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Original Research Article

Effect of Nuangong zhitong decoction on primary dysmenorrhea in mice

Ya-Zhen Xie[1], Jian-Qiang Qian[1], Qi-Bin Lu[2]*
[1]Taicang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Taicang 215499, [2]Department of Gynaecology, The Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, China

For correspondence:* ***Email:* *xyz8102@sina.com;* ***Tel:*** *+86 25 8661 7141*

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Abstract

Purpose: *To investigate the therapeutic effect of Nuangong Zhitong decoction (NZD) on primary dysmenorrhea (PD) in mice, and its mechanism of action.*
Methods: *Dysmenorrhea was established in female PD mice by intraperitoneal injection of oxytocin following estradiol benzoate pretreatment. The effects of NZD and its active principles (cinnamic aldehyde and cinnamic acid) on PD were determined using body twist method. Serum levels of prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2α) in mice were measured using ELISA.*
Results: *The results showed that NZD dose-dependently reduced oxytocin-induced writhing responses ($p < 0.05$). Moreover, cinnamic aldehyde and cinnamic acid reduced oxytocin-induced writhing responses in a concentration-dependent manner, with maximal inhibitions of 65.01 and 70.67 %, respectively, and also decreased serum levels of PGE2 and PGF2α in PD mice ($p < 0.05$).*
Conclusion: *These results indicate that NZD mitigates oxytocin-induced uterine tetanic contraction in mice. Thus, NZD has a potential for development into an anti-dysmenorrheal drug for use in humans.*

Keywords: *Nuangong Zhitong decoction, Prostaglandin, PGE2, PGF2α, Primary dysmenorrhea*

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INTRODUCTION

Dysmenorrhea is one of the most common gynecological symptoms caused by uterine contractions, with serious effects on quality of life of patients [1]. There are two types of dysmenorrhea: primary dysmenorrhea (PD) and secondary dysmenorrhea (SD). Primary dysmenorrhea refers to recurrent menstrual cramps that are not due to other diseases. In contrast, secondary dysmenorrhea refers to menstrual cramping pain caused by an organic pathology, such as endometriosis, adenomyosis and uterine fibroids [1,2]. Primary dysmenorrhea occurs in up to approximately 50 % of menstruating females reportedly suffer from PD. The pain associated with PD is extremely severe in 15 % patients, and results in psychological distress such as anxiety and depression [2]. Moreover, the pain in PD is accompanied by nausea, vomiting, fatigue, and even diarrhea [3]. Currently, the main pharmacological treatments for PD include oral contraceptives or non-steroidal anti-inflammatory drugs (NSAIDs) [1,4]. However, NSAIDs and oral contraceptives cause unwanted side effects [5]. As an

alternative, Chinese herbal medicine is used for treating PD due to its fewer adverse effects and lower recurrence, relative to pharmacological therapies [6-9].

Wen jing decoction is a traditional Chinese prescription that widely applied in clinical treatment of dysmenorrhea [10]. *Nuangong zhitong* decoction (NZD) was developed from *Wen jing* decoction and is composed of *Cinnamomi ramulus*, evodiamine, asarum, *Radix linderae* and *Rhizoma corydalis* at ratio of 1:2:1:2:2. Cinnamic Cinnamaldehyde and cinnamic acid, two major constituents of *Cinnamomi ramulus*, have been reported to suppress oxytocin-induced uterine contractions [11,12]. Other components of NZD such as evodiamine, asarum, *Radix linderae* and *Rhizoma corydalis* warm the meridians and dissipate cold.

The aim of this study was to investigate the therapeutic effect of NZD on PD, and the underlying mechanisms in a PD model mice.

EXPERIMENTAL

Chemicals, reagents and animals

Estradiol benzoate was purchased from MedChemExpress (NJ, USA). Oxytocin was purchased from SPH No1 Biochemical and Pharmaceutical Co., Ltd (Shanghai, China). *Yueyueshu* was purchased from Zhong Jing Wan Xi Pharmaceutical Co. Ltd (Henan, China. Prostaglandin 2 ELISA Kit was purchased from R&D Systems, Inc (MN, USA), while PGF2α ELISA Kit was product of Cayman Chemical Company (MI, USA). Female KM mice (25 ± 5 g, 6 – 8-week-old) were obtained from Cavens Laboratory Animal Co., Ltd (Changzhou, China). All experiments were approved by Ethics Committee of Taicang Hospital of traditional Chinese Medicine.

Preparation of *Nuangong zhitong* decoction and its active principle components

Nuangong zhitong decoction was composed of 10 g of *cinnamomi ramulus*, 5 g of evodiamine, 3 g of asarum, 10 g of *Radix linderae* and 10 g of *Rhizoma corydalis*. The herbs were mixed and decocted with 2000 mL of water for 30 min. The decoction was performed twice to maximize NZD extract yield, and the extract was concentrated and filtered through a membrane filter to obtain NZD at a concentration of 1g/mL. Cinnamic aldehyde and cinnamic acid (Shanghai Yuan Ye Biotechnology Co., Ltd (Shanghai, China) were dissolved in DMSO and diluted with normal saline prior to use.

Animal experiments

Asthenia cold type mice model was established through intragastric administration of a 4 g/mL decoction made from gypsum, gentiana, *Phelladendron chinensis* and *Rhizoma anemarrhena* (mixed in a ratio of 2:1.2:1:1.5) to 50 Balb/c mice for 14 days. The mice were then subcutaneously injected with estradiol benzoate (2 mg/kg) daily for 12 days so as to improve the sensitivity of the mice uterine tissues to oxytocin. On the 12th day, the mice were intraperitoneally injected with oxytocin (20 U/kg) to induce severe uterine contraction. Seven days after establishment of PD model, the mice were randomly divided into 5 groups (10 mice/group). One group received *yueyueshu* (1 g/kg; positive control group), while another group was given normal saline (PD group). The other 3 were NZD groups: low dose NZD group (3 g/kg), medium dose NZD group (9 g/kg), and high dose NZD group (30.00 g/kg). All treatments lasted for 7 days. 10 Balb/c mice which were intragastrically administered normal saline for 13 days (0.1 mL/10 g) as normal control group. On the 13th day, writhing reaction was induced through intraperitoneal injection of oxytocin (33 U/kg).

Studies on the effects of components of NZD on PD followed the same design used for NZD. The mice were randomly divided into the following groups (10 mice/group): normal control group, PD group (normal saline), positive control group (*yueyueshu,* 1 g/kg), low dose cinnamic acid group (10.00 mg/kg), medium dose cinnamic acid group (20.00 mg/kg), high dose cinnamic acid group (40.00 mg/kg), low dose cinnamaldehyde group (10.00 mg/kg), medium dose cinnamaldehyde group (20.00 mg/kg), and high dose cinnamaldehyde group (40.00 mg/kg).

Writhing test

The mice were placed in a box and intraperitoneally injected with oxytocin. The number of writhes in 30 min was counted. Analgesia (A) was calculated as in Eq 1.

$$A\ (\%) = \{(P - N)/P\}100 \quad \ldots\ldots\ldots\ldots (1)$$

where P and N are the number of writhes in PD and NZD groups, respectively.

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected from the eyeball of each mouse. Serum levels of PGE2 and PGF2α were

measured using ELISA according to the kit's protocol.

Statistical analysis

SPSS 19.0 software (IBM Corp, Armonk, NY, USA) was used for statistical analysis. All data are expressed as mean ± standard deviation (SD). The differences between two groups were analyzed by Student's t-test. One-way ANOVA was performed for comparisons of multiple groups. Values of $p < 0.05$ was deemed statistically significant.

RESULTS

Effect of NZD on writhing frequency

To evaluate the effect of NZD on PD, mouse model of PD was established using estradiol benzoate and oxytocin. As shown in Table 1, the writhing frequency of PD group was 60-fold higher than that of normal control group, indicating the successful establishment of PD model. Compared with PD group, the writhing frequency of NZD at all doses decreased significantly, with analgesia of 33.77, 35.24 and 69.49 %, respectively.

Effect of NZD on serum PGE2 and PGF2α

As shown in Figure 1, the levels of serum PGE2 and PGF2α were significantly increased in PD group. However, *nuangong zhitong* decoction reduced the serum levels of PGE2 and PGF2α in a dose-dependent manner. Moreover, the magnitude of reduction was higher in PGE2 than in PGF2α.

Effect of cinnamic acid and cinnamaldehyde on writhing frequency

As shown in Table 2, compared with PD group, the number of writhes of mice in the low dose cinnamaldehyde group (10.00 mg/kg), medium dose cinnamaldehyde group (20.00 mg/kg) and high dose cinnamaldehyde group (40.00 mg/kg) were significantly decreased, and the percentage analgesia reached 19.43, 48.23 and 65.01, respectively. Cinnamic acid also significantly and dose-dependently suppressed writhing responses in PD mice, with percentage analgesia values of 27.38, 46.47 for low, medium and high dose mice groups, respectively.

Effect of cinnamic acid and cinnamaldehyde on serum PGE2 and PGF2α levels

Cinnamic acid and cinnamaldehyde, at all doses, significantly reduced the level of PGE2 (Figure 2). Moreover, cinnamic acid and cinnamaldehyde dose -dependently reduced the level of serum PGF2α. The level of reduction of PGE2 by cinnamic acid and cinnamaldehyde was significantly higher that of PGF2α.

DISCUSSION

In Traditional Chinese Medicine (TCM), PD is thought to result from uterine blockage by blood stasis [12]. Herbal medicine has been used for a long time for treating PD in China [13,14].

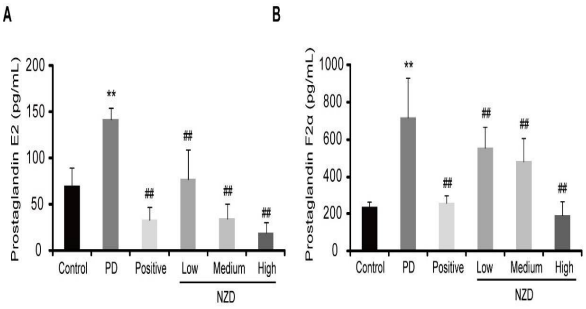

Figure 1: Decreased serum PGE2 and PGF2α levels by NZD in PD mice. Data are presented as mean ± SD (n = 10); $^{**}p < 0.01$ versus normal control group; $^{\#\#}p < 0.01$ versus PD group

Table 1: Effect of NZD on writhing reaction and analgesia in PD mice (n = 10)

Group	Writhing frequency (30 min)	Analgesia (%)
Normal control	0.00±0.00	100.00
PD	61.30±9.56**	0.00
Positive control	23.40±13.60$^{\#\#}$	61.82
NZD low (3.00 g/kg)	40.60±19.69$^{\#\#}$	33.77
NZD medium (9.00 g/kg)	39.70±15.17$^{\#\#}$	35.24
NZD high (30.00 g/kg)	18.70±11.78$^{\#\#}$	69.49

$^{**}P < 0.01$versus normal control group; $^{\#\#}p < 0.01$ versus PD group

Table 2: Decreased writhing reaction and increased analgesia by cinnamic acid and cinnamaldehyde in PD mice (n = 10)

Group	Writhing frequency (30 min)	Analgesia (%)
Normal control	0.00±0.00	100.00
PD	56.60±13.85**	0.00
Positive control	20.4±11.82##	63.95
Cinnamaldehyde (10 mg/kg)	45.60±6.57#	19.43
Cinnamaldehyde (20 mg/kg)	29.30±10.48#	48.23
Cinnamaldehyde (40 mg/kg)	19.80±7.87##	65.01
Cinnamic acid (10 mg/kg)	41.10±7.46##	27.38
Cinnamic acid (20 mg/kg)	30.3±10.13##	46.47
Cinnamic acid (40 mg/kg)	14.60±10.37##	70.67

**P < 0.01 versus normal control group; #p < 0.05, ##p < 0.01 versus PD group

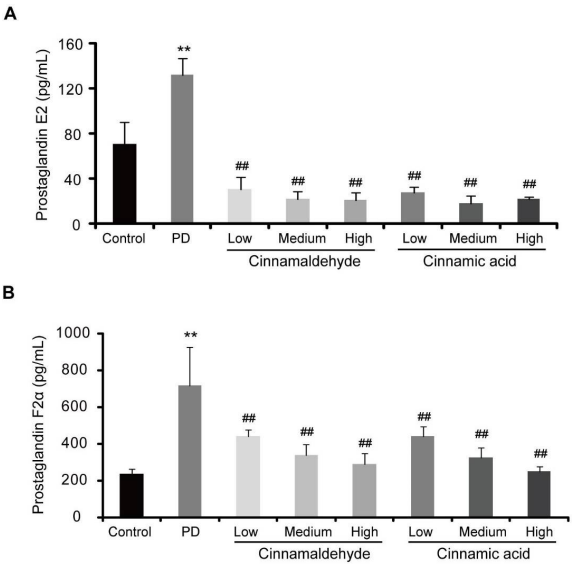

Figure 2: Decreased serum PGE2 and PGF2α levels by cinnamic acid and cinnamaldehyde in PD mice. Results are presented as mean ± SD (n = 10); **p < 0.01 versus normal control group; ##p < 0.01 versus PD group

Wenjing decoction is a traditional Chinese prescription used for the treatment of PD and first introduced by the Chinese sage Zhongjing Zhang in the book of *Jingui Yaolue*. The NZD used in the present study was developed from *wenjing decoction*. Compared with *Wenjing decoction*, NZD has added asarum, *Radix linderae* and *Rhizoma corydalis*, which increase its analgesic effect. A PD mice model in the present study was used to study the effect of NZD on PD and established via intraperitoneal injection of oxytocin.

The results obtained in this study showed that NZD concentration-dependently reduced oxytocin-induced writhing response. The analgesic effect of high dose of NZD was similar to that of *yueyue shu*, a traditional Chinese prescription used for the treatment of PD. Cinnamic acid and cinnamaldehyde are the two major constituents of *Cinnamomi ramulus* in NZD. It has been reported that cinnamic acid and cinnamaldehyde suppress oxytocin-induced uterine contractions [11,12]. In this study, it was found that cinnamic acid and cinnamaldehyde significantly reduced oxytocin-induced writhing response. The analgesic effects of cinnamic acid and cinnamaldehyde in the high-dose groups were even better than those of NZD. These data indicate that cinnamic acid and cinnamaldehyde are the active principles in NZD. Since *nuangong zhitong* decoction consists of five herbs, the roles of the other herbal components need to be investigated.

Prostaglandins (PGs) which are produced and released during menstruation cause abnormal uterine contractions, and sensitize spinal neurons to pain [15]. Prostaglandin 2 and PGF2α, two naturally occurring PGs, are regarded as the most crucial pain factors in PD [16]. They increase uterine contractility by binding to their receptors on the spiral arterioles [16]. The present study demonstrated that mice exposed to oxytocin had elevated PGE2 and PGF2α levels, which were suppressed by NZD, suggesting that PGE2 and PGF2α pathways may be involved in the relaxant effect of NZD. Moreover, it was demonstrated that cinnamic acid and cinnamaldehyde suppressed the levels of PGE2 and PGF2α in oxytocin-exposed mice, indicating that cinnamic acid and cinnamaldehyde may suppress oxytocin-induced writhing responses via inhibition of the effects of PGE2 and PGF2α. Since cyclooxygenase (COX)

catalyzes the biosynthesis of PGs [17,18], there is need to investigate whether COX is involved in the relaxant effect of NZD. Previous studies have demonstrated that oxytocin receptor (OTR) is up-regulated in PD patients and contributes to uterine contractions by binding to oxytocin [19,20]. In addition, it has been known that uterine contraction could be induced by intracellular Ca^{2+} overload in the uterine smooth muscles, leading to the occurrence of PD [21, 22]. Further studies will also investigate whether OTR and Ca^{2+} signaling are involved in the relaxant effect of NZD.

CONCLUSION

The findings of the present study indicate that NZD concentration-dependently reduces oxytocin-induced writhing responses in mice. Moreover, *Nuangong zhitong* decoction suppresses oxytocin-induced writhing responses possibly through modulating the levels of PGE2 and PGF2α. These findings suggest that NZD can potentially be used to treat PD in humans.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this study was done by the authors named in this manuscript and all liabilities pertaining to claims relating its content will be borne by the authors. Qi-Bin Lu designed all the experiments and revised the manuscript. Ya-Zhen Xie and Jian-Qiang Qian performed the experiments, while Ya-Zhen Xie wrote the manuscript.

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