Acupoint selection and acupuncture in the treatment of premature ovarian failure by promoting the secretion of exosome protein NELL2

Keywords
premature ovarian failure, exosome, kidney-deficiency liver-depression and blood stasis type, acupoint selection and acupuncture therapy

Abstract
Introduction
To explore the mechanism and clinical effect of acupoint selection and acupuncture therapy on premature ovarian failure.

Material and methods
A total of 100 cases of POF were collected and divided into POF group and POF+acupoint selection and acupuncture therapy (POF+ACU) group. Cerebrospinal fluid samples from patients in each group were extracted by lumbar puncture, and exosomes were isolated and purified. The expression level of exosome protein NELL2 was analyzed by western blot, and the morphology of exosomes was observed by transmission electron microscopy (TEM). The changes of sex hormone levels were detected by TOSOH AIA 1800 automatic chemiluminescence analyzer. Follicular development and ovulation were monitored by B-ultrasound.

Results
The expression of protein CD9, CD81 and NELL2 in POF+ACU group all increased compared with POF group. TEM images showed that the exosomes were oval or round membranous vesicles. After 3 months of continuous acupoint selection and acupuncture therapy, it was found that the level of testosterone (T) and estradiol (E2) in the POF+ACU group were significantly increased, while the follicular stimulating hormone (FSH) and luteinizing hormone (LH) were significantly decreased, showing a significant difference compared with the POF group. B-ultrasound observation of the endometrial thickness and the maximum follicle diameter showed that the values of POF+ACU group increased, showing no statistical difference compared with the NC group. Ovulation occurred in 20% of the 50 patients in the POF group and 70% in the POF+ACU treatment group.

Conclusions
Acupoint selection and acupuncture therapy can treat POF by promoting the secretion of exosome protein NELL2
Acupoint selection and acupuncture in the treatment of premature ovarian failure by promoting the secretion of exosome protein NELL2

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Abstract

Objective To explore the mechanism and clinical effect of acupoint selection and acupuncture therapy on premature ovarian failure (POF). Methods A total of 100 cases of POF patients were collected and divided into POF group and POF+acupoint selection and acupuncture therapy (POF+ACU) group. 50 healthy women were selected as normal control (NC) group. Blood samples from patients in each group were collected and exosomes were isolated and purified. The expression level of exosome protein NELL2 was analyzed by western blot. The changes of sex hormone levels were detected by TOSOH AIA 1800 automatic chemiluminescence analyzer. Follicular development and ovulation were monitored by B-ultrasound. Results The expression of NELL2 protein in POF+ACU group increased compared with POF group. After 3 months of continuous acupoint selection and acupuncture therapy, it was found that the level of testosterone (T) and estradiol (E2) in the POF+ACU group were significantly increased, while the follicular stimulating hormone (FSH) and luteinizing hormone (LH) were significantly decreased, showing a significant difference compared with the POF group. B-ultrasound found that ovulation occurred in 20% of the 50 patients in the POF group and 70% in the POF+ACU treatment group. Conclusion Acupoint selection and acupuncture therapy can treat POF by promoting the secretion of exosome protein NELL2.

Keywords: premature ovarian failure; exosome; kidney-deficiency liver-depression and blood stasis type; acupoint selection and acupuncture therapy

1. Introduction

Premature ovarian failure (POF) refers to a group of diseases in women with ovarian function decline before 40 years old. POF results in amenorrhea, infertility and perimenopausal syndrome, and accompanies by low estrogen and high gonadotropin status [1]. Patients with POF have hot flushes, sweating, facial flushing, low sexual desire and so on [2]. It is reported that the morbidity of POF in the general population is about 1-3% and POF accounts for 4-18% of women with secondary amenorrhea. In recent years, the incidence of this disease has been on the rise. POF has become a difficult gynecological disease with complicated etiology and difficult treatment, which seriously threatens women's physical and mental health [3].
The exact cause of POF has not been found in western medicine, and it has been widely believed that it may be related to heredity, immunity, iatrogenic injury, enzyme deficiency, radiotherapy and chemotherapy, environmental harmful factors and smoking \[^{[4]}\]. At present, there is no clear and effective method to restore ovarian function in treatment. Hormone replacement therapy, immunotherapy, ovulation induction, assisted egg donation technology are mainly performed according to fertility requirements of the patients. However, the therapeutic effect is not ideal, and there are certain side effects \[^{[5]}\]. Traditional Chinese medicine syndrome differentiation of POF characterizes as kidney deficiency, liver depression or liver stagnation, and blood stasis. Therefore, the treatment is mainly to tonifying the kidney and filling the essence, dredging the liver and tongren \[^{[6]}\].

POF is a common symptom for acupuncture \[^{[7]}\]. In clinical practice, acupoint selection of acupuncture are generally Shenyu, Guanyuan, Qihai, Zusanli, Taixi, Sanyinjiao, Taichong, Pishu, Zhongwan, Zhongzhong, Xuehai, Yinlingquan and so on. It can refer to the Chinese medicine disease name "infertility" or "amenorrhea" for syndrome differentiation treatment. Hence, in this study, acupoint selection and acupuncture therapy is utilized to treat patients with POF, and the mechanism is investigated based on the secretion of exosome protein NELL2.

2. Clinical data and methods

2.1 Cases of source

A total of 100 cases of POF patients with kidney deficiency and liver depression were selected and treated in the gynecological outpatient department of the Affiliated Hospital of Heilongjiang Academy of Traditional Chinese Medicine from January 2018 to December 2020. The ages of all patients ranged from 24 to 35 years old.

2.1.1 Diagnostic criteria

According to the diagnostic criteria of POF in Obstetrics and Gynecology: amenorrhea of more than 4 months appears before the age of 40, accompanied by symptoms of perimenopausal syndrome; follicular stimulating hormone (FSH) > 40 IU/L, estradiol (E2) < 73.2 pmol/L, luteinizing hormone (LH)>30 IU/L.

2.1.2 Inclusion criteria
It met the above diagnostic criteria and syndrome differentiation criteria; (2) Age < 40 years old; (3) The patients did not use hormone therapy in the past 3 months and did not use traditional Chinese medicine therapy in the past 1 month; (4) This study was approved by the Medical Ethics Committee of our hospital, and all patients agreed to cooperate with the treatment and obtained informed consent.

2.1.3 Exclusion criteria

(1) Amenorrhea caused by congenital genital organ abnormality and acquired genital organ injury due to genetic problems; (2) Temporary menopause during pregnancy and lactation; (3) Allergic to multiple drugs or known to be allergic to drug ingredients used in this study; (4) Complicated with heart, liver, kidney and hematopoietic system serious diseases, mental illness.

2.2 Grouping and treatment

A total of 100 cases of POF was divided into 2 groups. One group was received conventional treatment (POF), while another group was conducted acupoint selection and acupuncture therapy (POF+ACU) on the basis of conventional treatment. In addition, 50 healthy women of the same age were included as the normal control group (NC).

Acupuncture therapy method: Shenye, Guanyuan, Qihai, Zusanli, Taixi, Sanyinjiao, Taichong, Pishu, Zhongwan, Zhongzhong, Xuehai and Yinlingquan were selected for acupuncture treatment once a day, and treatment was stopped during menstruation for 3 consecutive months.

2.3 Separation and purification of exosomes

The blood samples of patients were collected in the morning, and the blood volume was 5-6 mL. The plasma was separated within 2 hours, and the whole blood was centrifuged at room temperature for 10 minutes to collect the lower red blood cells. Exosomes were extracted from the supernatant of erythrocyte suspension by supercentrifugation. Erythrocyte suspension was centrifuged at 300×g, 4 °C for 10 min. The supernatant was transferred to a new centrifuge tube at 2000 g at 4 °C for further cell removal. The supernatant was absorbed into a clean polypropylene centrifuge tube, centrifuged 30 min at 4 °C to remove fine cell debris and large vesicles. The precipitate
was centrifuged at 100000×g, 4 °C for 70 min. The precipitates are exosomes and are stored at -80 °C.

2.4 Western blot

Protein sample (1 µL) and SDS-PAGE protein loading buffer (4 µL) were mixed. The protein was fully denatured by heating at 100 °C for 10 min. The supernate was obtained by centrifuging at 5000×g for 1 min. The supernate was added to the sample well and changed the sample volume to ensure the same amount of protein in each lane. The proteins were separated by SDS-polyacrylamide gel electrophoresis. After the protein being transferred and sealed, the primary antibodies (CD9, CD81, NELL2 and β-actin) were incubated at 1∶1000 dilution for 2 h at room temperature. After TBST washing, HRP-labeled secondary antibodies were incubated at room temperature for 1 h. CD9, CD81, NELL2 in each group were scanned with analyzed using Image J software.

2.5 Observation of exosome morphology

Exosomes (20 μL) was taken and added to equal volume of 4% paraformaldehyde. After mixing completely, the mixture was added to para glue and completely covered with copper mesh to stand at room temperature for 5-10 min. Then, filter paper was used to wipe the excess liquid from the side, and 50 µL phosphotungstic acid (20 g/L) was added and incubated at room temperature for 3 min. They were then placed under an incandescent lamp and roasted for 3-5 min. Morphology of exosome was characterized on H7600 TEM (Hitachi, Japan).

2.6 Changes in sex hormone levels

Patients in this research were guaranteed adequate sleep before examination. Fasting venous blood of 4 mL from patients in each group was taken as test samples on the examination day. After serum separation, six sex hormones in each group were detected by TOSOH AIA 1800 automatic chemiluminescence analyzer. Six sex hormones include testosterone (T), prolactin (PRL), progesterone (P), estradiol (E2), follicular stimulating hormone (FSH) and luteinizing hormone (LH). The specific detection method was
performed according to the requirements in the instruction manual, and the results were determined according to the corresponding standards in the kit.

2.7 Follicular development and ovulation

Follicular development and ovulation changing situation is assisted by the gynecology B-ultrasound department of Heilongjiang Provincial Hospital of Traditional Chinese Medicine. According to the number of days of menstrual cycle, combined with the patient's self-feeling and the results of ovulation test paper, transvaginal B-ultrasound examination was performed before ovulation. Generally, the monitoring began on the 10th day of the menstrual cycle. When the diameter of the main follicle was 10-15 mm, the monitoring was changed to once every other day by B-ultrasound. When the diameter of the follicle was $\geq 16$ mm, the monitoring was changed to once a day until ovulation occurred. The maximum follicle diameter and endometrial thickness during ovulation were measured by B-ultrasound. If no follicles with a diameter $>1.0$ cm were detected until the menstrual cycle or the follicle diameter increase of less than 1 mm was detected for three consecutive times, the detection was stopped.

2.8 Statistical analysis

All experiments were replicated independently at least three times. The data were analyzed using one-way analysis of variance (ANOVA) and are presented as the mean $\pm$ standard deviation (SD). Statistical significance was defined as $P < 0.05$.

3. Results

3.1 Age and course of disease

Patients in POF group were aged from 24 to 35 years old, and the course of disease was as short as 5 months and as long as 36 months. Patients in POF+ACU group were aged from 25 to 35 years old, and the course of disease was as short as 5 months and as long as 35 months. There was no statistical significance between age and course of disease in the two groups.

3.2 Western blot
The expression of CD9, CD81, NELL2 proteins were detected by western blot, as shown in Figure 1. CD9, CD81 protein in POF+ ACP group all increased compared with POF group, ***$P < 0.001$. The expression levels of CD9, CD81 were highest in POF+ACU group than those of POF group and NC group. The expression level of NELL2 protein was higher in POF+ACU group compared with POF group. NELL2 protein was not found in NC group. Compared with POF group, *$P < 0.05$.

![Western Blot Images](image)

**Figure 1.** The expression levels of CD9, CD81, NELL2 proteins in different groups. ***$P < 0.001$, *$P < 0.05$ was considered with significant difference compared with POF group.

### 3.3 Observation of exosome morphology

Figure 2 showed the electron microscope images of exosomes. It could be seen that exosomes are elliptic or round membranous vesicles, which contain a large amount of contents. They are consistent with the structural characteristics of exosomes. The diameter
of exosomes is between 50–100 nm, which is in line with the size of exosomes. The secretion of exosomes were obvious in POF group and POF+ACU group.

Figure 2. Electron microscope image of exosomes in different group. Scale bar: 100 nm.

3.4 Changes in sex hormone levels

After 3 months of continuous acupuncture treatment, it was found that the levels of T and E2 were significantly decreased in the POF group, while, the levels of FSH and LH were significantly increased. The contents of LH and FSH in POF group were significantly higher than those in NC group, and the content of T in POF group was significantly lower than that in NC group, with significant difference, **P < 0.01, *P < 0.05. However, after acupuncture treatment, the level of T and E2 were significantly increased, while LH and FSH were significantly decreased, showing a significant difference compared with POF group, ##P < 0.01, #P < 0.05. PRL and P had no difference in NC, POF and POF+ACU group (Table 1). It suggested that prolactin and progesterone had little effect on POF patients.

Table 1. Changes of sex hormone levels in each group (n=50).

<table>
<thead>
<tr>
<th>Group</th>
<th>T(nmol/L)</th>
<th>E2(pmol/L)</th>
<th>LH(mIU/mL)</th>
<th>FSH(mIU/mL)</th>
<th>PRL(uIU/mL)</th>
<th>P (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.99±0.53</td>
<td>628.20±228.67</td>
<td>5.12±1.62</td>
<td>5.20±1.20</td>
<td>363.18±158.61</td>
<td>2.27±1.04</td>
</tr>
<tr>
<td>POF</td>
<td>0.94±0.26**</td>
<td>292.78±130.64**</td>
<td>9.21±3.13**</td>
<td>8.48±2.26</td>
<td>353.39±160.45</td>
<td>2.36±1.07</td>
</tr>
<tr>
<td>POF+ACU</td>
<td>1.72±0.47##</td>
<td>546.59±215.19##</td>
<td>5.91±3.10##</td>
<td>6.19±1.94##</td>
<td>301.85±169.84</td>
<td>2.63±1.14</td>
</tr>
</tbody>
</table>

Note: compared with NC group, **P<0.01, *P<0.05; compared with POF group, ##P<0.01, #P<0.05.
3.5 Follicular development and ovulation

The endometrial thickness and the maximum ovarian diameter of each group were observed by B-ultrasound. It was found that the value of POF group was the smallest, which was significantly different from that of NC group, \( **P < 0.01 \). However, after acupuncture treatment, endometrial thickness and maximum follicle diameter increased, showing no statistical difference compared with the NC group, and significant difference compared with POF group, \( ##P < 0.01 \). Ovulation in each group was monitored by B-ultrasound and the ovulation rate was analyzed. It was found that there were no ovulatory patients in the NC group, but in the POF group, the number of non-ovulatory patients accounted for 80% and the number of ovulatory patients was 20%. In the POF+ACU group, anovulation accounted for 30% and ovulation accounted for 70% (Table 2). Therefore, it confirms that acupoint selection and acupuncture therapy can relieve premature ovarian failure.

Table 2. Comparison of the endometrial thickness and the maximum follicle diameter in different groups (n=50).

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>POF</th>
<th>POF+ACU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial thickness</td>
<td>1.32±0.19</td>
<td>0.67±0.18**</td>
<td>1.27±0.24##</td>
</tr>
<tr>
<td>Maximum follicle diameter</td>
<td>1.87±0.63</td>
<td>1.04±0.32**</td>
<td>1.70±0.38##</td>
</tr>
<tr>
<td>Ovulation rate</td>
<td>100%</td>
<td>20%</td>
<td>70%</td>
</tr>
</tbody>
</table>

Note: compared with NC group, \( **P<0.01 \); compared with POF group, \( ##P<0.01 \).

4. Discussion

The expression of CD9 and CD81 in POF and POF+ACU group were detected by western blot. At the same time, the morphology of exosomes was shown by TEM. Exosomes are elliptic or round membranous vesicles, which are consistent with the structural characteristics of exosomes. It indicated that the samples secretion extracted by differential centrifugation method was exosomes. Exosomes are cystic vesicles with a diameter of 30-100 nm bilayer membrane secreted by budding cells \[8-10\]. Exosomes carry a large number of bioactive substances, including messenger RNA, proteins, enzymes and signal molecules. They can carry nucleic acids, proteins and other molecules to target cells through fusion with target cells, and play an important role in regulating the function and
signal of target cells \[11-13\]. When an exosome fuses with a target cell, it delivers and releases important biomolecules to the target cells \[14-16\].

The maturity of follicles and whether being smoothly discharged are of great significance to the conception of women. Generally, the maximum follicle diameter of dominant follicles $\geq 1.8$ cm is considered to be mature follicles \[17\]. As the follicles continue to develop, the endometrium will also thicken. When the follicles mature, the endometrium thickness before ovulation is usually $>0.8$ cm. Monitoring endometrial thickness can indirectly determine the level of E2 in human body and the development of follicles, thus helping to treat the phenomenon of POF \[18\]. The study found that POF patients had a much lower endometrial thickness than NC patients. At the same time, the maximum follicle diameter was only 1.04, which also indicated that follicles in patients with POF were not well developed. However, endometrial thickness and maximum follicle diameter increased significantly in patients treated with acupuncture, and the results were no different from those in the normal group. In a word, the experiment effectively showed that acupuncture can treat POF and promote the secretion of related hormones.

As for the mechanism of acupuncture treatment for POF, it may be related to hypothalamic gonadotropin-releasing hormone (GnRH). The regulation mechanism of functional activity of GnRH neurons is very complex, and many factors affect GnRH secretion. In recent years, studies have found that the exosome protein NELL2 may affect the secretion of GnRH through regulating glutamate secretion or other mechanisms \[19\]. NELL2 may be an important mediator of glutamic acid regulating the secretion of GnRH in neurons and thus affecting sexual development \[20\]. NELL2 plays an important role in nerve growth, nerve differentiation, maintenance of neuroplasticity and synaptic transport \[21, 22\]. In this study, the expression of protein NELL2 was significantly increased in POF patients after 3 months of acupuncture treatment. We speculated that acupoint selection and acupuncture therapy promoted thalamic secretion of NELL2, and NELL2 may be involved in the regulation of GnRH on sexual development and the treatment of POF by regulating glutamic acid.

5. Conclusion
The expression of exosome protein NELL2 increased in POF patients after 3 months of acupoint selection and acupuncture therapy. Compared with POF group, T and E2 were significantly higher, but LH, FSH were significantly lower in POF+ACU group. Endometrial thickness and maximum follicle diameter also increased in POF+ACU group. This suggests that the mechanism of acupoint selection and acupuncture treatment for POF is that acupuncture promotes thalamic secretion of NELL2.

Declarations

Ethics approval and consent to participate

The ethic approval was obtained from the Ethic Committee of General Hospital of Ruijin Rehabilitation Hospital and Written informed consent was obtained from all patients.

Consent to publish

All of the authors have Consented to publish this research.

Availability of data and materials

The data are free access to available upon request.

Competing interests

All authors declare no conflict of interest.

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None.

Authors' contributions

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.
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Reference


