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

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Abstract

Introduction: To explore the mechanism and clinical effect of acupoint selection and acupuncture therapy on premature ovarian failure (POF).
Material and methods: A total of 100 cases of POF patients were collected and divided into the POF group and POF + acupoint selection and acupuncture therapy (POF + ACU) group. Fifty healthy women were selected as a 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 a TOSOH AIA 1800 automatic chemiluminescence analyzer. Follicular development and ovulation were monitored by B-ultrasound.
Results: The expression of NELL2 protein in the POF + ACU group was higher than in the POF group. After 3 months of continuous acupoint selection and acupuncture therapy, it was found that the levels of testosterone (T) and estradiol (E2) in the POF + ACU group were significantly increased, while the follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels 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.
Conclusions: Acupoint selection and acupuncture therapy can treat POF by promoting the secretion of exosome protein NELL2.

Key words: premature ovarian failure, exosome, kidney-deficiency liver-depression and blood stasis type, acupoint selection and acupuncture therapy.

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 is accompanied 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

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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, and 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 it as kidney deficiency, liver depression or liver stagnation, and blood stasis. Therefore, the treatment is mainly to the treatment is mainly to tonify the kidney, fill the essence, drain the liver, and clear the ligaments [6].

Premature ovarian failure is a common symptom for acupuncture [7]. In clinical practice, acupoint selection of acupuncture is 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.

Material and methods

Cases

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 between January 2018 and December 2020. The ages of all patients ranged from 24 to 35 years old.

The diagnostic criteria of POF in Obstetrics and Gynecology were followed: 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.

Inclusion criteria: (1) The patients met the above diagnostic criteria and syndrome differentiation criteria. (2) Age < 40 years old. (3) The pa-

tients 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 provided informed consent.

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 serious heart, liver, kidney and hematopoietic system diseases, mental illness.

Grouping and treatment

A total of 100 cases of POF were divided into 2 groups. One group received conventional treatment (POF), while another group underwent 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: Shenyu, 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.

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 h, and the whole blood was centrifuged at room temperature for 10 min to collect the lower red blood cells. Exosomes were extracted from the supernatant of the erythrocyte suspension by supercentrifugation. The 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, then centrifuged for 30 min at 4°C to remove fine cell debris and large vesicles. The precipitate was centrifuged at 100 000 × g, 4°C for 70 min. The precipitates were exosomes and were stored at –80°C.

Western blot

The 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 supernatant was obtained by centrifuging at 5000 × g for 1 min. The supernatant was

added to the sample well and the sample volume was changed to ensure the same amount of protein in each lane. The proteins were separated by SDS-polyacrylamide gel electrophoresis. After the protein was 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 and analyzed using Image J software.

Observation of exosome morphology

Exosomes (20 μl) were taken and added to an 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 of 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 exosomes was characterized on a H7600 TEM (Hitachi, Japan).

Changes in sex hormone levels

Patients in this research were guaranteed adequate sleep before examination. 4 ml of fasting venous blood from patients in each group were taken as test samples on the examination day. After serum separation, six sex hormones in each group were detected by a TOSOH AIA 1800 automatic chemiluminescence analyzer. The six sex hormones were 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.

Follicular development and ovulation

Follicular development and the ovulation changing situation were assisted by the Gynecology B-ultrasound Department of Heilongjiang Provincial Hospital of Traditional Chinese Medicine. According to the number of days of the menstrual cycle, combined with the patient's self-feeling and the results of the 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 folli-

cle was ≥ 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 a follicle diameter increase of less than 1 mm was detected three consecutive times, the detection was stopped.

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 ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Results

Age and course of disease

Patients in the POF group were aged 24–35 years old, and the course of disease ranged 5–36 months. Patients in the POF + ACU group were aged 25–35 years old, and the course of disease ranged 5–35 months. There was no statistically significant difference in age or course of disease between the two groups.

Western blot

The expression levels of CD9, CD81, NELL2 proteins were detected by western blot, as shown in Figure 1. CD9 and CD81 protein levels in the POF + ACU group were higher than in the POF group, ***$p < 0.001$. The expression levels of CD9, CD81 were higher in the POF + ACU group than the POF group and NC group. The expression level of NELL2 protein was higher in the POF + ACU group than the POF group. NELL2 protein was not found in the NC group. Compared with the POF group, *$p < 0.05$.

### Observation of exosome morphology

Figure 2 shows the electron microscope images of exosomes. It can 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 in the range 50-100 nm, which is in line with the size of exosomes. The secretions of exosomes were obvious in the POF group and POF + ACU group.

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 the POF
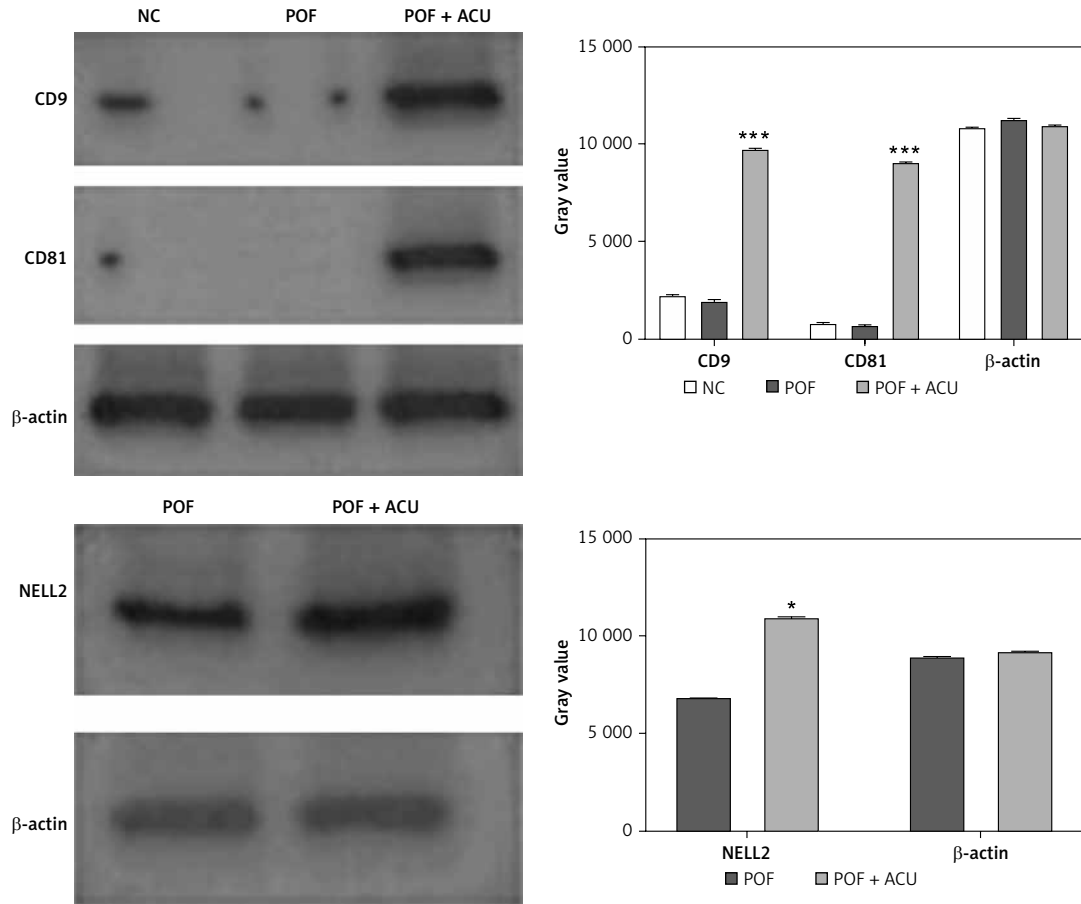
Figure 1. Expression levels of CD9, CD81, NELL2 proteins in different groups

***p < 0.001, *p < 0.05 are considered significant differences compared with POF group. NC – normal control group, POF – premature ovarian failure, ACU – acupoint selection and acupuncture therapy.
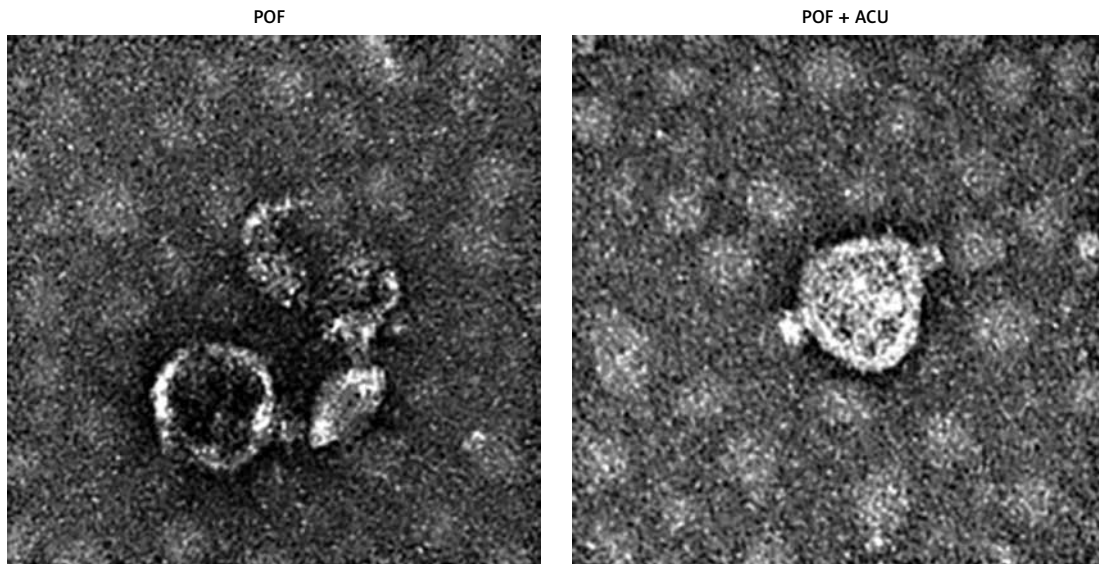
Figure 2. Electron microscope image of exosomes in different group. Scale bar: 100 nm

POF – premature ovarian failure, ACU – acupoint selection and acupuncture therapy.

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

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 the POF group was the smallest, and was significantly different from that of the NC group, $**p < 0.01$. However, after acupuncture treatment, endometrial thickness and maximum follicle diameter increased, showing no significant difference compared with the NC group, and a significant difference compared with the 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 percentage of ovulatory patients was 20%. In the POF + ACU group, anovulation accounted for 30% and ovulation accounted for 70% (Table II). Therefore, it confirms that acupoint selection and acupuncture therapy can relieve premature ovarian failure.

Discussion

The expression levels of CD9 and CD81 in POF and POF + ACU groups 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 the differential centrifugation method consisted of exosomes. Exosomes are cystic vesicles with a 30–100 nm diameter 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 signaling 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 they are 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 characteristic for 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 the 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 cm, 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 not different from those in the normal group. In a word, the experiment effectively

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

Group	T [nmol/l]	E2 [pmol/l]	LH [mIU/ml]	FSH [mIU/ml]	PRL[μIU/ml]	P [nmol/l]
NC	1.99 ±0.53	628.20 ±228.67	5.12 ±1.62	5.20 ±1.20	363.18 ±158.61	2.27 ±1.04
POF	0.94 ±0.26**	292.78 ±130.64**	9.21 ±3.13**	8.48 ±2.26*	353.39 ±160.45	2.36 ±1.07
POF + ACU	1.72 ±0.47##	546.59 ±215.19##	5.91 ±3.10##	6.19 ±1.94#	301.85 ±169.84	2.63 ±1.14

Compared with NC group, $**p < 0.01$, $*p < 0.05$; compared with POF group, $##p < 0.01$, $#p < 0.05$. T – testosterone, E2 – estradiol, LH – luteinizing hormone, FSH – follicular stimulating hormone, PRL – prolactin, P – progesterone, NC – normal control group, POF – premature ovarian failure, ACU – acupoint selection and acupuncture therapy.

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

Parameter	NC	POF	POF + ACU
Endometrial thickness	1.32 ±0.19	0.67 ±0.18**	1.27 ±0.24##
Maximum follicle diameter	1.87 ±0.63	1.04 ±0.32**	1.70 ±0.38##
Ovulation rate (%)	10	20	70

Compared with NC group, $**p < 0.01$; compared with POF group, $##p < 0.01$. NC – normal control group, POF – premature ovarian failure, ACU – acupoint selection and acupuncture therapy.

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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 shown 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 speculate that acupoint selection and acupuncture therapy promoted thalamic secretion of NELL2, and NELL2 may be involved in the regulation of GnRH in sexual development and the treatment of POF by regulating glutamic acid.

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

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

References

1. Liu MY, Qiu Y, Xue ZW, et al. Small extracellular vesicles derived from embryonic stem cells restore ovarian function of premature ovarian failure through PI3K/AKT signaling pathway. Stem Cell Res Ther 2020; 11: 3.
2. Zhang H, Qin FF, Liu A, et al. Electro-acupuncture attenuates the mice premature ovarian failure via mediating PI3K/AKT/mTOR pathway. Life Sci 2019; 217: 169-75.
3. Yang WL, Wang YW, Shan WQ, et al. Progress on the clinical applications of stem cells for premature ovarian failure. Nano Life 2018; 8: 1841003.
4. Coswami D, Conway GS. Premature ovarian failure. Hum Reprod Update 2005; 11: 391-410.
5. Gong P, Zhang MM. Progress in treatment of premature ovarian failure by traditional Chinese medicine and western medicine. Study of Tradit Chin Med 2010; 2: 321-4.
6. Zhuang Y. Effect of supplementing kidney, dispersing liver and promoting blood method combined with artificial cycle methods on the treatment of premature ovarian failure with kidney-deficiency liver-depression and blood stasis type. J Pract Tradit Chin Med 2018; 34: 1074-5.
7. Kong Y, Xu Z, Bu YQ, Du GZ. Application of frequently-used acupoints for POF. J Clin Acupunct Moxibustion 2016; 32: 62-4.
8. Barile L, Moccetti T, Marbán E, Vassalli G. Roles of exosomes in cardioprotection. Eur Heart J 2017; 38: 1372-9.
9. Jung JH, Fu X, Yang PC. Exosomes generated from iP-SC-derivatives: new direction for stem cell therapy in human heart diseases. Circ Res 2017; 120: 407-17.
10. Lucio B, Vincenzo L, Elisabetta C, et al. Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. Cardiovasc Res 2017; 103: 530-41.
11. Boelens MC, Wu TJ, Nabet BY, et al. Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. Cell 2014; 159: 499-513.
12. Zhou W, Fong M, Min Y, et al. Cancer-secreted mi R-105 destroys vascular endothelial barriers to promote metastasis. Cancer Cell 2014; 25: 501-15.
13. Roccaro AM, Antonio S, Patricia M, et al. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. J Clin Invest 2013; 123: 1542-55.
14. Le Q, Jin D, Cheng C, et al. Exosome-transmitted lncARSR promotes sunitinib resistance in renal cancer by acting as a competing endogenous RNA. Cancer Cell 2016; 29: 653-68.
15. Gezer U, Özgür E, Cetinkaya M, Isin M, Dalay N. Long non-coding RNAs with low expression levels in cells are enriched in secreted exosomes. Cell Biol Int 2014; 38: 1076-9.
16. Mohankumar S, Patel T. Extracellular vesicle long non-coding RNA as potential biomarkers of liver cancer. Brief Funct Genomics 2016; 15: 249-56.
17. Zhou YC, Guo WN. Ultrasonic medicine. Scientific and Technical Documentation Press, Beijing 1999; 819.
18. Wang H. Clinical application of urinary luteinizing hormone combined with B-type ultrasound monitoring ovulation. J Yunan Coll Tradit Chin Med 1998; 17: 32.
19. Jeong JK, Kim HR, Hwang SM, Park JW, Lee BJ. Region- and neuronal phenotype-specific expression of NELL2 in the adult rat brain. Mol Cells 2008; 26:186-92.
20. Nelson BR, Matsuhashi S, Lefcort F. Restricted neural epidermal growth factor-like like 2 (NELL2) expression during muscle and neuronal differentiation. Gene Expr Patterns 2002; 2: 7-15
21. Nelson BR, Claes K, Todd V. NELL2 promotes motor and sensory neuron differentiation and stimulates mitogenesis in DRG in vivo. Dev Biol 2004; 270: 322-35.
22. Ha CM, Choi J, Choi EJ, Costa ME, Lee BJ, Ojeda SR. NELL2, a neuron-specific EGF-like protein, is selectively expressed in glutamatergic neurons and contributes to the glutamatergic control of GnRH neurons at puberty. Neuroendocrinology 2008; 88: 199-211.