Original Article

Effects of Electroacupuncture on Expression of PI3K/Akt/Foxo3a in Granulosa Cells from Women with Shen (Kidney) Deficiency Syndrome Undergoing *in vitro* Fertilization-Embryo Transfer*

WU Hai-cui[1,2], ZHANG Jian-wei[1], SUN Zhen-gao[2], XIANG Shan[1], QIAO Yan[1], and LIAN Fang[2]

ABSTRACT   **Objective**: To observe the effects of electroacupuncture (EA) on reproductive outcomes in women with Shen (Kidndy) deficiency syndrome after *in vitro* fertilization-embryo transfer (IVF-ET), and explore the underlying molecular mechanism. **Methods**: Sixty-six infertile patients with Shen deficiency syndrome undergoing IVF-ET were divided into EA or control groups according to a random table, 33 cases in each group. Before undergoing IVF, patients in the EA and control groups received EA therapy and placebo needle puncture, respectively, for 3 menstrual cycles. Shen deficiency syndrome scores were assessed. Other outcome measures included the number of retrieved oocytes and fertilization, high-quality embryo and clinical pregnancy rates. Follicular fluid was collected on the day of oocyte retrieval, and granulosa cell expression of phosphatidylinositide 3-kinases (PI3K), serine-threonine kinase (Akt) and forkhead box O3 (Foxo3a) mRNA were measured by reverse transcribed and quantitative real-time polymerase chain reaction. **Results**: Syndrome scores for pre- versus post-treatments decreased significantly (16.53 ± 1.75 to 8.67 ± 1.61) in the EA group ($P<0.05$), but showed no significant change in the control group (17.18 ± 1.58 to 14.74 ± 1.58). A significant difference in score change was found between the EA and control groups ($P<0.05$). High-quality embryo and clinical pregnancy rates were both increased in the EA group compared with the control group [69.15% (195/282) *vs.* 60.27% (176/292) and 66.67% (22/33) *vs.* 42.42% (14/33), respectively, $P<0.05$]. The fertilization rate was equivalent in EA and control groups. No difference was found in the number of retrieved oocytes between the two groups. Granulosa cell expression levels of PI3K and Akt mRNA were significantly increased in the EA group compared with the control group, while the expression of Foxo3a was reduced (all $P<0.05$). **Conclusions**: For infertile patients with Shen deficiency syndrome undergoing IVF, EA for tonifying Shen as an adjunct treatment may alleviate clinical symptoms and improve the high-quality embryo rate. The EA-induced mechanism may involve regulation of PI3K/Akt/Foxo3a expression in granulosa cells to improve the developmental microenvironment of oocytes and inhibit granulosa cell apoptosis, possibly contributing to the improved clinical pregnancy rate (Registration No. ChiCTR 1800016217).
KEYWORDS   *in vitro* fertilization-embryo transfer, Shen deficiency, electroacupuncture, granulosa cells, PI3K/Akt/Foxo3a

Infertility is a global issue for childbearing couples unable to conceive.[1] The success of assisted reproductive technology (ART) procedures, although offering a treatment option and hope to patients, may be restrained by poor oocyte quality and other challenges.[2] At present, ART treatments may have undesired side effects and a low pregnancy rate, or lead to the development of ovarian hyperstimulation. Improving oocyte quality in infertile patients with Shen (Kidney) deficiency syndrome is an important goal for Chinese medicine (CM) researchers of ART.

"Shen deficiency" is a CM term meaning the body's essential essence is insufficient. From the

theory of CM, Shen deficiency is the main pathogenic factor of infertility. Acupuncture has been used to treat reproductive disorders for thousands of years.[3] Electroacupuncture (EA), a modern therapy derived from traditional acupuncture, has received more attention in recent years.[4,5] Previous studies showed that EA coinciding with an early stage of oocyte recruitment could improve the quality of oocytes and promote embryo development.[6,7] This study investigated the effect of EA on outcomes of *in vitro* fertilization (IVF) for infertile women and explored its mechanism by investigating the expression of PI3K, Akt, and Foxo3a mRNA expression in granulosa cells.

METHODS

Study Design

This was a randomized, placebo-controlled study. The study was approved by relevant health authorities and ethics committees (No. SDSZYYSZ20170216). The trial was registered at the China National Center for clinical trial registration (No. ChiCTR 1800016217).

Diagnosis

Western Medicine Diagnosis

Infertility is defined as failure of a couple of reproductive age to conceive after one year or more with regular coitus and without using contraception.[8] Tubal infertility may be caused by fallopian tube resection or tubal obstruction, confirmed through laparoscopic or hysterosalpingography.

CM Diagnosis

Diagnosis of infertility with Shen deficiency syndrome may include the following primary symptoms: infertility, delayed menarche, heavy menstrual bleeding (>80 mL) or light menstrual bleeding (<5 mL), delayed or irregular menstrual cycle, amenorrhea, sore waist and knees, dizziness and tinnitus.[9] Secondary symptoms may include lusterless, being tired and weary, chills, a cold belly, clear urine, diarrhea, leukorrhagia, and low sexual desire.[10-12] Typical tongue and pulse manifestation: pale tongue with a white coating, and a deep and thin pulse. The diagnosis of Shen deficiency is made if there are 3 or more of the 4 primary symptoms and 2 or more secondary symptoms. Syndrome identification was performed by 2 associate professors independently examining each patient, which were included in the study if the syndrome diagnosis was consistent from both professors.

Chinese Symptom Scoring

According to the 1st to 4th symptom levels (1st: continued symptoms that affect daily life; 2nd: repeated symptoms; 3rd: occasional symptoms; 4th: no symptoms), the primary symptoms scores were 6, 4, 2, and 0, and the secondary symptoms scores were 3, 2, 1, and 0 for the 1st to 4th symptom levels, respectively.

Inclusion Criteria

Criteria for inclusion included married women aged 25–40 years old diagnosed with Shen deficiency syndrome, those diagnosed with tubal infertility, no infertility factor in their male partner, clear communication skills with hospital researchers, and normal range for hormonal test result.

Exclusion Criteria

Criteria for exclusion were that patients (1) had received hormonal therapy in the latest 3 months; (2) could not endure pregnancy because of severe diseases; (3) had severe mental health disorders, acute urogenital system inflammation, or sexually transmitted diseases; (4) had hereditary disease and were not allowed to have a baby, drug abuser or having a harmful addiction, exposed to radiation, toxins, drugs within a period that could cause a malformation. The patients who had received Chinese acupuncture and herbal medicine had to withdraw in this trial.

Subjets

All 66 infertile patients were recruited from the Integrative Medicine Research Centre of Reproduction and Heredity, Affiliated Hospital of Shandong University of Traditional Chinese Medicine from September 2017 to August 2018. The 66 patients were randomly assigned to the EA or control groups using a random number table. All participants gave written consent prior to entering the trial.

The sample size was estimated by the following formula for population rate hypothesis testing in a completely random design:

$$n_1=n_2=\frac{[Z_\alpha\sqrt{2\overline{P}(1-\overline{P})}+Z_\beta\sqrt{P_1(1-P_1)+P_2(1-P_2)}]^2}{(P_1-P_2)^2}$$

P1 and P2 are 0.70, 0.30, respectively, and the estimated value is 31 cases. When the patients were included, 33 cases were included in each group, which

all satisfied the results of sample size estimation. A nurse was in charge of registration and assignment.

Treatment Protocol

Treatment Regimen

Before undergoing the IVF procedure, patients from the EA and control groups received EA therapy and placebo needle puncture, respectively, for 3 menstrual cycles. Acupoints [Zhongji (CV 3), Qihai (CV 6), Guilai (ST 29), Sanyinjiao (SP 6), Hegu (LI 4)] were selected according to the dominating distribution of ovarian nerves, mainly in the abdomen, the inside of the calf and forearm (Th12–L2, S2–S4).[13,14] Disposable single-use sterile stainless steel needles (0.30 mm × 40 mm in size) were inserted to produce local muscle contractions without discomfort. Needles were attached to an electro-acupuncture treatment instrument (HANS-100A, Nanjing Gensun Medical Technology Co. Ltd., China) and stimulated electrically with low-frequency EA (20–25 mA, 2 Hz). Each treatment lasted 25 min and was given every 2 days. Acupuncture points and electrical stimulation were the same for all women in the EA group.

The control group received the placebo acupuncture at the same acupoints. A placebo needle used had the same specification as that for the treatment group, but was cut to 20 mm, and the end section was polished and attached to a piece of rubber. This acupuncture method allowed the patient to have a tactile sensation without puncturing the skin. The needle body was fixed by tape. The electrode line was connected and the needle was left in place for 25 min.

Controlled Ovarian Hyperstimulation

All subjects received controlled ovarian hyperstimulation following a long protocol [gonadotropin-releasing hormone agonist (GnRHa), follicle-stimulating hormone (FSH)-r, human menopausal gonadotrophin (hMG), and human chorionic gonadotrophin (hCG) protocol].

On approximately day 21 (in the luteal phase) of the last menstrual cycle prior to the IVF procedure, all subjects received a single dose of 10,000 IU hCG (Livzon Pharmaceutical Group Inc., Zhuhai, China, batch No. 090515B) after confirmation of ⩾ 3 follicles with a size ⩾18 mm by vaginal ultrasound, endometrium thickness 8–10 mm, and cervical mucus wire drawing ⩾10 cm. About 36 h after hCG

injection, oocyte retrieval (OR) was conducted under transvaginal ultrasound. *In vitro* fertilization was performed and fertilized embryos were cultured *in vitro* for 3 days. Two high-quality embryos were selected and transferred on day 3 after OR. After embryo transfer (ET), luteal phase support was provided with progesterone for up to 14 days when urine or serum hCG tested positive, defined as biochemical pregnancy, or until confirmation of clinical pregnancy, which was defined by at least one intrauterine gestational sac with a fetal heartbeat detected by transvaginal ultrasound 5 weeks after ET.

Syndrome Scoring

According to the standard scoring for the Chinese syndrome of Shen deficiency, the symptoms were scored and recorded on the recruitment day (pre-treatment) and the OR day (post-treatment) to evaluate the effect of EA on the syndrome.

Morphology Grading Standard Maturity

Fertilization rate (%) = sperm number/total oocyte number × 100%. Embryo quality was classified on a scale of 1 to 5 as described by Puissant, et al,[15] and a high-quality embryo was defined as an embryo with a score ⩾3. High-quality embryo rate (%) = high-quality embryo number/blastomere number × 100%.

Collection of Granulosa Cells

Six samples per group were processed within 1 h after collection to avoid post-aspiration cell death. Cells were sequentially washed by Ficoll-Paque (GE Healthcare, Sweden) and then stored at –80 ℃ for long-term storage prior to RNA extraction.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRIzol Reagent (Ambion, Life Technologies, USA). The 260/280 absorbance ratio was measured to verify the purity of RNA. Total RNA was reverse transcribed and quantitative real-time polymerase chain reaction conducted using the TUREscript 1st stand cDNA synthesis kit (Aidlab, Beijing, China). The primers used to amplify cDNA sequences and the fluorescence-labeled probes, all obtained from Lian Chuan Technologies (Hangzhou, China) are listed in Table 1. Analysis of relative gene expression data was performed using the $2^{-\triangle\triangle CT}$ method. $\triangle Ct$ = Ct (target gene) – Ct (reference gene) and $\triangle\triangle Ct = \triangle Ct$ (control

Table 1. Primers Used in Real-Time RT-PCR

Primers	Primer sequence	Size of the fragment (bp)
GAPDH	F: CGGAGTCAACGGATTTGGTC	20
	R: CGGTGCCATGGAATTTGCCA	20
PI3K	F: ACAGGAGAAGAAGGATGAA	19
	R: TGATGAGCAGGGTTTAGA	18
pAKT	F: CTATTGTGAAGGAGGGTTG	19
	R: AAGGTGCCATCATTCTTG	18
Foxo3a	F: GAGTCCATTATCCGTAGTG	19
	R: TTCAAACCAACAACATTCTG	20

group) $-$ $^{\triangle Ct}$ (EA group).

Adverse Reactions and Safety Outcome Measures

Patients were observed and queried for adverse reactions including routine tests of blood, urine and stool, liver and kidndy functions and electrocardiogram during the treatment period.

Statistical Analysis

All experiments were performed 3 times and data were expressed as the mean $\pm$ standard deviation ($\bar{x} \pm s$). Statistical analysis was performed using SPSS 22.0 for Windows. The t-test was used to compare the mean value of the measured data between pre- and post-treatments in the same group. Categorical data were analyzed with the Chi-square criterion. $P<0.05$ was considered to be statistically significant.
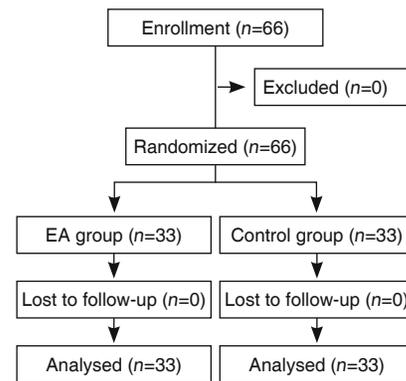
RESULTS

Baseline Characteristics

No significant differences were found for the general characteristics between the two groups ($P>0.05$, Table 2). All 66 patients completed the study without major protocol violations and all were included in the outcome analyses. The flow chart is shown in Figure 1.

Table 2. Patient Characteristics for Two Groups

Group	Case	Age (Year, $\bar{x} \pm s$)	Disease course (Year, $\bar{x} \pm s$)	Medical history (Case)	
				Primary infertility	Secondary infertility
EA	33	31.76 $\pm$ 4.26	3.76 $\pm$ 3.00	18	15
Control	33	33.73 $\pm$ 5.40	3.33 $\pm$ 2.19	14	19

Comparison of Shen Deficiency Syndrome Scores between Groups

Comparing pre-treatment with post-treatment, the Shen deficiency syndrome score was significantly lower at post-treatment in the treatment group

Enrollment (n=66)
↓
Excluded (n=0)
↓
Randomized (n=66)
↓
EA group (n=33) | Control group (n=33)
↓ | ↓
Lost to follow-up (n=0) | Lost to follow-up (n=0)
↓ | ↓
Analysed (n=33) | Analysed (n=33)

Figure 1. Flow Chart of Patients with Shen Deficiency Syndrome Undergoing IVF-ET with EA Treatment

($P<0.05$), while there was no significant difference in the control group. There was a significant difference in the syndrome score change (from pre- to post-treatment) between the treatment and control groups ($P<0.05$, Table 3).

Table 3. Comparison of Syndrome Scores between Groups ($\bar{x} \pm s$)

Group	Case	Time	Syndrome score	Change from pre-treatment
Treatment	33	Pre-treatment	16.53 $\pm$ 1.75	$-8.12 \pm 2.12$$^{\triangle}$
		Post-treatment	8.67 $\pm$ 1.61*	
Control	33	Pre-treatment	17.18 $\pm$ 1.58	-2.43 ± 1.96
		Post-treatment	14.74 $\pm$ 1.58	

Notes: *$P<0.05$, compared with pre-treatment in the same group; $^{\triangle}P<0.05$, compared with the control group

Comparison of Oocyte Retrieval, Fertilization, High-Quality Embryo and Clinical Pregnancy Rates between Groups

The average number of retrieved oocytes per patient was similar between the two groups. The fertilization rate was higher in the EA treatment group but the difference was not statistically significant ($P>0.05$). The high-quality embryo rate was higher in the EA treatment group than the control group ($P<0.05$). The clinical pregnancy rate was 66.67% (22/33) in the EA group, higher than the control group [42.42% (14/33), $P<0.05$, Table 4].

Comparison of Expression of PI3K, Akt and Foxo3a mRNA

Figure 2 shows the expressions of PI3K, Akt and Foxo3a mRNA in granulosa cells from the two groups. Compared with the control group, the expression levels of PI3K and Akt mRNA in the EA group were significantly increased, while the expression of Foxo3a mRNA was reduced (all $P<0.05$).

Table 4.   Comparison of Oocyte Number Per Patient, and Fertilization, High-Quality Embryo and Clinical Pregnancy Rates between Groups

Group	Case	Oocyte number per patient ($\bar{x} \pm s$)	Fertilization rate (%)	High-quality embryo rate (%)	Clinical pregnancy rate (%)
EA	33	11.42 ± 6.62	79.05 (298/377)	69.15 (195/282)*	66.67 (22/33)*
Control	33	12.24 ± 6.86	74.51 (301/404)	60.27 (176/292)	42.42 (14/33)

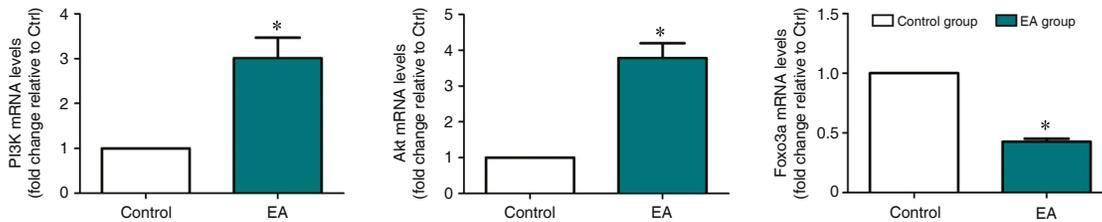Note: *P<0.05, compared with the control group.

Figure 2.   Expression of PI3K (A), Akt (B) and Foxo3a (C) mRNA in Two Groups
Note: *P<0.05, compared with the control group.

Adverse Reaction

No obvious adverse reactions were found in either group during the entire study duration. Routine tests of blood, urine and stool, liver and kidney functions and electrocardiogram found normal range results after treatment.

DISCUSSION

Shen governing reproduction, one of the classic CM theories, is a summary describing understanding of human reproductive function, physiology and pathology. The production of reproductive essence depends on the congenital essence of Shen. The essence of Shen is the guarantee of the normal growth and development of follicles, and a follicular developmental disorder is believed to be mostly caused by Shen insufficiency. The development and application of modern ART has expanded the connotation of "Shen governing reproduction". Descriptions of the sperm, egg, and embryo make the theory more specific and visualized. The quality and developmental potential of sperm, eggs, and embryos are inseparable from the characteristics of parents.

Acupuncture, one of the main treatment methods of CM, has been investigated in long-term clinical practice. Because of its advantages, such as beneficial therapeutic effects with small side effects, and convenience, it has been increasingly accepted by patients in more countries. EA has developed from traditional acupuncture, and the needles can deliver quantifiable micro-currents close to human bio-electricity, combining two kinds of stimulation via needle and electricity to treat diseases.[16] Research

on acupuncture in the field of assisted reproduction is currently expanding.[17]

The basic functional unit of the ovary is the follicle, which begins to develop in the embryonic phase. Folliculogenesis requires a carefully orchestrated cross-talk between the oocyte and the surrounding somatic cells. However, only a small proportion of follicles mature to ovulation in the lifetime of women, and most follicles are lost during the developmental path, mainly due to apoptosis and autophagy of granulosa cells.[18] In general, follicular atresia is a physiological process that maintains ovarian function, but excessive atresia can lead to disorders of follicular development.[19] Granulosa cells secrete paracrine and autocrine factors, which include growth factors, cytokines, and intracellular proteins, that play key roles in the maturation, meiosis and energy supply of the oocyte.[20,21] Studies have shown that apoptosis of granulosa cells via multiple signaling pathways is an important mediator of follicular atresia.

PI3K is a key member of the growth factor superfamily signaling pathway, and its downstream signaling molecule is Akt. The Akt signaling pathway is mainly responsible for the transmission of biological information initiated by PI3K.[22] Akt plays an important role in various biological processes, such as cell metabolism, cell cycle regulation, and cell growth and apoptosis. Studies found that the PI3K/Akt signaling pathway mediates oocyte apoptosis and that the forkhead transcription factor, Foxo3a, is a key regulator controlling early follicle development in neonatal rodent ovaries.[23,24] Accelerated initiation of

follicular development occurred in FoxO3a-/- mice, which resulted in premature follicular depletion and ovarian failure.[25] Matsuda, et al[25] also reported that FoxO3a was expressed in porcine follicles and induced during granulosa cell apoptosis. Genetic and biochemical studies have shown that FOXOs are key downstream substrates of the PI3K/Akt signaling pathway in mammalian cells.[25] Wang, et al[27] found that BimEL induced porcine granulosa cell apoptosis during follicular atresia, and its expression was regulated by FSH via the PI3K/Akt/FoxO3a pathway.

The selection of acupoints was carried out according to the affiliated meridian. The CV 3 is believed to have the effect of tonifying Shen and regulating the Meridian of Chong-Ren. The CV 6 is believed to tonify the Shen and regulate the qi and blood. The SP 6 is the intersection of the foot three yin, and this acupuncture may benefit the Gan and Shen, strengthening the Pi (Spleen) and Wei (Stomach), and regulating the qi. The ST 29 is the acupoint of Wei in the small abdomen, and this acupuncture may play a role in warming and reducing coldness, and regulating menstruation. The LI 4 is the original point of the Large Intestine, and this acupuncture may regulate qi and blood.

Our study shows that the high-quality embryo and clinical pregnancy rates were both elevated in the EA group compared with the control group. Expression levels of PI3K and Akt mRNA were significantly increased in the EA group compared with control group, while the expression of Foxo3a was reduced. One possible underlying mechanism by which EA improves embryo quality and the subsequent pregnancy rate may involve the EA-induced central secretion of β-endorphin, which further affects the GnRH pulse generator to stimulate the secretion of gonadotrophins and steroids.[28-30] These overall effects may lead to over-repression of PI3K/Akt in ovarian granulosa cells and reduction of abnormal follicular atresia, thereby stimulating the developmental potential of oocytes. For infertile patients with Shen deficiency syndrome undergoing IVF, EA for tonifying the Shen as an adjunct treatment may alleviate clinical symptoms and improve the high-quality embryo rate. The mechanism may involve regulation of PI3K/Akt/Foxo3a expression in granulosa cells to improve the developmental microenvironment of oocytes and inhibit granulosa cell apoptosis, leading to improvement in the clinical pregnancy rate.

EA may improve the quality of oocytes and embryos in infertile patients with Shen deficiency. However, the current study examined a small number of patients, and there is a need for verification using a randomized control trial with a larger sample size to further investigate CM theory. Considerable research is needed to allow more people to accept acupuncture and to explore the molecular mechanisms behind the efficacy of acupuncture.

Conflicts of Interest

The authors declare that they have no conflict of interest with the contents of this manuscript.

Author Contributions

Wu HC designed the research, collected patient information and wrote the manuscript. Zhang JW treated the patients, collected the information and critically revised the manuscript. Sun ZG collected the information and did statistical analysis. Xiang S and Qian Y collected ovarian granulosa cells. Lian F critically revised the manuscript. All the authors have seen and approved the final version.

Acknowledgements

REFERENCES

1. Xia JF, Inagaki Y, Zhang JF,Wang L, Song PP. Chinese medicine as complementary therapy for female infertility. Chin J Integr Med 2017;23:245-252.

2. Bjelica A, Nikolic S. Development and achievements of assisted reproductive technology. Med Pregled 2015;68:353-357.

3. Zhou J, Qu F. Treating gynaecological disorders with traditional Chinese medicine: a review. Afr J Tradit Complement Alternat Med 2009;6:494-517.

4. Johansson J, Redman L, Veldhuis PP, Sazonova A, Labrie F, Holm G, et al. Acupuncture for ovulation induction in polycystic ovary syndrome: a randomized controlled trial. Am J Physiol Endocrinol Metabol 2013;304:E934-E943.

5. Qian Y, Xia XR, Ochin H, Huang C, Gao C, Gao L, et al. Therapeutic effect of acupuncture on the outcomes of *in vitro* fertilization: a systematic review and meta-analysis. Arch Gynecol Obstetr 2017;295:543-558.

6. Li J, Cui W, Sun W, Zhang QY, Guan Q. Effect of electro-acupuncture on the spindle and oocytes quality in patients with PCOS. Chin J Integr Tradit West Med (Chin) 2015;35:304-309.

7. Jo J, Lee YJ. Effectiveness of acupuncture in women with polycystic ovarian syndrome undergoing *in vitro* fertilisation or intracytoplasmic sperm injection: a systematic review and meta-analysis. Acupunct Med 2017;35:162-170.

8. Xie X, Gou W, eds. Obstetrics and gynecology. 8th ed. Beijing: People's Medical Publishing House; 2013;369.

9. Munro MG, Critchley HO, Broder MS, Fraser IS, FIGO Working Group on Menstrual Disorders. FIGO classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nongravid women of reproductive age. Int J Gynaecol Obstet 2011;113:3-13.

10. Zheng XY, ed. Guidelines for Clinical Research of New Chinese herbs for Treatment of Irregular Menstruation, Amenorrhea and Infertility. 2002 ed. Beijing: China Medical Science and Technology Press; 2002:388-390.

11. Diagnostic and Treatment Standards for TCM Diseases. Beijing: State Administration of Traditional Chinese Medicine;1994:258-259.

12. Tan Y, ed. Gynecology in traditional chinese medicine. 2nd ed. Beijing: China Traditional Chinese Medicine Press; 2007:324-332.

13. Jedel E, Labrie F, Oden A, Holm G, Nilsson L, Janson PO, et al. Impact of electro-acupuncture and physical exercise on hyperandrogenism and oligo/amenorrhea in women with polycystic ovary syndrome: a randomized controlled trial. Am J Physiol Endocrinol Metabol 2011;300:E37-E45.

14. Stener-Victorin E, Jedel E, Janson PO, Sverrisdottir YB. Low-frequency electroacupuncture and physical exercise decrease high muscle sympathetic nerve activity in polycystic ovary syndrome. Am J Physiol Regul Integr Comparat Physiol 2009;297:R387-R395.

15. Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F. Embryo scoring as a prognostic tool in IVF treatment. Human Reproduct 1987;2:705-708.

16. Han JS, Ho YS. Global trends and performances of acupuncture research. Neurosc Biobehav Rev 2011;35:680-687.

17. Qu F, Li R, Sun W, Lin G, Zhang R, Yang J, et al. Use of electroacupuncture and transcutaneous electrical acupoint stimulation in reproductive medicine: a group consensus. J Zhejiang Univ Sci B 2017;18:186-193.

18. Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. J Reproduct Develop 2012;58:44-50.

19. Tiwari M, Prasad S, Tripathi A, Pandey AN, Ali I, Singh AK, et al. Apoptosis in mammalian oocytes: a review. Apoptosis 2015;20:1019-1025.

20. Almeida CP, Ferreira MCF, Silveira CO, Campos JR, Borges IT, Baeta PG, et al. Clinical correlation of apoptosis in human granulosa cells—a review. Cell Biol Int 2018;42:1276-1281.

21. Uyar A, Torrealday S, Seli E. Cumulus and granulosa cell markers of oocyte and embryo quality. Fertil Steril 2013;99:979-997.

22. Brazil DP, Yang ZZ, Hemmings BA. Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem Sci 2004:29:233-242.

23. Liu H, Luo LL, Qian YS, Fu YC, Sui XX, Geng YJ, et al. FOXO3a is involved in the apoptosis of naked oocytes and oocytes of primordial follicles from neonatal rat ovaries. Biochem Biophys Res Commun 2009;381:722-727.

24. Hosaka T, Biggs WH 3rd, Tieu D, Boyer AD, Varki NM, Cavenee WK, et al. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A 2004;101(9):2975-2980.

25. Matsuda F, Inoue N, Maeda A, Cheng Y, Sai T, Gonda H, et al. Expression and function of apoptosis initiator FOXO3 in granulosa cells during follicular atresia in pig ovaries. J Reproduct Develop 2011;57:151-158.

26. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 2003;301:215-218.

27. Wang XL, Wu Y, Tan LB, Tian Z, Liu JH, Zhu DS, et al. Follicle-stimulating hormone regulates pro-apoptotic protein Bcl-2-interacting mediator of cell death-extra long (BimEL)-induced porcine granulosa cell apoptosis. J Biol Chem 2012; 287:10166-10177.

28. Stener-Victorin E, Waldenstrom U, Wikland M, Nilsson L, Hägglund L, Lundeberg T. Electro-acupuncture as a peroperative analgesic method and its effects on implantation rate and neuropeptide Y concentrations in follicular fluid. Human Reproduc 2003;18:1454-1460.

29. Chang R, Chung PH, Rosenwaks Z. Role of acupuncture in the treatment of female infertility. Fertil Steril 2002;78:1149-1153.

30. Bedwell C, Dowswell T, Neilson JP, Lavender T. The use of transcutaneous electrical nerve stimulation (TENS) for pain relief in labour: a review of the evidence. Midwifery 2011;27:e141-e148.