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Articulo Original / Original Article

Time-sensitive effects of Zhuang medicated thread moxibustion on estrogen level in female perimenopausal model rats

[Efectos sensibles al tiempo de la moxibustión con hilo medicado de Zhuang sobre el nivel de estrógeno en ratas modelo perimenopáusicas hembras]

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Abstract: The present study was conducted to ascertain the estrogenic effect of Zhuang Medicated Thread Moxibustion (ZMTM) and explore its time-sensitive impact on estradiol (E2) in female perimenopausal rats. 40 female rats were randomized into four groups: the control, model, ZMTM, and acupuncture groups. The perimenopausal syndrome was induced in the last three groups with a daily subcutaneous dose of 80 mg/kg of 4- vinylcyclohexene diepoxide for 15 days. Afterward, rats in the model and control groups were fed routinely, while rats in the ZMTM and acupuncture groups were treated with six ZMTM and acupuncture courses, respectively. Results of the study suggested that following the six courses of treatment, the E2 level in the model group was significantly the lowest, while the regular group was the highest ($P < 0.05$). There was also a gradual increase in the E2 level of the ZMTM group compared to the model and acupuncture groups, e.g. after the 5th and 6th courses of treatment, their E2 level was significantly higher than the model and acupuncture groups. The ZMTM group was better than the model and acupuncture groups. In summary, ZMTM can improve perimenopausal induced rats' estrogen level.

Keywords: ZMTM; Perimenopausal syndrome; Estrogen; Time-sensitiveness; Chinese medicine.

Resumen: El presente estudio se llevó a cabo para determinar el efecto estrogénico de la moxibustión con hilo medicado Zhuang (ZMTM) y explorar su impacto sensible al tiempo en el estradiol (E2) en ratas hembras perimenopáusicas. Se dividió al azar una muestra de 40 ratas hembras en cuatro grupos: control, modelo, ZMTM y acupuntura. El síndrome perimenopáusico se indujo en los últimos tres grupos con una dosis subcutánea diaria de 80 mg/kg de diepóxido de 4-vinilciclohexeno durante 15 días. Después, las ratas en los grupos modelo y control fueron alimentadas rutinariamente, mientras que las ratas en los grupos ZMTM y acupuntura recibieron seis cursos de ZMTM y acupuntura, respectivamente. Los resultados del estudio sugieren que después de los seis cursos de tratamiento, el nivel de E2 en el grupo modelo fue significativamente más bajo, mientras que el grupo regular fue más alto (p<0,05). También hubo un aumento gradual en el nivel de E2 del grupo ZMTM en comparación con los grupos modelo y acupuntura, por ejemplo, después del quinto y sexto cursos de tratamiento, su nivel de E2 fue significativamente más alto que los grupos modelo y acupuntura. El grupo ZMTM fue mejor que los grupos modelo y acupuntura. En resumen, el ZMTM puede mejorar el nivel de estrógeno de las ratas inducidas por la perimenopausia.

Palabras clave: ZMTM; Síndrome perimenopáusico; Estrógeno; Sensibilidad al tiempo; Medicina China.

INTRODUCTION

Perimenopausal syndrome (PPS) is also known as climacteric syndrome, whose main symptoms include endocrine dyscrasia and neurasthenia induced by the degeneration of ovarian functions (Ye *et al*., 2013; Yaoping *et al*., 2017). It is also known as a perimenopausal disorder in traditional Chinese medicine. As a disease in women, PPS mainly occurs around the time of menopause. According to a study by Lijuan et al., the probability of having PPS in women is the highest (92.10%) among ages 45 to 55 years, and PPS seriously affects their quality of life and living standard (Li *et al*., 2010; Lijuan *et al*., 2018). The perimenopausal syndrome is a disease with high morbidity. It can have severe physical and psychological effects on perimenopausal women. According to modern medicine, the diseases significant causes include the central nervous system and the autonomic nervous system. The primary treatment is hormone replacement therapy, which can relieve climacteric symptoms and negative emotions, but it has side effects (Chaohui, 2017). Other research shows that long-term hormone therapy increases the risk of breast cancer, endometrial cancer, ovarian cancer, and other related diseases. Therefore, the exploration of new treatments is vital for improving the quality of life of women.

Estrogen is of great significance in the regular operation of the female endocrine system (Birzniece & Ho, 2021). Estrogen exerts its effect through responding receptors. It is interesting to note that estrogen's primary receptor, ER-α, is closely linked to neuron growth, function, and structure. Contemporary literature shows that E2's positive influence on gonadotropin secretion occurs primarily in the hypothalamus; however, it negatively influences the pituitary gland (Vrtačnik *et al*., 2014; Gonsioroski *et al*., 2020;). The leading causes of PPS include low estrogen levels and degeneration of ovarian function. Therefore, an increase in estrogen levels provides a foundation for treating PPS (Lindzey *et al*., 2006).

Moxibustion is a traditional Chinese medicine technique that involves the burning of mugwort plant (*Artemisia vulgaris* or *Artemisia argyii*), a small and spongy herb, to promote healing. Zhuang Medicated Thread Moxibustion (ZMTM) is a characteristic surgical procedure established on the theory of "yin-yang basis", the man-nature theory featured by the synchrony of triple initial energy, and the theory of "Sandao lianglu" which is concerned with the tract for the digestion and absorption of food, tract of water, tract of energy, tract of blood, and sensing tract (Jinming & Chen, 2006).

The procedure for ZMTM entails a ramie thread (No. 2 thread with a diameter of 70 mm), which is firstly soaked in Chinese herbs and then ignited and applied on the patient's special acupoints to stimulate those acupoints through heat and medicine. Therefore, energy can be regulated after treatment on the body surface (or acupoints) and the blood and the sensing tract (Meichun *et al*., 2012).

According to the literature, the expression of specific factors can be regulated, and cell apoptosis can be improved through ZMTM (Yaoping *et al*., 2017). When ovariectomized rabbits are treated with ZMTM, the interleukin-2 (IL-2) level in their serum can be increased. The high rates of apoptosis in splenocytes can be improved, their immunity can be enhanced, and the negative influence on the uterus shape and ER-α expressions can be improved (Gang *et al*., 2011; Yaoping *et al*., 2017). Based on previous studies, this study was designed from the perspective of time-sensitive effects of ZMTM on the level of estrogen in perimenopausal model rats. Therefore, the aim is to investigate the influence of ZMTM on the changes of estrogen in PPS and related features, which can thus provide a reference and favorable implication for clinical application.

MATERIALS AND METHODS

This prospective and randomized study was conducted at the Dept. of Medicine of Hubei Minzu University and Guangxi University of Chinese Medicine, performed for two years from 1 September 2018 - 1 September 2020. The Institutional Review Board and the local ethical committee have approved the study after registration with the Research Center (protocol Gui Ke AD1924518). This study followed the criteria as declared by the ARRIVE guidelines 2.0.

Experimental animals

Female Sprague-Dawley rats (40 in number) (180 – 220 g) about six weeks old were acquired from the Changsha Topgene Biotechnology Co., Ltd; (certification: SCXK (Xiang) 2014-001. The animals had been housed under managed conditions with a $22 \pm 2°C$ controlled temperature, $50 \pm 5\%$ air humidity, 12 h light–12 h dark cycle, and fed

routinely with access to tap water ad libitum and allowed to acclimatize for one week before the commencement of the experimental study. The maintenance and treatment of rats was carried out strictly following the Care and Use of Laboratory Animals' guidelines published by the U.S. National Academies Press (Eighth Edition, update, 2011). The committee approved the experimental protocols on the Ethics of Animal Experiments of the School of Zhuang Medicine, Guangxi University of Chinese Medicine.

Randomization of consecutive rats was done using the slot method, and the rats were divided into four groups: the standard control group (n = 10), the model group (n = 10), the acupuncture group (n = 10), and ZMTM group (n = 10). A single operator performed all procedures on the groups' rats under standard aseptic conditions and protocols to remove bias. The First operator and second operator (readings) were double-blind. And a third operator was used for evaluating the data.

Zhuang medicated thread moxibustion, alcohol lamp, glass slides, normal saline, 1 mL syringe, and cotton swabs were all provided by the School of Zhuang Medicine laboratory, Guangxi University of Chinese Medicine. 4-vinyl cyclohexene dioxide (4-VCD), Rat E2 ELISA KIT (Shanghai Meilian Biotech Co., Ltd.), inverted research microscope (DM18, Leica, Germany), multifunctional microplate reader (M200 TECAN, Switzerland), and high-speed freezing centrifuge (3K15 Sigma, Germany).

The standard control and model groups received no treatment. They were routinely fed and had access to water. However, for rats in the ZMTM group, they received ZMTM. After the thread was ignited and the acupoints were located, the operator used his wrist and fingers to press the thread's ignited tip on the acupoints swiftly. When the glow was extinguished, the whole operation could be seen as one "Zhuang." Typically, each acupoint was acupunctured for one "Zhuang." Again, the no.2 thread was selected, which was 70 mm in diameter, and when the ignited thread was held over the acupoints, a 1 - 2 cm tail of the thread should be left intact. Disinfection of acupoints was not necessary before applying ZMTM because of the heat. Typical acupoints include Xiaguanyuan, Qizhousi acupoints, and Beiba acupoints, which are characteristic in Zhuang medicine, mainly for treating miscellaneous gynecological diseases. Acupoints on rats were located according to Experimental Acupuncture Science. The ZMTM was applied to acupoints once per day, 2 Zhuang per acupoint, for six courses. Rats in the acupuncture group were treated with 0.28 filiform needles with the same acupoints, and the treatment time was similar to the ZMTM group. Vaginal smears collection time was strictly based on the menstrual cycle of female rats. Typically, one course of treatment lasts for one menstrual cycle.

Model preparation and evaluation

The model, acupuncture, and ZMTM groups received a daily subcutaneous injection of 4-VCD at 80 mg/kg for 15 consecutive days. The 200 g rats were given a volume of 0.2 ml (80 mg/mL), i.e., 0.001 mL/g, for 15 consecutive days to induce the perimenopausal model. After five days of treatment, rat vaginal smears were collected from each group for five consecutive days. The estrous cycle of rats was confirmed by microscopic evaluation of cells in vaginal smears. In all three groups, besides the standard control group, rat vaginal smears showed estrous symptoms, i.e., a significant amount of leukocytes with some epithelial cells and keratinocytes were present, which implies that the perimenopausal model was induced successfully. No mortality was seen during the induction of the perimenopausal phase.

Sample collection

2 mL venous blood from each rat's eye socket was collected in the 1st, 3rd, 5th, and 6th courses of treatment and was kept in estrogen receptor tubes at 4°C. When blood coagulation occurred, the tubes were placed in a centrifuge. After centrifugation, the supernatant was collected and kept in a freezer at -20°C. Rat vaginal smears were collected simultaneously for H & E staining, which was done in Guangxi National Hospital and observed by the microscope. All rats were sacrificed by cervical dislocation under anesthesia.

Measurement of serum Estradiol (E2)

ELISA kit was used, and the standard operation was completed according to instructions.

Statistical analysis

SPSS 19.0 software was used for analyzing results. Data were calculated as mean ± SD (standard

deviation). Intragroup data were compared using a one-factor analysis of variance (ANOVA). The differences are statistically significant when the *p*-value is less than 0.05.

RESULTS

H & E staining of rat vaginal smears from each group and vaginal smears of rats in estrous cycle.

In pre-estrus period: a great number of nucleated epithelial cells and some cornified epithelial cells are present. In estrus period: almost all cells are cornified cells without cell nucleus, with some epithelial cells present. In late estrus period: mostly cornified cells and leukocytes are present. In estrus interval period: most leukocytes and some mucosa and epithelial cells are present (Figure No. 1).

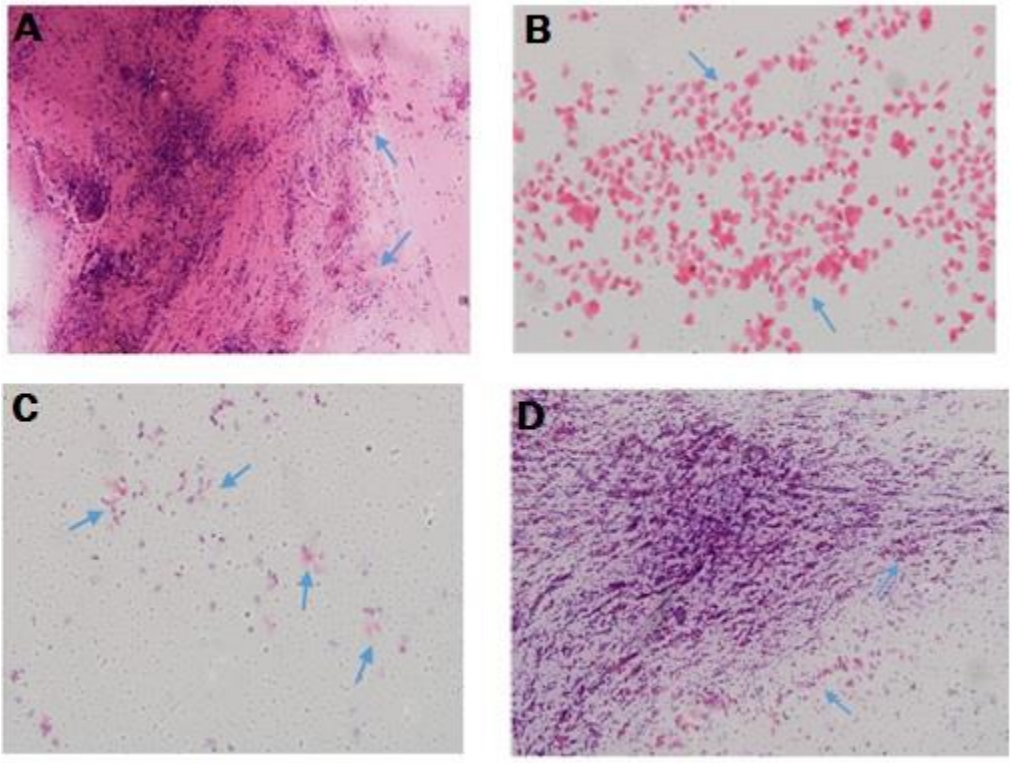

Figure No. 1

H & E staining of rat vaginal smears from each group (100x) and vaginal smears of rats in estrous cycle. A: Pre-estrus: a great number of nucleated epithelial cells and some cornified epithelial cells are present; B: Estrus period: almost all cells are cornified cells without cell nucleus, with some epithelial cells present; C: Late estrus: mostly cornified cells and leukocytes are present; D: Estrus interval: most leukocytes and some mucosa and epithelial cells are present

Comparing the general condition of rats in each group after six courses of treatment

The mental state, disposition, feed intake, reaction, and general well-being of all rats among all groups were examined at the end of the treatment course. The standard control rats were generally stable, mentally agile, and had a regular feed intake and disposition. On the contrary, the model group rats were restless, with slow reaction, inadequate feed intake, and physically weak. However, the treatment group, i.e., ZMTM, showed the improved ($p<0.05$) mental status of the rats and feed intake, and the fur returned to smooth and supple, the hair grew again, the diet increased, the activity increased, the temperament returned to normal. Although the rats' disposition was docile, there was a general

improvement in the condition of the rats. The results obtained for the ZMTM group appeared similar to those of the acupuncture group (Table No. 1).

Table No. 1
General condition of rats in each group following treatment

Groups	Mental state	Reaction	Disposition	Feed intake	Conditions after treatment
Normal group	Good	Agile	Docile	Normal	Normal
Model group	Bad	Slow	Restless	Reduced	Poor
ZMTM group	Very good	Basically normal	Docile	Normal	Good
Acupuncture group	Average	Average	Docile	Basically normal	Very good

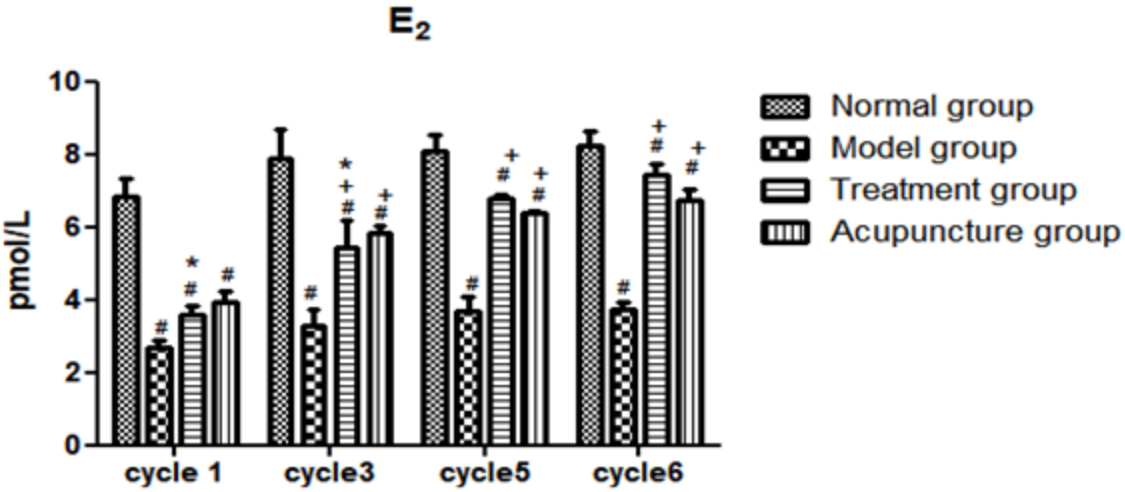

Figure No. 2
Time-sensitiveness of E2 in rats after perimenopausal model induction

The effect of ZMTM on E2 of perimenopausal model rats

The results of E 2 levels are shown in Figure No. 2 and Figure No. 3. As seen from Figure No. 2, the level of E2 in the normal control group was the highest from cycle 1 to cycle 6. After modeling, the E2 value of perimenopausal model group was significantly lower than that of normal group (t 'value = 25.9173, #p<0.05). After moxibustion with medicinal thread, the E2 level of the treatment group in the first cycle (t 'value = 22.9647, +p1<0.0000, the 3rd cycle（t 'value = 7.1713, +p3<0.05）, the 5th cycle（t 'value = 37.108, +p5<0.05, the 6th cycle（t 'value = 67.4292, +p6<0.0000）were all significantly higher than that of the model group (p<0.05)), and the sixth cycle is the highest. After moxibustion with medicinal thread, the E2 level in the treatment group was equivalent to that in the acupuncture group in the 3rd cycle (t 'value = 1.0679, *p3>0.05), but significantly higher than that in the

acupuncture group in the 5th cycle (t 'value = 56.85, **p*5<0.05) and 6th cycle (t 'value = 3.3333, **p*6<0.05).

Figure No. 3 gives an insight into the E2 level in the 6th cycle. It can be seen here that the E2 level in the ZMTM treatment was significantly higher than that of perimenopausal model group (t 'value = 67.4292, +*p*6=0.0000<0.05), significantly higher than that of normal control group (t 'value = 6.8917, #*p*6<0.05), and better than that of acupuncture group (t' value = 3.3333, **p*6<0.05).

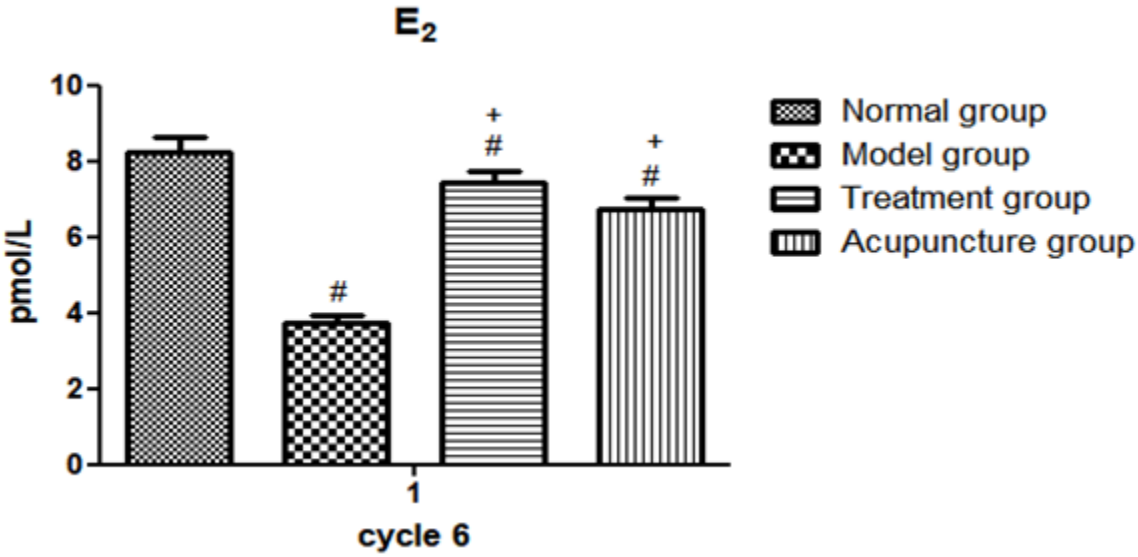

Figure No. 3
Comparison of E2 levels in rats after perimenopausal model induction on the sixth cycle

DISCUSSION

A perimenopausal syndrome is a common female disease typified by neurasthenia and neuropsychological symptoms, mainly caused by an alteration in the hormone levels around menopause (Zheng *et al*., 2011; Ma *et al*., 2019). According to traditional Chinese medicine, PPS falls into the category of hysteria and perimenopausal disorder. The leading causes include deficiency of kidney qi, stagnation of liver qi, internal disturbance of seven emotions, and stress, injuring qi and blood. There are many therapeutic approaches to handle PPS (Tang *et al*., 2020; Wang & Yu, 2021). According to the researches, standard acupuncture and electro-acupuncture can relieve the symptoms of menopause. According to Zhuang medicine, "heiq" (qi) and "lwed" (blood) play an essential role in female growth and development. When women are in perimenopause, it means the degeneration of their physical quality and body function. The decline in "heiq" (qi) and "lwed" (blood) will cause the disorder of qi and blood and finally lead to PPS (Zheng *et al*., 2009; Geng *et al*., 2021). As a traditional external therapy, ZMTM can regulate qi and blood through acupoints to balance qi and blood, relieve symptoms, improve body health, and finally show good therapeutic efficacy (Lijuan *et al*., 2018). Essential therapy tools include medicated thread made from many Zhuang herbs, e.g., *Santalum album*, musk, frankincense, and *Argyreia acuta*. It has a delicate smell and can achieve good therapeutic effects (Meichun *et al*., 2011). Qizhousi acupoints, Beiba acupoints, and other selected acupoints are characteristic acupoints in Zhuang medicine, mainly treating various gynecological diseases (Cao *et al*., 2017).

Perimenopausal syndrome correlates with changes in sex hormones. Therefore, the difference in

sex hormones should be given close attention during treatment by the treating physicians (Ma & Chen, 2015; Cao *et al*., 2017; Shen *et al*., 2018; Caihua, 2019). Furthermore, the physicians should examine the levels of luteinizing hormone (L.H.), stimulating follicle hormone (FSH), E2, and other hormones to assist in the diagnosis and treatment of PPS (Cao *et al*., 2017; Caihua, 2019). This study indicates that rat estrogen levels decreased after perimenopause model induction and improved after ZMTM treatment. This effect of ZMTM was significant right from the 1st to the 6th cycle. However, its influence became notable from the 3rd cycle and was most meaningful and useful between the 5th and 6th cycles. E2 level in the ZMTM group was significantly higher (p<0.05) than that of the acupuncture group and model group, although slightly lower than that of the standard group.

CONCLUSION

This study confirms that ZMTM can improve perimenopausal model rats' estrogen levels; this curative effect is most effective at the 3rd cycle and tends to become better in further treatment cycles. Although the mechanism of action of ZMTM is yet to be explored, making further human trials necessary, however, this study has laid a foundation for its clinical research and application.

Abbreviations

ZMTM: Zhuang Medicated Threads; PPS: Perimenopausal Syndrome; E: Estradiaol; IL-2: Interleukin 2.

Ethics approval and consent to participate

All animal experiments were authorized by the Guangxi Zhuang Yao Medicine Center of Engineering and Technology, Guangxi University of Chinese Medicine and Hubei Minzu University Animal Ethics Committee and carried out in compliance with the institutional guidelines.

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