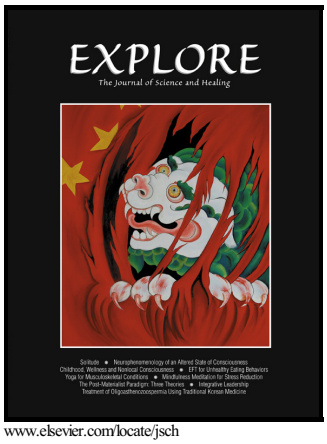

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TRANSCUTANEOUS ELECTRICAL ACUPOINT STIMULATION IMPROVES THE OUTCOMES OF IN VITRO FERTILIZATION: A PROSPECTIVE, RANDOMIZED AND CONTROLLED STUDY

Fan Qu, Fang-Fang Wang, Yan Wu, Jue Zhou, Nicola Robinson, Paul J. Hardiman, Jie-Xue Pan, Yi-Jing He, Yu-Hang Zhu, Han-Zhi Wang, Xiao-Qun Ye, Ke-Lin He, Long Cui, Hong-Li Zhao, Ying-Hui Ye

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Transcutaneous electrical acupoint stimulation improves the outcomes of in vitro fertilization: A prospective, randomized and controlled study.

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Transcutaneous electrical acupoint stimulation improves the outcomes of in vitro fertilization: A prospective, randomized and controlled study.

Abstract

Objectives: To explore whether transcutaneous electrical acupoint stimulation (TEAS) can improve the outcomes of in vitro fertilization (IVF).

Design: A prospective, randomized and controlled study.

Setting: IVF center in a university hospital.

Participants: Four hundred and eighty-one infertile patients with bilateral tubal blockage who were referred for IVF. Patients were randomized into four groups.

Intervention: TEAS was administered for 30 min, respectively at 24 h before TVOR and 2h before ET. The acupoints included SP10 (Xuehai, bilateral), SP8 (Diji, bilateral), LR3 (Taichong, bilateral), ST36 (Zusanli, bilateral), EX-CA1 (Zigong, bilateral), RN4 (Guanyuan), PC6 (Neiguan, bilateral) and RN12 (Zhongwan). Based on different frequencies of TEAS, patients were grouped into a TEAS-2 Hz group, a TEAS-100 Hz group and a TEAS-2/100 Hz group. Patients in the control group only received routine IVF treatment and no TEAS were applied on them.

Primary and secondary outcome measures: The number of mature oocytes, normally fertilized oocytes and good-quality embryos were used to evaluate oocyte developmental competence of the patients. Data of clinical pregnancy rate (CPR), implantation rate (IR) and live birth rate (LBR) were also obtained. The levels of neuropeptide Y (NPY), transforming growth factor alpha and granulocyte colony-stimulating factor in the follicular fluids were measured with enzyme-linked immunosorbent assay (ELISA).

Results: No significant differences were found between the control, TEAS-2 Hz, TEAS-100 Hz and TEAS-2/100 Hz groups on the numbers of metaphase II oocytes, normally fertilized zygotes, early cleavage embryos or good quality embryos (P>0.05). However, the CPR, IR and LBR of the TEAS-2/100 Hz group were significantly higher than those of the other groups, respectively (P<0.05). The NPY levels in the follicular fluids of TEAS-2/100 Hz group were significantly higher than those of the other groups (P<0.05).

Conclusion: TEAS using a frequency of 2/100 Hz could help to improve the IVF outcomes partly by increasing NPY levels in the follicular fluids.

Key words: Transcutaneous electrical acupoint stimulation (TEAS), in vitro fertilization (IVF), clinical pregnancy rate (CPR), implantation rate (IR), live birth rate (LBR).[1]

TEAS: Transcutaneous electrical acupoint stimulation; IVF: In vitro fertilization; CPR: Clinical pregnancy rate; IR: Implantation rate; LBR: Live birth rate; NPY: Neuropeptide Y; ELISA: Enzyme-linked immunosorbent assay; HSG: Hysterosalpingogram; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; E2: Estradiol; TT: Total testosterone; PRL: Prolactin; ART: Assisted reproductive technology; WHO: World Health Organization; CONSORT: Consolidated Standards of Reporting Trials; STRICTA: Standards for Reporting Interventions in Clinical Trials of Acupuncture; TVOR: Trans-vaginal oocyte retrieval; ET: Embryo transfer; COH: Controlled ovarian hyperstimulation; HCG: Human chorionic gonadotropin; TGF-alpha: Transforming growth factor alpha; G-CSF: Granulocyte colony-stimulating factor; GnRH: Gonadotropin-releasing hormone; CSF3: Colony-stimulating factor 3.

Introduction

In vitro fertilization (IVF) is an important treatment for infertility, and over 300,000 cycles of IVF are performed each year in Europe.[1] In recent years, although some equipment, techniques and drug therapies have been developed for improving the outcomes of IVF, the progress in developing safe and effective therapies has been limited.[2,3] Many infertile women have now turned to complementary and alternative medicine as an adjuvant therapy to improve the outcomes when they undergo IVF treatment.[4] Acupuncture, as an effective non-pharmacological therapy, has been chosen by many infertile couples undergoing infertility treatment.[3,5-8] It is reported that "fertility problems" is the second most common health condition for which people choose acupuncture treatment in the UK.[9]

Acupuncture could improve the reproductive outcomes of IVF.[10,11] However, in the traditional acupuncture treatment, patients' skin is pierced with the needles, which can lead to certain adverse effects and can affect acupuncture utilization. One study on the incidence of adverse effects during acupuncture therapy showed hemorrhage was the main side effect with a rate of 2.9%.[12] Another study found that bleeding (6.1%), pain (1.7%) and vegetative symptoms (0.7%) were common adverse effects of acupuncture.[13] These side effects may be reasons for dropout acupuncture in clinical studies[14] or for decreased utilization in clinical practice.

Transcutaneous electrical acupoint stimulation (TEAS) uses self-adhesive electrodes instead of the needles to stimulate acupoints with electric current.[15] Different frequencies of TEAS can generate different biological effects. Because of the advantages of noninvasiveness and painlessness, TEAS recently has been a popular treatment in many conditions, including pain,

depression, cancer, and withdrawal syndrome.[16-19] The present prospective, randomized and controlled trial was designed to investigate whether TEAS can improve IVF outcomes for infertile women with bilateral tubal blockage and to explore the possible underlying mechanism.

Methods

Subjects

Over a five-month period (May 1, 2014 to September, 30, 2014), we screened 657 infertile women with bilateral tubal blockage who were referred to a university hospital for IVF. Among the patients screened, 162 women did not meet the inclusion criteria. Eleven women did not consent to treatment, and three women were excluded for other reasons. Finally, 481 infertile women with bilateral tubal blockage were included in the study. The subjects were randomized into a control group, a TEAS-2 Hz group, a TEAS-100 Hz group or a TEAS-2/100 Hz group using computer generated randomization that randomized numbers into four groups with a proportion of 1:1:1:1. The random allocation sequence was concealed until the interventions were assigned. A nurse enrolled the participants and assigned them to their groups. The IVF clinicians and laboratory staff were blinded to the group assignment. Ethical permission to conduct the study was obtained from the Institutional Review Board of Reproductive Medicine. The aim and methodology of the study were explained to the patients in detail. Voluntary participation was requested and informed written consents were obtained from all of the participants.

The subjects included in the study were infertile women with bilateral tubal blockage who were referred to our department for IVF. The bilateral tubal blockage was determined by hysterosalpingogram (HSG). Subjects were otherwise healthy women with regular menstrual cycles and normal sex hormone levels [luteinizing hormone (LH): 3.80-20.00 IU/L; follicle stimulating hormone (FSH): 3.80-17.20 IU/L; estradiol (E_2): 100.00-275.00 pmol/L; total testosterone (TT): 0.30-3.00 nmol/L; prolactin (PRL): 5.00-30.00 ng/mL] and no other pelvic pathology.. No structural abnormalities of uterus and ovaries were found by vaginal ultrasound or laparoscopy. None of the women had received salpingectomy or assisted reproductive technology (ART) therapy before. We excluded patients with neurologic, mood or psychiatric disorders; patients who were taking any tranquilizers; patients who were receiving acupressure or acupuncture therapy; patients with a history of smoking or drinking; and patients who underwent preimplantation genetic diagnosis or preimplantation genetic screening. All the partners of the women had normal spermiograms and sperm morphology according to the World Health Organization (WHO) criteria.

The present study adheres to the Consolidated Standards of Reporting Trials (CONSORT) statement,[20] and Standards for Reporting Interventions in Clinical Trials of Acupuncture (STRICTA).[21,22] The TEAS treatment was performed by two professional acupuncturists. The TEAS protocol was developed based on the clinical experience in our hospital, the literature and consultation with six experts in Chinese medicine. The TEAS devices (HANS-100B) used in the study were provided at a regular price without any direct involvement of the manufacturer (Jisheng Medical Technology Co., Ltd, Nanjing, China).

Group and administration

Thirty-minute TEAS were administered at 24 hours before trans-vaginal oocyte retrieval (TVOR) and 2 hours before embryo transfer (ET) respectively. Acupoints of SP10 (Xuehai, bilateral), SP8 (Diji, bilateral), LR3 (Taichong, bilateral), and ST36 (Zusanli, bilateral) were chosen before TVOR, and acupoints of EX-CA1 (Zigong, bilateral), RN4 (Guanyuan), PC6 (Neiguan, bilateral) and RN12 (Zhongwan) were chosen before ET. TEAS was applied through self-adhesive surface electrodes. The standard electrodes affiliated to the TEAS device were applied to the denuded skin and the intensity was set strong enough to elicit visible muscle contraction.

Generally, 2 Hz is chosen as a representative for low frequency, 100 Hz for high frequency, and 2/100 Hz for alternate low and high frequencies. According to the different frequencies of TEAS, patients were divided into three TEAS groups: TEAS-2 Hz group, TEAS-100 Hz group and TEAS-2/100 Hz group. In each group, the frequency of TEAS used before TVOR and ET was consistent. Patients in the control group followed the routine procedure of IVF treatment and no TEAS was applied. According to our pilot experiment, two treatments (before TVOR and ET) were sufficient to provide therapeutic benefits. Since patients could know whether they received treatment and detect the frequencies of TEAS, they were not blinded.

Collection of the follicular fluids

The long agonist protocol for controlled ovarian hyperstimulation (COH) was used as previously described.[23] Briefly, COH was performed by administration of recombinant follicle stimulating hormone (r-FSH, Gonal-F, Serono International S.A., Geneva, Switzerland) after pituitary suppression with triptorelin (Serono) started in the midluteal phase of the

preceding cycle. The dosages of gonadotrophins were individualized according to E_2 levels and trans-vaginally ultrasonic measurements of the follicles. When at least three follicles had reached the diameters of 16–18 mm, ovulation was induced by administration of 10,000 IU human chorionic gonadotropin (HCG, Libao Biochemistry Co., Zhuhai, China). Trans-vaginal oocyte aspiration was performed with ultrasound guidance under general anesthesia 36 h after injection of HCG. The follicular fluids were sampled by trans-vaginal ultrasound-guided puncture and aspiration of the follicles with diameters of 16–18 mm. Those follicular fluids samples that did not contain any visible blood contamination were used in this study. The follicular fluids samples were immediately centrifuged for 10 min at 550g. The supernatants were stored at -80°C until analysis.

Index and method

The oocytes' developmental competence was evaluated by obtaining the numbers of metaphase II oocytes, normally fertilized zygotes and good quality embryos. The zygotes were checked 17-20 hours after fertilization, for two pronuclei (2PN) and polar bodies to assess fertilization. The good quality embryo was defined as: 7-9 cells on Day 3, <10% fragmentation and equally sized mononucleated blastomeres. The clinical pregnancy rate (CPR), the primary outcome, was defined as the presence of at least one gestational sac or fetal heartbeat, confirmed by trans-vaginal ultrasound. Implantation rate (IR, defined as the number of gestational sacs per number of transferred oocytes) and live birth rate (LBR, defined as the presence of a baby born alive after 24 weeks gestation) for all the patients were also obtained. The levels of neuropeptide Y (NPY), transforming growth factor alpha (TGF-alpha) and granulocyte colony-stimulating factor (G-CSF) in the follicular fluids were

measured with enzyme-linked immunosorbent assay (ELISA, RayBio, GA, U.S.A).

Sample size and data analysis

The sample size was analyzed using Power Analysis and Sample Size (PASS 11.0). According to the records, a CPR of 0.38 was estimated in the control population and a CPR of 0.40, 0.42 and 0.57 was respectively estimated in TEAS-2 Hz population, TEAS-100 Hz population, and TEAS-2/100 Hz population. A sample size of 393 achieves 90% power to detect an effect size (W) of 0.19 using a 3 degrees of freedom Chi-Square Test with a significance level (alpha) of 0.05.

Data were analyzed by an independent statistician using Statistical Package for Social Sciences (SPSS 19.0 for Windows). One-way analysis of variance (ANOVA) was used to evaluate statistical significances of continuous data. Chi-square test was used to compare categorical data. For all analyses, significance was set at $P<0.05$.

Results

The sample population and baseline characteristics

After the included 481 infertile women with bilateral tubal blockage were randomized, the control group had 120 cases, TEAS-2 Hz group 121 cases, TEAS-100 Hz group 119 cases and TEAS-2/100 Hz group 121 cases. The ET treatment was conducted three days after TVOR. In the statistical analysis, 11, 13, 8 and 7 cases respectively in the control group, TEAS-2 Hz group, TEAS-100 Hz group and TEAS-2/100 Hz group were excluded, as these patients did not complete ET. As shown in Table 1, there were no significant differences between the control group, TEAS-2 Hz group, TEAS-100 Hz group and TEAS-2/100 Hz group on all the

baseline characteristics (*P*>0.05).

Table 1. The baseline characteristics of the participants

Items	Control group (n=109)	TEAS-2 Hz group (n=108)	TEAS-100 Hz group (n=111)	TEAS-2/100 Hz group (n=114)
Ages (years)	29.81±6.17	31.22±5.92	30.44±5.82	31.16±6.09
BMI (kg/m^2)	21.53±6.28	22.97±6.59	21.77±5.98	23.14±6.55
Duration of infertility (year)	6.49±4.17	5.84±3.99	6.30±4.01	5.92±4.49
Day-3 LH(nmol/L)	5.65±2.83	6.12±3.04	5.97±2.96	6.25±3.39
Day-3 FSH (nmol/L)	7.42±3.15	6.97±2.82	7.08±3.11	7.33±2.90
Day-3 TT(pmol/L)	1.41±0.08	1.41±0.11	1.42±0.09	1.43±0.11
Day-3 E2(pmol/L)	157.81±49.24	146.12±43.89	150.97±46.25	155.67±45.04
Cycle length (days)	33.54±8.51	34.92±7.92	32.87±8.53	35.73±8.82
rFSH administered (IU)	2409.36±786.73	2327.05±801.05	2419.55±835.67	2431.18±822.17
Induction length (days)	9.98±2.58	9.82±2.37	10.32±2.89	10.17±2.74
Number of oocytes	15.32±7.05	14.87±6.98	15.02±6.73	14.58±7.67
Number of follicles >14mm	13.55±5.64	12.98±6.02	13.84±6.29	13.22±5.83
Education level				
High	71 / 109 (65.14%)	68 / 108 (62.96%)	70 / 111(63.06%)	73 / 114 (64.04%)
Medium	30 / 109 (27.52%)	28 / 108 (25.93%)	31 / 111 (27.93%)	29 / 114 (25.44%)
Low	8 / 109 (7.34%)	12 / 108 (11.11%)	10 / 111 (9.01%)	12 / 114 (10.53%)

Note: Data were shown as mean ± S.D. TEAS: transcutaneous electrical acupoint stimulation; BMI: body mass index; Day-3: the 3rd day of spontaneous menstrual cycle; LH: luteinizing hormone; FSH: follicle stimulating hormone; TT: total testosterone; E2: estradiol; r-FSH: recombinant FSH.

The oocytes developmental competence

As shown in Table 2, there were no significant differences between the control, TEAS-2 Hz, TEAS-100 Hz and TEAS-2/100 Hz groups on the numbers of metaphase II oocytes, normally fertilized zygotes or good quality embryos (*P*>0.05).

Table 2: The oocytes developmental competence and the outcomes of in vitro fertilization.

Items	Control group (n=109)	TEAS-2 Hz group (n=108)	TEAS-100 Hz group (n=111)	TEAS-2/100 Hz group (n=114)
Number of metaphase II oocytes	11.67±4.19	11.85±4.78	10.95±4.34	11.21±4.59
Number of normally fertilized zygotes	7.62±5.73	8.04±6.11	7.90±5.88	7.48±6.02
Number of good quality embryos	3.49±2.23	3.31±2.19	3.62±2.05	3.54±1.99
Clinical pregnancy rate (%)	46 / 109 (42.20%)	46 / 108 (42.59%)	47 / 111 (42.34%)	64 / 114 (56.14%) * # $
Implantation rate (%)	54 / 217 (24.88%)	55 / 219 (25.11%)	53 / 223 (23.77%)	82 / 231 (35.50%) * # $
Live birth rate (%)	38 / 109 (34.86%)	38 / 108 (35.19%)	39 / 111 (35.14%)	55 / 114 (48.25%) * # $

Note: Data were shown as mean ± S.D. *P<0.05, compared with the control group; #P<0.05 compared with the TEAS-2 Hz group; $$P$<0.05 compared with the TEAS-100 Hz group. TEAS: transcutaneous electrical acupoint stimulation.

IVF outcomes

As shown in Table 2, the CPR, IR and LBR of TEAS-2/100 Hz group were significantly higher than those of the other three groups respectively (P<0.05). No significant differences existed between the control, TEAS-2 Hz and TEAS-100 Hz groups on the CPR, IR or LBR (P>0.05) (Table 2).

NPY, TGF-alpha and G-CSF levels in the follicular fluids

As shown in Table 3, the NPY levels in the follicular fluids of the TEAS-2/100 Hz group were significantly higher than those of the other three groups respectively (P<0.05). In the TEAS-100 Hz group, the NPY levels in the follicular fluids were significantly higher than those of the control and the TEAS-2 Hz groups (P<0.05) and no marked differences existed between the control and the TEAS-2 Hz groups (P>0.05). There were no significant

differences between all the groups on the TGF-alpha or G-CSF levels in the follicular fluids (P>0.05).

Table 3: The levels of neuropeptide Y, transforming growth factor alpha and granulocyte colony-stimulating factor in the follicular fluids.

Items	Control group (n=109)	TEAS-2 Hz group (n=108)	TEAS-100 Hz group (n=111)	TEAS-2/100 Hz group (n=114)
NPY(ng/ml)	23.70±7.95	25.66±8.19	34.07±9.10*#	49.70±13.53*#$
TGF-alpha (pg/ml)	29.13±6.38	30.61±7.23	30.04±6.76	29.93±6.42
G-CSF (pg/ml)	84.71±12.96	85.32±13.08	83.76±12.88	85.07±13.19

Note: Data were shown as mean ± S.D. *P<0.05, compared with the control group; #P<0.05 compared with the TEAS-2 Hz group; $$P$<0.05 compared with the TEAS-100 Hz group. TEAS: transcutaneous electrical acupoint stimulation; NPY: neuropeptide Y; TGF-alpha: transforming growth factor alpha; G-CSF: granulocyte colony-stimulating factor.

Adverse events

No side effects of the treatment were reported in any of the groups during the research and the follow-up period.

Discussion

In the present study, 481 infertile patients with bilateral tubal blockage referred for IVF were included to investigate whether TEAS can improve IVF outcomes. Patients were divided into four groups: TEAS-2 Hz group, TEAS-100 Hz group, TEAS-2/100 Hz group and control group. We found the CPR, IR, LBR and NPY levels of the TEAS-2/100 Hz group were significantly higher than those of the other groups. However, there were no significant differences of the numbers of metaphase II oocytes, normally fertilized zygotes, early cleavage embryos and good quality embryos among the four groups.

TEAS is a new technique integrating traditional electro-acupuncture and new knowledge from advancing science. It can activate nerve endings and generate action potentials by

electrical stimulation. These signals, transmitted to spinal cord and brain, appear to stimulate the central nervous system to generate specific chemical substances to produce physiological effects. [24,25] Effective frequency ranges from 2 Hz to 100 Hz for TEAS, and stimulation with different frequencies may generate totally different biological effects.[26] Previous studies showed different frequencies of TEAS could generate totally different biological effects: low-frequency (2 Hz) electrical stimulation promoted the release of enkephalins, endorphins and endomorphin in the brain to interact with the μ- and δ-opioid receptors; high-frequency (100 Hz) promoted the release of dynorphin in spinal cord to interact with the κ opioid receptors; and alternate low and high frequencies (2/100 Hz) promoted the release of all four kinds of endogenous opioid peptides.[27] TEAS treatment was found to improve CPR in the patients with decreased ovarian reserve during IVF.[28] In the present study, we found TEAS using a frequency of 2/100 Hz significantly improved the CPR, IR and LBR of the IVF women.

The impact of acupuncture and electro-acupuncture on the clinical outcomes of IVF has been well studied. Positive effects have been shown in modulating the ovarian blood flow and the functions of the hypothalamus-pituitary-ovary system.[29-31] One randomized, prospective, controlled clinical study found that clinical pregnancy rate and ongoing pregnancy rate more than doubled by luteal-phase acupuncture.[32] Another study showed acupuncture on the day of ET increased clinical pregnancy and ongoing pregnancy rates by about 50%.[11] However, because the acupoints, acupuncturists, patients and treatment time in these studies were varied, it was difficult to compare acupuncture with TEAS.

For patients who are unwilling to receive invasive or painful acupuncture treatment, TEAS is

potentially a good choice. In the TEAS treatment, the skin electrodes are placed on the patients' acupoints, instead of piercing the skin with the traditional acupuncture needles. TEAS can also increase the reproducibility of the acupuncture-like stimulation.[24,25] Compared with acupuncture, TEAS is easier to use: patients can even potentially use TEAS on their own under the guidance of acupuncturists. We did not find any adverse events in any group during the research and the follow-up period. This indicates TEAS is a safe intervention to improve the outcomes of IVF.

In the study, the levels of NPY, TGF-alpha and G-CSF in the follicular fluids of the IVF patients were measured to explore the potential mechanism underlying TEAS improving the outcomes of IVF. NPY may represent a link between nutrition and reproduction at the level of the central nervous system,[33] and may serve as one of the molecular signlas integrating the control of metabolism and reproduction.[34] NPY may play a role in positive regulation of gonadotropin-releasing hormone (GnRH) throughout the neural axis and also up-regulate the LH cells in the pituitary.[35] NPY is also a key factor during the follicular growth, ovulation and regulating the functions of the hypothalamic-pituitary-ovarian axis.[36-41] Electro-acupuncture was found to significantly improve the levels of NPY in the follicular fluids of the patients.[42] The result is consistent with the present study.

TGF-alpha plays an important role in regulating the growth of follicles and the maturation of oocyte.[43-45] G-CSF, also known as colony-stimulating factor 3 (CSF3), is a biomarker of human oocyte developmental competence for embryo implantation.[46-49] It has a higher discriminatory power to predict the ongoing pregnancy in multivariate logistic regression analysis for G-CSF levels in the follicular fluids, compared with embryo

morphology.[50] It was found that the quality of porcine oocyte and embryonic viability were significantly improved when adding the human recombination G-CSF into porcine in vitro maturation medium.[51] In the present study, the NPY levels in the follicular fluids of the TEAS-2/100 Hz group were significantly higher than those of the other groups, and there were no significant differences between all the four groups on the TGF-alpha or G-CSF levels in the follicular fluids. This could indicate that NPY, as opposed to one of the othese other molecules examined, may be the primary mediator of the improvement in outcomes of the IVF patients.

Although it has been reported that TEAS may improve IVF outcomes, the underlying mechanisms are not clear. Several mechanisms have been suggested to explain the role of acupuncture in IVF, such as increasing blood flow and ovarian steroid hormone secretion, reducing anxiety of patients, etc. In the present study, we investigated whether TEAS may improve IVF outcomes by influencing oocyte developmental competence, which is defined as the ability of oocyte to complete meiosis and undergo fertilization, embryo genesis and term development. We did not find any differences between the control, TEAS-2 Hz, TEAS-100 Hz and TEAS-2/100 Hz groups on the numbers of metaphase II oocytes, normally fertilized zygotes, early cleavage embryos or good quality embryos. Moreover, follicular fluid levels of TGF-alpha (reflecting oocyte maturation) and G-CSF (a biomarker of human oocyte developmental competence) showed no significant differences between all the four groups, indicating that TEAS influences IVF outcomes through mechanisms other than improving oocyte quality.

In conclusion, TEAS using a frequency of 2/100 Hz could help to improve the IVF outcomes

partly by increasing the NPY levels in the follicular fluids. These findings provide preliminary evidence to suggest that TEAS may be a new option for clinicians and infertile women during IVF treatment. Limitations of this study include lack of a placebo group, small sample size, short research period and the fact that patients included were only those with bilateral tubal blockage. A larger randomized, multicenter, double-blinded and placebo-controlled trial should be conducted in the near future to further confirm the effects of TEAS on the IVF outcomes and to further explore the potential underlying mechanism of this effect.

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Conflicts of interest

None.

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