Effect of transcutaneous electrical acupuncture point stimulation on endometrial receptivity in women undergoing frozen-thawed embryo transfer: a single-blind prospective randomised controlled trial

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ABSTRACT

Objective To evaluate the effect of transcutaneous electrical acupuncture point stimulation (TEAS) on endometrial HOXA10 protein expression and three-dimensional (3D) power Doppler ultrasound parameters as markers of endometrial receptivity in women undergoing frozen-thawed embryo transfer (FET).

Methods A total of 68 women undergoing FET were randomised to receive TEAS or mock TEAS at acupuncture points CV3, CV4 and SP6 and Zigong bilaterally. Both groups had six sessions per cycle for three menstrual cycles prior to the scheduled FET. Each session lasted 30 min and was repeated every other day. 3D power Doppler ultrasound parameters, HOXA10 protein expression and rates of embryo implantation, clinical pregnancy and live birth were compared.

Results There were no significant differences between the two groups in endometrial thickness or endometrial volume. The ultrasonographic endometrial triple-line pattern was present more often in the TEAS group (p=0.002). The TEAS group had a greater endometrial and subendometrial vascularisation index (VI) than the mock TEAS group (p=0.001 and p<0.001, respectively) on 3D ultrasound and increased endometrial HOXA10 expression (p=0.001) immediately prior to FET. Subsequently, the rates of embryo implantation, clinical pregnancy and live birth rates were all higher in the TEAS group than in the mock TEAS group (p=0.024, p=0.038 and p=0.033, respectively).

Conclusions In patients undergoing FET, TEAS may have beneficial effects on endometrial HOXA10 expression and ultrasound markers of endometrial receptivity. These findings may explain the improvement in clinical outcome of FET associated with the use of TEAS. Trial registration number ChiCTR-TRC-14004448.

INTRODUCTION

Since the birth of the first thawed human pre-embryo in 1983, frozen-thawed embryo transfer (FET) has become a fundamental component of assisted reproductive technology (ART).1 2 The advantages of FET include its relatively low costs and its safety profile—for example, a negligible incidence of ovarian hyperstimulation syndrome because it can be performed in a natural cycle.3–5 Despite improvements in the techniques of embryo thaw and transfer, there has not been a significant increase in embryo implantation rate, which has remained steady at around 30–50%.6 7 Successful implantation depends on a synchronised dialogue between high quality blastocysts and a receptive endometrium.8 Different strategies have been developed to assess endometrial receptivity including measurement of endometrial cytokines and ultrasound examination. The HOXA10 gene codes for a transcription factor that functions as a regulator of uterine organogenesis during the embryonic period. It is evident that HOXA10 also plays a pivotal role in embryo implantation by regulating downstream target genes. In addition, HOXA10 has been shown to play a role in endometrial

development such as pinopode formation and endometrial decidualisation. Defective HOXA10 expression in the endometrium during the mid-luteal phase has been observed in infertile women with hydrosalpinx, endometriosis and polycystic ovary syndrome. Three-dimensional (3D) power Doppler ultrasonography represents a unique non-invasive tool that can be used to identify a receptive uterus before FET. Several ultrasound parameters have been proposed as markers of endometrial receptivity including endometrial thickness, endometrial pattern, endometrial volume and endometrial/subendometrial vascularisation index (VI). Studies have shown that a triple-line pattern of the endometrium and higher endometrial or subendometrial VI measured on the day of human chorionic gonadotrophin (hCG) administration are associated with higher pregnancy rates in the context of ART.10

Acupuncture is an essential part of Traditional Chinese Medicine (TCM) which has been widely adopted for treating female infertility.11 12 Cumulative evidence from systematic reviews suggests that acupuncture may improve clinical pregnancy and/or live birth rates when used as an adjunct to ART, although much controversy remains.13 Possible mechanisms of action include modulation of the neuroendocrine system and increased uterine blood flow, which may lead to enhanced endometrial receptivity.14

To increase the effect of acupuncture-like stimulation and reduce the invasiveness, we chose to investigate transcutaneous electrical acupuncture point stimulation (TEAS) applied to acupuncture points using painless skin electrodes instead of manual needling. Compared with manual acupuncture or electroacupuncture, TEAS carries no risk of infective complications or visceral injury and avoids any fear which may be associated with needling.15 To our knowledge, the effects of TEAS on endometrial receptivity in FET have not previously been reported, although a large multicentre trial of its efficacy in FET have not previously been reported, knowledge, the effects of TEAS on endometrial receptivity in FET have not previously been reported,

TEAS and mock TEAS treatment
The TEAS group received TEAS at acupuncture points CV3, CV4 and SP6 and Zigong bilaterally using self-adhesive skin electrodes. Electrical acupuncture point stimulation was delivered via an electrostimulator (LH202H HANS, Huawei Co Ltd, Beijing, China) using dispersed-dense waves at 2 Hz frequency. The intensity was set to approximately 10–20 mA, which was the level of maximal tolerance without discomfort. Intensity was checked every 5 min and readjusted if necessary. Each treatment lasted for 30 min in total.

Women allocated to the control group had TEAS electrodes applied to the same sites and received intermittent 2 Hz (10 s on and 20 s off) TEAS at an intensity of 5 mA. Although not completely inert, this technique has been shown to constitute a successful placebo treatment in a previous study of functional brain networks.18 The stimulation is minimal but sensible and appears to be psychologically effective yet physiologically ineffective.

Frozen-thawed embryo transfer
All subjects underwent FET in hCG-induced natural cycles. Basal levels of follicle stimulating hormone (FSH) in serum were measured on day 3 of the first cycle using a chemiluminescent enzyme immunoassay (Beckman Coulter, USA). The development of the endometrium and dominant follicle were monitored by serial vaginal ultrasound examinations starting from day 10 of the cycle and repeated when necessary. Subsequently, ovulation was triggered by administration of hCG (Menotropins 5000 IU) in both groups as soon as an endometrial thickness of 8 mm or more was reached and a dominant follicle of 17 mm diameter was present. Ultrasound-guided FET was performed 5 days after hCG injection. A maximum of two embryos were transferred into the uterus transvaginally. Patients in both groups received vaginal

METHODS
Trial design and subjects
A prospective randomised controlled trial was conducted in women undergoing hCG-induced natural cycle FET at the Reproductive Medicine Center of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine between January and April 2013. To be eligible, patients had to be subfertile, aged between 25 and 40, have regular menstrual cycles (25–34 days), a previous unsuccessful pregnancy outcome following IVF-embryo transfer (IVF-ET) and ≥3 frozen-thawed embryos of quality score ≥6.17 Patients were excluded if they had received hormonal therapy within 3 months of enrolment, had previously used acupuncture, or were known to have genital tract anomalies or endocrine or gynaecological pathology.

Sixty-eight subjects were assigned randomly using opaque sealed envelopes according to a computer-generated randomisation list to one of two groups: TEAS or mock TEAS (n=34 each). Physicians, sonographers and pathologists were blind throughout the study, although it was not possible to blind the acupuncturists providing the treatments as muscle contractions at the site of stimulation were visible.

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progesterone (Utrogestan 200 mg; three separate doses daily) starting on the day after hCG administration. The serum β-hCG level was measured 12 days after the transfer. Clinical pregnancy was defined as the presence of an intrauterine gestational sac and fetal pole with cardiac activity 5 weeks after embryo transfer by transvaginal ultrasound examination.

Assessment of ultrasound parameters
The ultrasound examination was carried out within 1 h of FET in all participants using LOGIQ S6 equipment (General Electric, USA). The machine was equipped with a transvaginal 3–9 MHz volume transducer.

The endometrial thickness and endometrial pattern were visualised in two-dimensional B-mode. The maximum thickness was measured between the interfaces of the endometrial–myometrial junction. The endometrial pattern was classified as a triple-line pattern (a central hyperechogenic line surrounded by two hypoechogenic layers) or a homoechogenic pattern (hyperechogenic endometrium). After completion of the B-mode examination, the ultrasound probe was kept steady and the 3D power Doppler volume mode was switched on, with the volume angle preset to 70°. Once the region of interest (ROI) had been identified, the volume box was superimposed. Thereafter, the data were analysed using the virtual organ computer-aided analysis (VOCAL) imaging program. The manual trace mode of the VOCAL program was used to cover the whole 3D volume of the ROI, with 15° rotation steps. Hence, 12 contour planes were analysed for each ROI. After obtaining the total volume of the ROI, the program automatically calculated the ratio of colour index to all the voxels to determine the endometrial/subendometrial VI.

Evaluation of immunohistochemistry
Endometrial biopsy was performed in the mid-luteal phase just before the FET cycle, corresponding to the window of implantation. Endometrial biopsy specimens were preserved in neutral formalin. The paraffin slides were kept in an oven at 65–70°C for 1 h. The slides were then deparaffinised and rehydrated through a series of xylene and grades of alcohol in distilled water, rinsed with phosphate-buffered solution (PBS) three times (3 min each). Next, epitope retrieval was performed by microwave processing with 10 mM sodium citrate buffer for 20 min followed by natural cooling to 37°C. The slides were then rinsed in PBS three times (3 min each) and endogenous peroxidase was quenched with 3% hydrogen peroxide (H₂O₂) for 10 min followed by washing with PBS three times (3 min each). Non-specific binding was blocked with 3% normal horse serum in PBS for 2 h at 37°C. Excess normal horse serum was drained. Following this, slides were then incubated overnight at 4°C with anti-human monoclonal HOXA10 antibody (Abcam, UK) at a dilution of 1:150. After being rinsed in PBS three times (3 min each), slides were incubated with secondary antibody, polyclonal goat anti-rabbit (Abcam), in a 1:200 dilution at 37°C for 60 min. On the following day, slides were washed with PBS three times (3 min each) and then incubated in substrate diamobenzidine for 5–8 min until the colour developed, and counterstained with haematoxylin for 5 min. Finally, slides were dehydrated in grades of ethanol and cleared for 20 min in xylene.

To compare HOXA10 protein expression in the endometrium obtained from both groups, the intensity of HOXA10 protein expression was measured by semiquantitative analysis using an immunohistochemistry (IHC) score (percentage of immunostained cells×intensity of nuclear staining). Immunostained cells were assessed in the epithelial and stromal compartments. The intensity of nuclear staining was stratified using a three-point scale from 1 to 3 where 1=weak, 2=moderate and 3=strong intensity. Five randomly chosen fields from each slide were evaluated and then averaged.

Statistical analysis
Statistical analyses were conducted using SPSS V19.0 for Windows. All data are presented as mean±SD or percentages. Continuous variables were compared using the Student t test or Mann–Whitney U test for independent samples, where appropriate. Comparison of categorical variables was carried out by χ² analysis or the Fisher exact test, if required. A two-tailed p value <0.05 was considered to be significant.

RESULTS
Sixty-eight subjects were randomised according to a computer-generated randomisation list: 34 received TEAS and 34 received mock TEAS. None of the 68 participants dropped out or withdrew from the study.

The baseline characteristics of all patients are shown in Table 1. No significant differences were observed between the two groups in age, body mass index, duration or type of infertility or serum basal FSH concentration.

Table 2 shows the results of the 3D power Doppler ultrasonography and immunohistochemistry between the two study groups. The endometrial triple-line pattern was present more often in the TEAS group than in the mock TEAS group (p<0.01). No significant differences were observed between the two groups in terms of endometrial thickness or endometrial volume (p>0.05). Endometrial and subendometrial VI were higher in the TEAS group than in the mock TEAS group (both p<0.001). HOXA10 protein expression in the endometrial samples from the TEAS group appeared to be increased relative to those from the mock TEAS group (Figure 1) and, accordingly, the mean IHC score of the immunohistochemical image
was significantly higher in the TEAS group than in the mock TEAS group.

Of the 68 patients attending our institution for FET, the rates of embryo implantation, clinical pregnancy and live birth were all higher in the treatment group than in the control group (p<0.05, table 3).

**DISCUSSION**

Implantation of the human embryo within the uterine cavity is a complex process, and a non-receptive endometrium is considered to be a crucial limiting factor. A mathematical model indicated that uterine receptivity may contribute to a successful conception by as much as 31–64%. Unfortunately, little is known about the precise biological mechanisms that directly influence endometrial proliferation and differentiation, and consequently implantation.

Table 2 Comparison of the ultrasound parameters and IHC score of study groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>TEAS group (n=34)</th>
<th>Mock TEAS group (n=34)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial thickness (mm)</td>
<td>11.26±1.50</td>
<td>10.74±1.54</td>
<td>0.112*</td>
</tr>
<tr>
<td>Endometrial pattern (n, %)</td>
<td>28 (91.2)</td>
<td>16 (41.2)</td>
<td>0.002†</td>
</tr>
<tr>
<td>Triple-line</td>
<td>6 (18.2)</td>
<td>3 (8.8)</td>
<td></td>
</tr>
<tr>
<td>Homoechogenic</td>
<td>6 (18.2)</td>
<td>3 (8.8)</td>
<td></td>
</tr>
<tr>
<td>Endometrial volume (mL)</td>
<td>3.44±0.84</td>
<td>3.15±0.77</td>
<td>0.148‡</td>
</tr>
<tr>
<td>Endometrial VI (%)</td>
<td>1.33±0.55</td>
<td>0.87±0.40</td>
<td>0.001*</td>
</tr>
<tr>
<td>Subendometrial VI (%)</td>
<td>1.91±1.02</td>
<td>1.05±0.70</td>
<td>0.000*</td>
</tr>
<tr>
<td>IHC score</td>
<td>2.21±1.44</td>
<td>1.13±0.73</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Values are means±SD or n (%).

In its role as a transcription factor, the HOXA10 gene has been shown to be indispensable for the process of embryo implantation and also for endometrial development in response to steroid hormones. In women, HOXA10 has been shown to participate in the regulation of multiple downstream target genes that are necessary for implantation such as β3-integrin and cyclooxygenase-2. Blitek et al reported that HOXA10 expression is present throughout the menstrual cycle but is upregulated dramatically in the mid-luteal phase (the window of implantation). Other studies have shown that women who lack HOXA10 expression suffer from infertility due to implantation failure. The HOXA10 gene is therefore a well-established marker of endometrial receptivity, and alterations in its expression may result in implantation defects.

The technique of 3D power Doppler ultrasound is an established method of assessing endometrial receptivity which enables the accurate assessment of various uterine parameters. The endometrial triple-line pattern and endometrial/subendometrial VI are important predictors of successful implantation. Previous studies have demonstrated that a triple-line pattern of the endometrium before FET is associated with higher implantation and pregnancy rates, and its absence might be a sign of premature secretory changes indicating that the time of optimal endometrial receptivity has already passed. Assessment of endometrial/subendometrial 3D power Doppler flow indices before FET adds a physiological dimension to the ultrasound assessment and has drawn much attention over recent years.

In the present study, all subjects undergoing FET were similar with respect to baseline characteristics and the number of transferred embryos when evaluated prior to the treatment. Thus, known predictors of outcome were balanced in all the participants, indicating effective randomisation. After TEAS at acupuncture points CV3, CV4 and SP6 and Zigong bilaterally, we observed a significantly higher rate of ultrasonographic triple-line pattern in the endometrium and HOXA10 expression level during the implantation window compared with the mock TEAS group. A significant increase in endometrial/subendometrial VI was also present following TEAS, which may reflect enhanced endometrial blood flow favouring embryo implantation. This study suggests that the use of TEAS as an adjuvant treatment in women undergoing FET produces better therapeutic results than those obtained with FET alone (represented by the mock TEAS group).

Acupuncture is an emerging therapy that has been shown to influence the menstrual cycle and upregulate the myoelectrical activities of the uterus, which may in turn impact reproductive function. Acupuncture may also improve reproductive capability through regulation of the neuroendocrine and immune
systems. It has previously been reported that low-frequency electroacupuncture, which can inhibit central sympathetic nerve activity and increase the level of β-endorphin and other substances in the cerebrospinal fluid, can reduce the impedance of the uterine artery and thereby improve the blood flow to the uterus around the time of implantation.

While knowledge of the mechanisms of action of TEAS is relatively limited, it can be assumed that it represents an analogous form of somatosensory stimulation with similar effects mediated via the nervous system.

To our knowledge, this study is the first to evaluate the effect of TEAS on endometrial receptivity in women undergoing FET. No TEAS-related complications or other side effects were observed, suggesting that TEAS therapy is clinically effective and also safe and acceptable to patients undergoing FET. Although TEAS protocols are growing in popularity in patients undergoing IVF due to its relative convenience, lack of adverse effects and unique therapeutic effects, most physicians agree that this remains a controversial area and that further studies are needed to ensure greater acceptance of TEAS in Western countries as part of ART protocols such as FET. Despite the positive results, the present study was ultimately limited by a relatively small number of participants and its single-centre design.

**CONCLUSIONS**

Our results indicate that TEAS may produce significantly higher rates of embryo implantation, clinical pregnancy and live birth following ART. A higher frequency of the ultrasonographic triple-line endometrial pattern, greater endometrial/subendometrial VI and increased HOXA10 protein expression were noted in the TEAS group in the present study. Based on these results, women with a regular menstrual cycle could be offered TEAS in the 3 months prior to FET as it appears to enhance endometrial receptivity. However, further research is required to confirm the apparent benefits of TEAS in women undergoing FET, ideally in the form of large-scale multicentre clinical trials. Further investigation into the underlying mechanisms of action of TEAS is also needed.

### Summary points

- Sixty-eight subfertile women were randomised to receive transcutaneous electrical acupuncture point stimulation (TEAS) or mock TEAS for 3 months prior to frozen-thawed embryo transfer (FET).
- Rates of embryo implantation, clinical pregnancy and live birth were significantly higher in the TEAS group than in the mock TEAS group (p<0.05).
- TEAS had a positive effect on several markers of endometrial receptivity including the ultrasonographic endometrial triple-line pattern, endometrial/subendometrial vascularisation index and endometrial HOXA10 expression.

### Table 3

<table>
<thead>
<tr>
<th>Variables</th>
<th>TEAS group (n=34)</th>
<th>Mock TEAS group (n=34)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation rate (n, %)</td>
<td>17 (50.0)</td>
<td>8 (23.5)</td>
<td>0.024</td>
</tr>
<tr>
<td>Clinical pregnancy rate (n, %)</td>
<td>15 (44.1)</td>
<td>7 (20.6)</td>
<td>0.038</td>
</tr>
<tr>
<td>Live birth rate (n, %)</td>
<td>14 (41.2)</td>
<td>6 (17.6)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

*Chi-square test. TEAS, transcutaneous electrical acupuncture point stimulation.

**Contributors** All authors participated in the study design. ZHS and FL were responsible for obtaining and analysing the data, drafting and revising the manuscript. PFL performed the statistical analysis. WXY performed the ultrasound examination.

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Competing interests None.

Patient consent Obtained.

Ethics approval The research was approved by the ethics committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine and met the standards stipulated in the Declaration of Helsinki for Good Clinical Practice.

Provenance and peer review Not commissioned; externally peer reviewed.

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