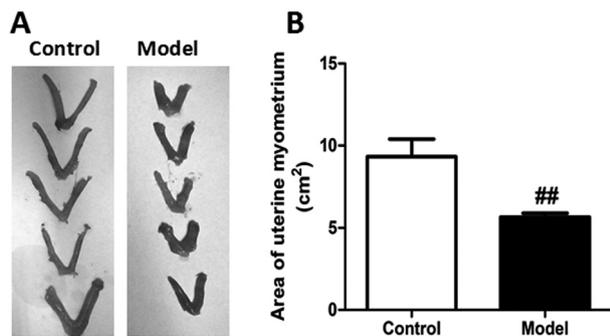


Fig. 2. Area of uterine myometrium in oxytocin-induced writhing mice. Pictures of uterine myometrium in control and model group (A). The areas of uterine myometrium were calculated by Quantity One software 4.6.2. (B) Data are presented as Means \pm SEM (n=5). Model group versus the control group ^{##} $P < 0.01$. Statistical significance was analyzed by using Student's *t*-test.

Table 1. Hemodynamic parameters of uterine vasculature evaluated by Doppler ultrasound

	Control	Model
Uterine artery		
VTI, mm	18.67 \pm 0.98	15.08 \pm 0.77 [#]
Maximum velocity, mm/s	211.5 \pm 5.44	154.8 \pm 6.90 ^{##}
Mean velocity, mm/s	135.5 \pm 7.03	104.4 \pm 1.93 ^{##}
Minimum velocity, mm/s	80.90 \pm 4.43	54.45 \pm 2.15 ^{##}
Maximum gradient, mm Hg	0.179 \pm 0.01	0.108 \pm 0.006 ^{##}
Mean gradient, mm Hg	0.0793 \pm 0.006	0.0435 \pm 0.002 ^{##}
PI	0.897 \pm 0.036	0.956 \pm 0.036
RI	0.618 \pm 0.017	0.646 \pm 0.016

VTI indicates velocity-time integral of Doppler signal; PI, pulsatility index; RI, resistance index. Data are presented as the mean \pm SEM (n=6). Model group versus control group [#] $P < 0.05$ ^{##} $P < 0.01$. Statistical significance was analyzed by using Student's *t*-test.

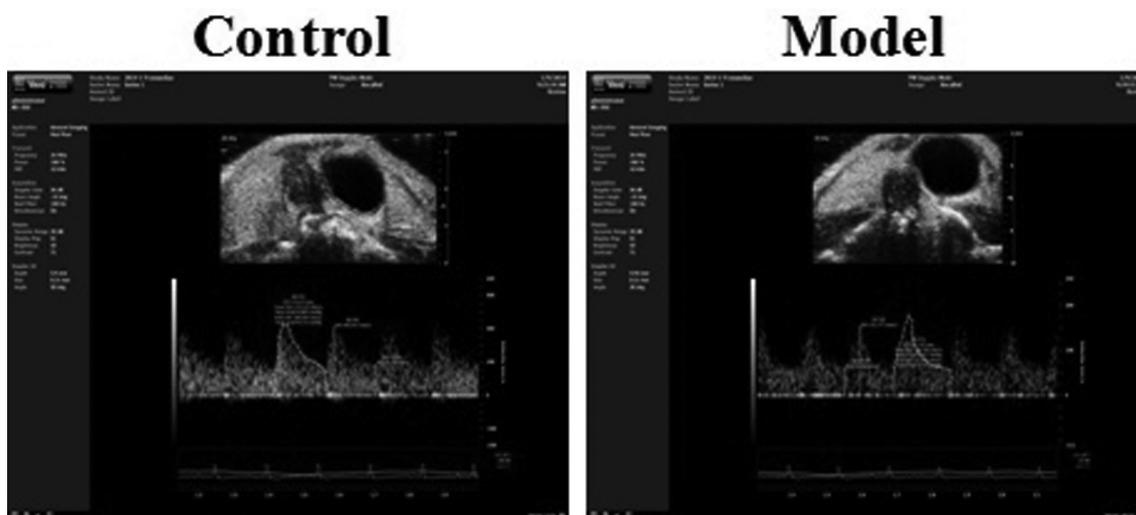


Fig. 3. Example uterine artery Doppler waveforms from normal mice and dysmenorrhea model mice. The uterine artery blood flow features in dysmenorrhea model mice.

with increased thickness.

Uterine artery blood flow features in oxytocin-induced writhing mice

As shown in Table 1, mean velocity of uterine artery blood flow decreased from 135.5 mm/s to 104.4 mm/s after intraperitoneal injection of oxytocin in model group, and the pulsatility index raised 11.7%. Figure 3 presented a typical image of uterine artery Doppler waveforms from dysmenorrhea model mice and control mice.

PGF_{2 α} /PGE₂ levels of oxytocin-induced writhing mice

The serum and uterine PGF_{2 α} /PGE₂ ratio also signifi-

cantly increased 53% and 46% in the model mice, respectively (Fig. 4).

TXA₂/PGI₂ levels of oxytocin-induced writhing mice

Figure 5 depicted that plasma TXB₂ (a metabolite of TXA₂)/6-keto-PGF_{1 α} (a metabolite of PGI₂) increased 36% in the model group compared with the control group ($P < 0.05$).

Histomorphology assessment of the uterus with H&E staining

As shown in Fig. 6A, the uterus histomorphology study of control groups showed no discernible patho-

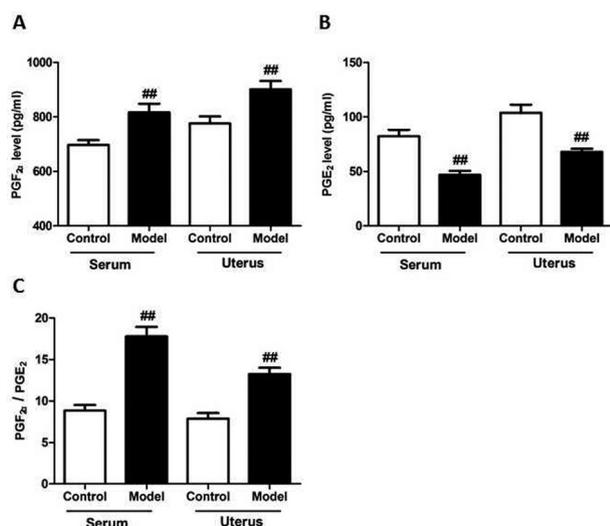


Fig. 4. Serum and uterine PGF_{2α}/PGE₂ levels of oxytocin-induced writhing mice. PGF_{2α} levels (A); PGE₂ levels (B); PGF_{2α}/PGE₂ levels (C). Data are presented as the Mean ± SEM (n=10). Model group versus control group ##*P*<0.01. Statistical significance was analyzed by using Student's *t*-test.

logical changes under the light microscope at × 100 objective, while the model groups showed moderate to severe edema in the endometrium lamina propria. Compared with the control group, the number of uterine glands has no obvious increase, but the amount of deep eosin dye homogeneous secretions presented in the glandular lumens in model mice. Fig. 6B showed the pathological score in control and model group. The scoring system of pathological change presented in the supplementary data 1.

Expressions of OTR, COX-2 and beta2-AR in uterus of oxytocin-induced writhing mice

A significant increase of oxytocin receptor (OTR, functional protein of uterine contraction) and a significant reduction of beta 2-adrenergic receptor (beta2-AR, functional protein of uterine relaxation) were observed in uterine tissue. The uterine cyclooxygenase-2 (COX-2) expression of oxytocin-induced model mice significantly increased by approximately 70% compared with the control mice (Fig. 7).

Discussion

As far as the existing PD models concerned, they are usually initiated by different stimulators, such as PGF_{2α} and oxytocin on isolated uterine horn [18, 20]. All of

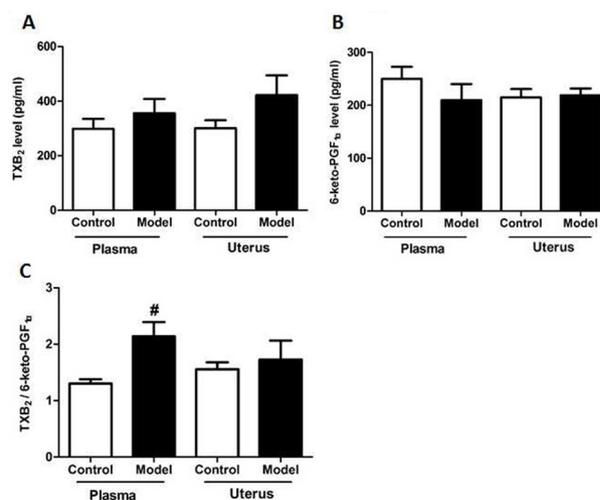


Fig. 5. Plasma and uterine TXA₂/PGI₂ levels of oxytocin-induced writhing mice. TXB₂ (a metabolite of TXA₂) levels (A); 6-keto-PGF_{1α} (a metabolite of PGI₂) levels (B); TXA₂/PGI₂ levels (C). Data are presented as the Mean ± SEM (n=10). Model group versus control group #*P*<0.05. Statistical significance was analyzed by using Student's *t*-test.

these isolated organ models focused on the local effects on uterus instead of the whole animal. Since the pathogenesis of PD related to hypothalamic-pituitary-ovarian axis [5, 11], thus the comprehensive therapeutic effects of the test drugs or the pathological mechanisms of PD were difficult to be elucidated by the isolated uterine horn model. An ideal animal model is expected to remedy the defects of the *in vitro* isolated uterine horn model. In China, the oxytocin-induced writhing mouse model, a frequently-used pain model in mice, was used to evaluate the anti-dysmenorrhea effects of Chinese herbal medicine, which has been used to prevent and cure PD since ancient times.

Although the complete PD disease process is not yet understood, PD in humans being due to uterine spasm contraction and focal ischemia has been recognized. Clinically, various studies of quantitative measurement of the area of myometrium at times of maximum and minimum pain in dysmenorrheic and eumenorrheic groups demonstrated that the area of the uterine myometrium, which represents the contraction ratio, significantly decreased during days 1–3 of the menstrual cycle in the dysmenorrheic group [17]. Oxytocin is a small peptide hormone to stimulate uterine contractions through functionally coupling to oxytocin receptor (OTR) [23]. OTR over expressed when activated by estrogen, and the concentrations of OTR determine the

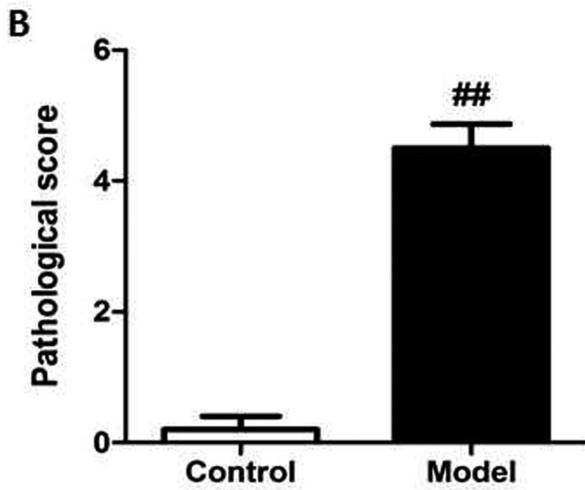
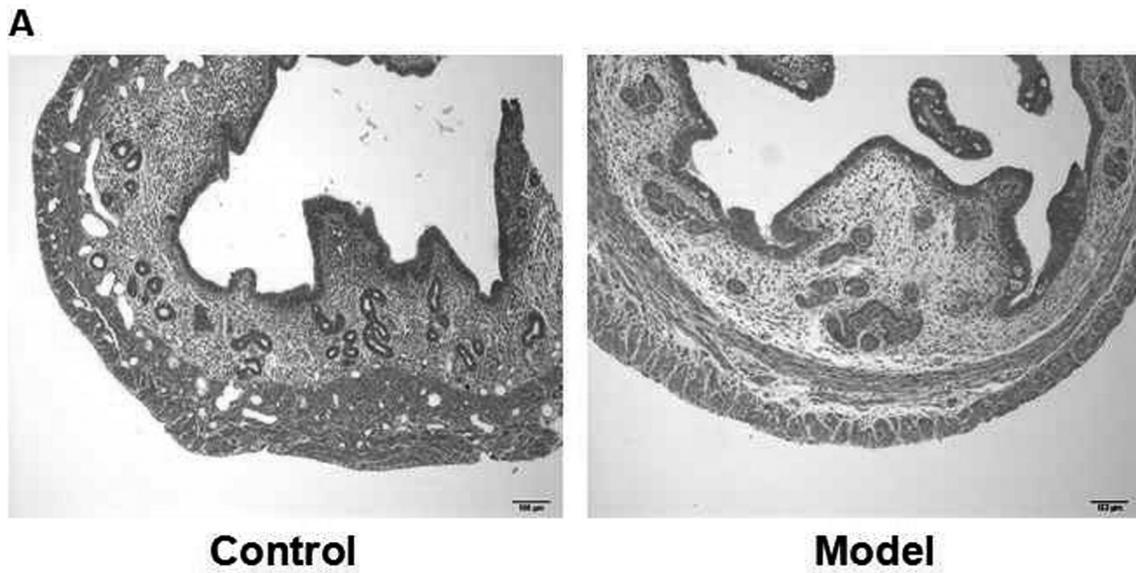


Fig. 6. Pathological change in uterus. (A) Example pathological section of the uterus; H&E staining. No discernible pathological changes were observed in the control group, some inflammatory pathological changes were observed in the model group. (B) Pathological score of uterus in control and model group. Data are presented as the Mean ± SEM (n=6). Model group vs control group ^{##}P<0.01. Statistical significance was analyzed by using Student's *t*-test.

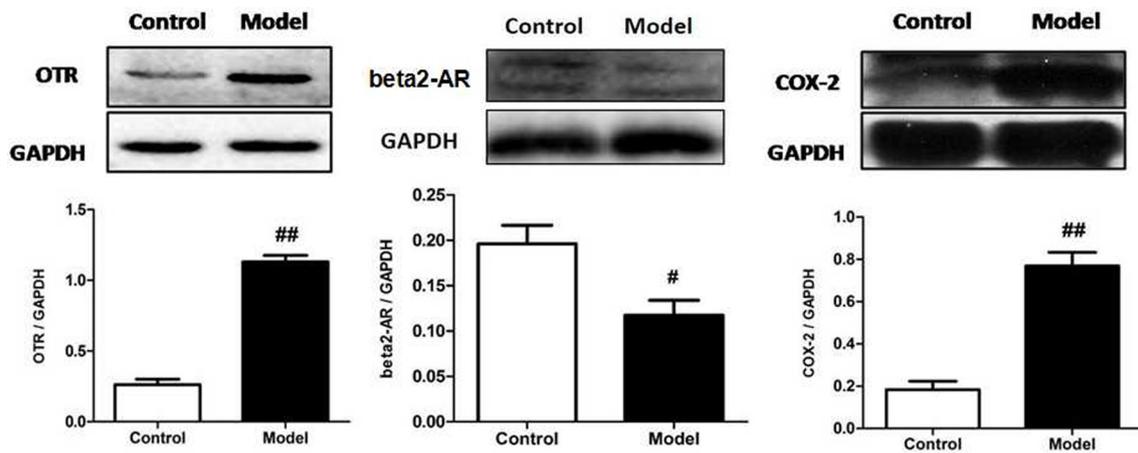


Fig. 7. Uterine oxytocin receptor, COX-2 and beta 2-AR protein expression in the oxytocin-induced writhing mice. Data illustrated on the graph bar represent the mean ± SEM for six uterus in each group (n=6). Model group vs control group ^{##}P<0.01. Statistical significance was analyzed by using Student's *t*-test.

sensitivity of endometrium to oxytocin stimulation [14]. In the present study, oxytocin-induced writhing responses only occurred in the female mice pretreated with estrogen, and a significant decrease of myometrium area was observed. Our results showed increased uterine OTR expression in mice model pretreated with estrogen and proved the writhing responses of this pain model in mice were caused by abnormal contraction of the uterus. The results were consistent with the clinical findings and further illustrated the contraction of the target organ *in vivo* as a symptom of PD.

Moreover, the clinical studies have shown that the pulsatility index and resistance index of both uterine arteries, as well as the arcuate artery are significantly higher on the first menstrual day in women with PD, suggesting the paradox of increased blood flow impedance and uterine vasoconstriction may be the cause of PD [10]. Reduced uterine blood flow, possibly caused by strong and abnormal uterine contractions in dysmenorrheic women, results in myometrial ischemia and produces cramp-like pain. A report on the measurement of blood flow in the uterine artery with Doppler US also indicated that uterine artery resistance was significantly higher in subjects with dysmenorrhea than in those asymptomatic controls, and thus supported the idea that ischemia is another cause of PD [19]. Our study demonstrated that oxytocin-induced uterine artery blood flow velocity dropped distinctly, while the pulsatility index and resistance index slightly increased in model mice, which were in accordance with the clinical features of PD patients.

In addition, Prostaglandins signaling have been proved to directly affect the uterine contraction, which are possibly the main reasons for PD [6, 21]. Clinical studies have demonstrated that $\text{PGF}_{2\alpha}$ levels of PD patients are much higher than those of asymptomatic controls, therefore the increased level of $\text{PGF}_{2\alpha}/\text{PGE}_2$ have been regarded as the indicator of clinical diagnosis for PD [9, 16]. Our study also demonstrated that the serum and uterine $\text{PGF}_{2\alpha}/\text{PGE}_2$ ratio significantly increased 53 and 46% in the model group.

In addition, clinical reports also suggested that increased oxytocin receptor (OTR) expression occurs in PD patients. OTR is functionally coupled to $\text{G}\alpha_{q/11}$ as well as $\text{G}\alpha_i$ in myometrial cells and mediates uterine contraction [1, 3, 26]. On the contrary, beta2-AR is predominantly a *Gas*-coupled receptor and mediates uterine relaxation via an increase in intracellular cAMP levels.

The mechanisms underlying crosstalk between the two receptors have been investigated and the relationship with $\text{PKC}\zeta$ was reported, which is involved in the OTR and beta2-AR mediating ERK1/2 activation, respectively [12]. Our data demonstrated that uterine OTR expression was significantly increased and beta2-AR expression was significantly decreased in the oxytocin-induced estrogen pretreated mice model, providing an explanation for the abnormal uterine contraction.

After confirmed the abnormal spasm contraction and ischemia of uterus in the oxytocin-induced estrogen pretreated mice model, further investigations were made through the histopathological and biochemical measurements. The results showed the model group presented moderate to severe edema in endometrium lamina propria, the number of uterine glands changed little, but the amount of deep eosin dye homogeneous secretions presented in the glandular lumens suggesting that the oxytocin-induced writhing model was possibly a visceral and inflammatory pain model. Prostaglandins and prostanoids are biosynthesized from arachidonic acid through the COX pathway after production of arachidonic acid from hydrolysis of phospholipids by phospholipase [22]. Myometrial cells could synthesize both $\text{PGF}_{2\alpha}$ and PGE_2 and be regulated by steroids through a transcription-independent manner, which modulated the effect of OT on COX-2 expression [8, 25]. Our study demonstrated that COX-2 protein was over expressed in oxytocin-induced mice model, providing some evidence that oxytocin-induced writhing response might be a COX-2 dependent inflammatory response.

Thromboxane A_2 (TXA_2) produced by activated platelets and has prothrombotic properties, stimulates activation of new platelets as well as accelerates platelet aggregation. Prostacyclin (PGI_2) was a potent vasodilator. We demonstrated that the plasma TXB_2 (a metabolite of TXA_2)/6-keto- $\text{PGF}_{1\alpha}$ (a metabolite of PGI_2) increased 36% in writhing animal mice model mice, which was related to the reduced blood flow velocity and the raised pulsatility index.

At last, what is particularly worth mentioning on this animal model is the fact that uterine ultrasound did not show any visible pelvic lesions, which is in line with the clinical diagnosis of PD.

Oxytocin-induced *in vivo* pain model in female mice with estrogen pretreatment recapitulates the pathophysiology and clinical features of PD, providing an experimental model to study dysmenorrheic abdominal pain.

However, the experimental models of disease have weaknesses and PD modeling is no exception. For example, it is an acute animal model and the writhing response only continued for about 30 min. It's impossible to observe the pharmacokinetic features of the test drugs by this model. There are limitations to this study, and in future, we will further observe more indicators to compare the similarity to human PD, such as the uterine peristalsis evaluated with Cine-Mode-Display MR Imaging.

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Conflicts of interest

The authors declare no conflict of interest.

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