Electroacupuncture decreases the progression of ovarian hyperstimulation syndrome in a rat model

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Abstract This study aimed to elucidate the effect of electroacupuncture treatment on preventing early ovarian hyperstimulation syndrome (OHSS) and the potential mechanisms involved using an induced rat model. The ovarian response was examined by measuring ovary weight, vascular permeability, levels of inflammation (interleukin-6, tumour necrosis factor alpha, chemokine ligand 2 (also known as monocyte chemoattractant protein 1), vascular endothelial growth factor and hormone concentrations (oestradiol, progesterone, testosterone and prolactin). Sprague–Dawley female rats underwent ovarian stimulation to induce OHSS. Hyperstimulated rats received consecutive electroacupuncture treatment from 3 days before the beginning of pregnant mare serum gonadotrophin treatment or the time point of pregnant mare serum gonadotrophin treatment respectively, and last until 3 days after HCG administration. Electroacupuncture treatment reduced ovary weight and vascular permeability in hyperstimulated rats. Electroacupuncture treatment also reduced the levels of serum steroid hormones (progesterone and testosterone), inflammatory cytokines (interleukin-6, tumour necrosis factor alpha and monocyte chemotactic protein 1) and vascular endothelial growth factor in hyperstimulated rats. The results indicate that electroacupuncture can modulate endocrine hormone secretion and affect the secretion of inflammatory cytokines and vascular endothelial growth factor, and thus prevent the progress of OHSS. Electroacupuncture may provide a simple and effective method for the prevention and treatment of OHSS.

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is a common iatrogenic complication of ovarian stimulation, occurring in about 0.3–5% of cycles (Schenker and Weinstein, 1978). The severe form of OHSS may cause death. The cause of OHSS remains elusive. Vascular endothelial growth factor (VEGF) is considered to be a prime causative agent in OHSS progression (Abramov et al., 1997). A recent study showed that inflammatory cytokines such as interleukin (IL)-2, IL-6 and tumour necrosis factor alpha (TNF-α) are the potential mediators in the pathogenesis of OHSS (Artini et al., 2002). Multiple methods have been used to prevent progression of OHSS, which include cycle cancellation, coating, intravenous albumin infusion during oocyte collection, low doses of HCG for oocyte triggering, replacing HCG with a gonadotrophin releasing hormone agonist to induce final follicular maturation, elective cryopreservation of all embryos, and the administration of cabergoline (Fatemi and Garcia-Velasco, 2015; Mathur et al., 2007). The simple, effective and optimal strategy to prevent OHSS during fresh embryo transfer has not yet been defined.

Acupuncture is a conventional technique in Traditional Chinese Medicine. Because of its few side-effects, convenience and unique effect on general wellbeing, acupuncture has gained significant popularity in Western countries. In reproduction, electroacupuncture has shown some advantages in treating polycystic ovary syndrome through multichannel and multi-targeted regulation of the reproductive and endocrine functions in the hypothalamic–pituitary–ovarian axis. Electroacupuncture can adjust woman’s ovulation and menstrual cycle by affecting or improving hypothalamic, pituitary and ovarian functions (Jedel et al., 2011; Manneras et al., 2009). In addition, evidence is growing that acupuncture can reduce the stress during IVF and embryo transfer, and improve the success rate (de Lacey et al., 2009; Kovarova et al., 2010). The first clinical trial was published in 1999 (Stener-Victorin et al., 1999), and suggested that acupuncture was effective in improving the clinical pregnancy rate of IVF.

Our previous unpublished study investigated the effect of acupuncture on endometrial receptivity. Ovarian volume was significantly smaller and the ascetic fluid was significantly decreased, compared with those in the control group. These interesting results imply that acupuncture might possess the potential to prevent the progress of OHSS. The aim of the present study, was to investigate the effect of different acupuncture methods on OHSS rat model, which provides valuable evidence for preventing the progress of OHSS during fresh embryo transfer.

Materials and methods

Animal model

Immature female Sprague–Dawley rats aged 22 days (D22), weighing 80–90 g, were obtained from the Animal Experiment Center of Nanjing Jinling Hospital. They were kept under a controlled 12:12 h light–dark cycle, with standard diet and free access to water. The study protocol was approved by the Committee of the Use and Care of Animals of Nanjing Jinling Hospital on 29 July 2014 (reference number 20140729).

A total of 28 rats were randomly assigned to four groups. Control group: rats (n = 7) were injected with 10 IU of pregnant mare serum gonadotrophin (PMSG) at 15:00 of day 27 (D27) and 10 IU of HCG after 48 h (15:00 on D29). OHSS group: the experimental OHSS models were established following Kitajima et al.’s protocol (2004). Rats (n = 7) received excessive doses of PMSG (50 IU/day) for 4 consecutive days (from D25 to D28, at 15:00), followed by 150 IU of HCG (D29, at 15:00). Electroacupuncture pretreatment plus OHSS (PEAO) group: rats (n = 7) received electroacupuncture treatment for 15 min per day (beginning at 12:00) for 3 days before the OHSS model was established and continued to D31. Electroacupuncture plus OHSS (EAO) group: hyperstimulated rats (n = 7) received acupuncture treatment for 15 min/day (beginning at 12:00) from D25 to D31.

Electroacupuncture stimulation

All animals were anaesthetized with ketamine. Then, in the PEAO and EAO groups, electroacupuncture stimulation was applied to the acupuncture points of unilateral Saninyijiao (SP6) and Guanyuan (CV4) by using an electroacupuncture stimulator instrument (Model KDZ-I; Yangzhou Kaida Medical Equipment Co., LTD, Yangzhou, China). In the control and OHSS groups, the rats were not treated with electroacupuncture. The depth and location of electroacupuncture were determined according to the concept of Traditional Chinese Medicine, the atlas of skeleton and acupoints of rat, and the anatomical location described in previous a study (Hua et al., 1991). SP6 is situated about 10 mm above the top of the medial malleolus and on the posterior border of the tibia. CV4 is located at the point of 3/5 down the ventral midline connecting the umbilicus to the pubic tubercle. Two 0.3-mm stainless steel acupuncture needles (Shanghai Taicheng Technology Development Co., LTD, Shanghai, China) that were connected with the output terminal were inserted at a depth of 2 mm into the above-mentioned acupuncture points. The stimulation with 2 or 15 Hz frequencies was generated at an intensity of muscle twitch threshold and lasted 15 min every day.

Measurement of peritoneal and ovarian capillary permeability

On the basis of Ujioka1’s protocol (1997), all rats (D31) were anaesthetized with 100 mg/kg ketamine and then kept on a thermal blanket to avoid hypothermia. A total of 0.2 ml of 5 mmol/l of Evans Blue dye (Sigma, USA, dissolved in distilled water) was injected via the tail vein. Thirty minutes after Evans Blue injection, the peritoneal cavity was injected with 5 ml of 0.9% saline (21°C, pH 6.0) and massaged for 30 s. Subsequently, to prevent tissue or vessel damage, the fluid was drained through an insemination catheter (Cook Medical Trade Co., LTD, Shanghai, China). The peritoneal fluid was recovered in tubes containing 0.05 ml of 0.1 mol/l sodium hydroxide to avoid any protein interference. After centrifugation at 900 × g for 15 min at room temperature, Evans Blue
concentration was measured at 620 nm on a spectrophotometer (Synergy HTX, BioTek Instrument, Inc. USA).

Ovaries were collected after blood collection. Two ovaries from each rat were weighed; the right one was frozen at −80°C, and the left one was incubated in 2 ml formamide at 37°C for 24 h. To evaluate the capillary permeability in the ovary, Evans Blue concentration in the formamide extract of the ovary was also measured. The dye concentration in the peritoneal irrigated fluid was presented as μg/100 g body weight, and Evans Blue content in the ovary was presented as μg/g tissue wet weight.

**Hormone assays**

After peritoneal irrigation, blood samples were immediately collected from orbital puncture. Blood samples were kept at room temperature for 10 min and then centrifuged at 900 g for 10 min. The serum was collected and stored at −80°C until assay. The levels of progesterone, oestradiol, testosterone, and prolactin were determined by chemiluminescent immunosorbent system (Backman Coulter, Inc., Brea CA, USA). The intra-assay and inter-assay coefficients of variation for progesterone, oestradiol, testosterone and prolactin were 8.18 and 7.89%, 5.13 and 6.23%, 2.56 and 5.19%, and 5.52 and 3.53%, respectively.

**Measurements of VEGF, TNF-α, IL-6 and MCP-1**

Ovaries in ice-cold phosphate buffered saline (0.01 mol/l, pH 7.0–7.2) were homogenized with ultrasonic cell crusher (SONICE, USA). Supernatant was obtained by centrifugation at 12,000 g for 30 min. Protein concentration was determined by the bicinchoninic acid method. Serum interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) levels were determined by enzyme-linked immunosorbent assay (ELISA). The VEGF and IL-6 levels in the ovary extract were determined by ELISA. The Kits for VEGF, IL-6, TNF-α and MCP-1 were purchased from Sigma (St Louis, MO, USA, for VEGF of ovary, IL-6, TNF-α and MCP-1 of serum), Cloud-Clone Co. (Houston, TX, USA, for IL-6 of ovary). The measurement range of serum TNF-α, MCP-1 and IL-6 ELISA kits was 82.30–20000 pg/ml, 24.67–18000 pg/ml and 40.96–10000 pg/ml, respectively; and with a sensitivity of 25 pg/ml, 15 pg/ml and 30 pg/ml, respectively. The measurement range of ovarian VEGF and IL-6 ELISA kits was 0.82–200 pg/ml and 1.56–100 pg/ml, respectively; and with a sensitivity of 2 pg/ml and 0.55 pg/ml, respectively.

**Statistical analysis**

Data were presented as mean ± standard error of mean (SEM). One-way ANOVA method was used for comparisons of means of normally distributed parameters, and the homogeneity of variance was tested before (with 0.1 as the standard test conducted Levene test). When equal variances were assumed, the least significant difference test was selected; otherwise, a Dunnett’s T3 test was carried out. Statistical analyses were conducted with the Statistical Package for the Social Sciences Version 13.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Effect of electroacupuncture on ovary enlargement**

Ovary weight was significantly increased in OHSS, PEAO and EAO groups (P < 0.01) compared with the control group. Only PEAO but not EAO significantly reduced ovary weight (P < 0.05) (Figure 1A), compared with the OHSS group.

**Measurement of Evans Blue in peritoneal irrigated fluid and ovarian capillary permeability**

Ovarian capillary permeability was assessed using Evans Blue dye. Compared with the control group, Evans Blue concentration in peritoneal irrigated fluid in the OHSS group was significantly increased (P < 0.01) (Figure 1B). Concentration of Evans Blue was significantly decreased by PEAO but not EAO in peritoneal irrigated fluid (P < 0.05) compared with OHSS group. Moreover, compared with EAO group, PEAO also obviously reduced Evans Blue concentration (P < 0.05) (Figure 1B). Compared with control group, Evans Blue dye content in the ovary was statistically significantly higher in the OHSS group (P < 0.01). In the OHSS group, PEAO caused a statistically significant reduction of ovarian Evans Blue content (P < 0.05) (Figure 1C).

**Effect of electroacupuncture treatment on the secretion of hormones**

Serum oestradiol and prolactin levels in rats experiencing OHSS were significantly higher than those in control rats (P < 0.01), whereas both PEAO and EAO slightly reduced oestradiol and prolactin levels in the rats experiencing OHSS, although the differences were not statistically significant (Figure 2A and 2B). Serum progesterone and testosterone levels in rats experiencing OHSS, compared with the control group, were increased 3.5-fold and 2.7-fold, respectively. Compared with the OHSS group, PEAO but not EAO significantly decreased serum progesterone and testosterone levels (P < 0.05) (Figure 2C and 2D).

**Effect of electroacupuncture treatment on serum MCP-1 and TNF-α levels and ovarian IL-6 expression**

Compared with the control group, levels of MCP-1 and TNF-α in serum were significantly higher in the OHSS group (P < 0.05 and P < 0.01, respectively) (Figure 3A and 3B). PEAO but not EAO significantly decreased levels of MCP-1 and TNF-α levels in serum, and PEAO also reduced ovarian IL-6 expression compared with OHSS group (P < 0.05) (Figure 3).

**Electroacupuncture treatment affects VEGF expression in the ovary**

Compared with the control group, the expression of VEGF in the ovary was significantly higher in the OHSS group (P < 0.01)
Electroacupuncture decreases the progression of OHSS in a rat model

Figure 1 Effects of electroacupuncture treatment on ovary weight and capillary permeability in rats induced to achieve ovarian hyperstimulation syndrome. (A) Comparison of rat ovary weight between groups; (B) comparison of Evans Blue concentration in peritoneal irrigated fluid; (C) comparison of Evans Blue concentration in the ovarian extract. Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01. EB, Evans Blue dye. EAO, electroacupuncture plus OHSS; OHSS, ovarian hyperstimulation syndrome; PEAO, electroacupuncture pretreatment plus OHSS.

Ujioka et al. (1997) first used immature female rats to produce a hyperstimulated manifestation. In the present study, an experimental rat model of OHSS based on previous literature (Kitajima et al., 2004) was established. Briefly, D25 rats were treated with consecutive 4-day PMSG and induced ovulation with high-dose of HCG. We found the ovaries in our model were significantly enlarged and showed increased capillary permeability. In particular, the local capillary permeability of ovary was significantly increased. Our results showed that the OHSS rat model in rats was successfully generated.

Discussion

Ujioka et al. (1997) first used immature female rats to produce a hyperstimulated manifestation. In the present study, an experimental rat model of OHSS was established. The ovaries in our model were significantly enlarged and showed increased capillary permeability. In particular, the local capillary permeability of ovary was significantly increased. Our results showed that the OHSS rat model in rats was successfully generated.

Electroacupuncture points are the locations where the needle can gain access to some deeper tissue components and mostly convey the impression along the meridians. Particular acupoints link with unique connective tissue location (Langevin and Yandow, 2002). In this study, the stimulated points were Sanyinjiao (SP6) and Guanyuan (CV4). According to the Traditional Chinese Medicine principles and modern acupuncture point theory, SP6 is considered as a classic acupoint for female disorders and can soften and harmonize the liver function and benefit the kidney Qi (Wang, 2003). CV4 is on the conception vessel, which can nourish the uterus to adjust the axis function (Wang, 2003). On the basis of a multitude of documents and materials, the SP6 and CV4 acupoints have been commonly used to clinically treat a series of diseases in the Department of Gynaecology in China. Therefore, the SP6 and CV4 acupoints were used as the experimental treatment choice in this study.

Fresh embryo transfer has been widely used in various reproductive medical centers, so it is important to prevent OHSS in clinic. According to our previous data (unpublished), we hypothesize that electroacupuncture is a potentially useful treatment to prevent OHSS. The start time, intensity and the last time of acupuncture, however, should be defined. Therefore, in the present study, we used two electroacupuncture methods (PEA and EA). This experimental design would propose basic research for further clinical application.

Some reports have found that patients who experience OHSS, and rats induced to achieve OHSS, demonstrate increased expression level of progesterone and testosterone (Gomez et al., 2006; Ozccakir et al., 2005; Yuen et al., 1979). These data imply that progesterone and testosterone contribute, at least in part, to the pathophysiology of OHSS. In our study, PEA showed a non-significant tendency to reduce serum oestradiol and prolactin levels and it significantly decreased the level of progesterone and testosterone. Chen (1997) summarized that electroacupuncture might regulate the dysfunction of hypothalamic–pituitary–ovarian axis in several ways, in which electroacupuncture could affect some gene expression in brain, thereby, regulate secretion of some hormones. Liu et al. (2009) punctured CV4 and SP6 in rats with primary dysmenorrhoea and observed the elevated serum FSH, LH and oestradiol levels, and decreased progesterone level. Jie Sun et al. (2013) observed that androstenedione and testosterone were significantly decreased after 14 consecutive days of electroacupuncture in rats with polycystic ovary syndrome. Combined with our results, acupuncture could attenuate the symptoms of OHSS through down-regulating progesterone and testosterone expression level.

The association between cytokines and the pathophysiology of OHSS is still unclear. Abramov et al. (1996), however, reported high concentration of cytokines in the ascitic fluid and plasma of patients with severe OHSS. Friedlander et al. (1993) observed a high level of IL-6 in plasma and ascitic fluid of patients experiencing OHSS. Chistyakova et al. (2014)
reported that the levels of TNF-α and IL-6 were significantly increased in patients with early and late OHSS. Because high MCP-1 is regarded as an early sign of vascular damage and cardiovascular disease, high level of MCP-1 in rats induced to achieve OHSS is associated with vascular damage, which is also consistent with high capillary permeability in these rats. These findings suggest a close relationship between inflammatory cytokines and the pathophysiology of OHSS. Our present study found that the levels of TNF-α and MCP-1 in serum and of IL-6 in ovary were significantly rescued by PEA. It has been reported that testosterone can increase TNF-α and MCP-1 expression in RAW264.7 macrophage in vitro (Zhu et al., 2009); testosterone enhances lipopolysaccharide-induced IL-6 and MCP-1 expression in 3T3-L1 adipocytes (Su et al., 2015). Therefore, we speculate that acupuncture can down-regulate the expression level of inflammatory factors, such as TNF-α, IL-6 and MCP-1, via reducing the testosterone level, which prevents the progression of OHSS.

It has been reported that the fundamental physiological change related to OHSS is characterized by increased vascular permeability, leading to the leakage of fluid from the vascular compartment, with third-space fluid accumulation and intravascular dehydration (Chen et al., 2012). VEGF, also known as vascular permeability factor, seems to be the critical mediator of OHSS by increasing vascular permeability (Ludwig et al., 1998; Mansour et al., 2005; Nargund et al., 2007; Rizk et al., 1997).

Gomez et al. (2002) reported a cause–effect relationship between increased vascular permeability and ovarian VEGF expression (Gomez et al., 2002). On the basis of indirect observations in women with enlarged ovaries and ascites, Itskovitz-Eldor et al. (1997) thought that the ovary may be the source and target of VEGF in humans. Artini et al. (1998) showed that the levels of serum and follicular fluid VEGF in patients with severe OHSS were significantly higher than those among controls. Our data also show that VEGF level in the ovary of OHSS rats was significantly higher than that in the control group. Moreover, increased VEGF levels in the ovary were consistent with increased capillary permeability in the ovary (high Evans Blue concentration) of rats with OHSS. In this experiment, we found that electroacupuncture pretreatment could reduce ovary weight and capillary permeability in the peritoneal irrigated fluid and ovary. Moreover, electroacupuncture pretreatment significantly decreased the production of VEGF from ovary. Wei et al. (2013) showed that IL-6 trans-signalling can induce vascular leakage and increased the expression of VEGF in OHSS (Wei et al., 2013). Zhang et al. (2009) observed that TNF-α can induce the human umbilical vein endothelial cell production of VEGF. This evidence supports the concept that electroacupuncture inhibits VEGF expression and reduces vascular permeability, which is achieved by down-regulating inflammatory factors, to reduce vascular permeability. On the other hand, our present study also found the expression of progesterone and VEGF was inhibited by electroacupuncture. Yuan et al. (2002) found that progesterone can also regulate the expression of VEGF. So we speculate that the low expression of VEGF may be affected by reduced progesterone.
This study, however, has several limitations. First, electroacupuncture pretreatment was only for 3 days before PMSG treatment and lasted for 10 days. Although electroacupuncture pretreatment exerted significant effect on inhibiting the progression of OHSS, the most appropriate therapeutic course for OHSS treatment still needs further investigation. Second, we only chose CV4 and SP6 as acupunc-
ture points. Whether other acupoints have good or even better effects still needs investigation. Third, the suitable clinical treatment protocol to prevent OHSS in those patients who have potential of high-response is still being investigated. We will observe the effect of PEA on preventing OHSS using randomized controlled trials in the future.

In conclusion, our data support that electroacupuncture can affect testosterone and progesterone expression and down-regulate the expression of inflammatory factors and VEGF, and thus cause restrained vascular permeability. Electroacupuncture, especially PEA, in clinical fresh embryo transfers, may provide a simple and effective treatment method to prevent the process of OHSS.

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References


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