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Effects of electroacupuncture on luteal regression and steroidogenesis in ovarian hyperstimulation syndrome model rat

Xuan Huang[a,1], Li Chen[a,1], You-Bing Xia[b,c,1], Min Xie[a], Qin Sun[a], Bing Yao[a,*]

[a] Reproductive Medical Center, Jinling Hospital Affiliated to Medical School of Nanjing University, Nanjing 210002, China
[b] State Key Laboratory of Reproductive Medicine of Nanjing Medical University, Nanjing 210029, China
[c] Second Clinical Medical School of Nanjing University of Traditional Chinese Medicine, China

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ABSTRACT

Aims: Electroacupuncture (EA) is an effective and safe therapeutic method widely used for treating clinical diseases. Previously, we found that EA could decrease serum hormones and reduce ovarian size in ovarian hyperstimulation syndrome (OHSS) rat model. Nevertheless, the mechanisms that contribute to these improvements remain unclear.

Materials and methods: HE staining was used to count the number of corpora lutea (CL) and follicles. Immunohistochemical and ELISA were applied to examine luteal functional and structural regression. Immunoprecipitation was used for analyzing the interaction between NPY (neuropeptide Y) and COX-2; western blotting and qRT-PCR were used to evaluate the expressions of steroidogenic enzymes and PKA/CREB pathway.

Key findings: EA treatment significantly reduced the ovarian weight and the number of CL, also decreased ovarian and serum levels of PGE2 and COX-2 expression; increased ovarian PGF2α levels and PGF2α/PGE2 ratio; decreased PCNA expression and distribution; and increased cyclin regulatory inhibitor p27 expression to have further effect on the luteal formation, and promote luteal functional and structural regression. Moreover, expression of COX-2 in ovaries was possessed interactivity increased expression of NPY. Furthermore, EA treatment lowered the serum hormone levels, inhibited PKA/CREB pathway and decreased the expressions of steroidogenic enzymes. Hence, interaction with COX-2, NPY may affect the levels of PGF2α and PGE2 as well as impact the proliferation of granulosa cells in ovaries, thus further reducing the luteal formation, and promoting luteal structural and functional regression, as well as the ovarian steroidogenesis following EA treatment.

Significance: EA treatment could be an option for preventing OHSS in ART.

1. Introduction

Electroacupuncture (EA) is an important, and conventional treatment in Traditional Chinese Medicine, which is becoming increasingly more popular in Western countries. EA has been shown to have beneficial effects on general wellbeing [1,2], and has been widely used to treat many clinical diseases without causing any side-effects [3,4]. According to a large number of published clinical studies on reproduction, EA has also proven useful for treating infertile patients [4–6].

OHSS is a serious, life-threatening, iatrogenic complication of ovarian stimulation in assisted reproduction technology (ART), that is characterized by enlarged ovaries with multiple corpora lutea (CL), and elevated sexual hormones in serum [7,8]. The luteinizing hormone (LH), prostaglandin E2 (PGE2) may accelerate the deterioration of OHSS [9,10]. In our previous study, we found that EA could efficiently block the progression of OHSS by reducing ovarian size and decreasing the levels of sexual hormones in serum as well as inflammatory factors [11]. Nonetheless, the exact physiological events occurring in ovaries after EA still remain unclear.

EA has effect on ovaries via the regulation of hypothalamus-pituitary-gonad (HPG) axis [12–14] and neuropeptides levels [6]. Specifically, low-frequency EA instigates the release of many neuropeptides, including nerve growth factor (NGF) and neuropeptide Y (NPY) that are distributed in the peripheral and central nerve systems, and are essential for inducing functional changes in different organ systems [15–17]. Furthermore, NPY can be released from sympathetic nerves via electrical stimulation [6,18,19]. It seems reasonable that NPY could be involved in the working principles of EA treatment in OHSS model rat.

* Corresponding author at: Jinling Hospital, Nanjing, Jiangsu, China.
E-mail address: yaobing@nju.edu.cn (B. Yao).
[1] These authors contributed equally to this work.

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COX-2 is the limited-rating enzyme for prostaglandins production that could affect the expression of luteolytic PGF2α and luteotropic PGE2, which are involved in the regulation of luteinization and luteal regression. COX-2 inhibitor, meloxicam, can decrease the incidence of OHSS in a rat model [20]. Nonetheless, it is still unknown if and how EA treatment could affect the expression of COX-2. The aim of the present study was to elucidate the mechanisms that lead to changes in ovarian size and steroidogenesis, and are probably accompanied with internal structural and functional alterations, following EA treatment.

2. Material and methods

2.1. Animals and study design

Immature female Sprague-Dawley rats 22 days old (D22) were obtained from the Animal Experiment Center of Nanjing Jinling Hospital, Jiangsu, China. All rats were housed in an environment with temperature of 22 ± 1 °C, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 h. In addition, rats were fed with a standard diet, allowed free access to water.

Rats were randomly divided into two groups ($n = 8$ per group) with a computer-generated randomisation value. The successful OHSS rat model was established as our previous study described, briefly, (1) the OHSS group, which received 50 IU of pregnant mare serum gonadotropin (PMSG, Ningbo N0.2 Hormone Factory) on a daily basis from D25 to D28 following with the injection of 150 IU human chorionic gonadotrophin (hCG) (Ningbo, China) on D29. All hormones were administered intraperitoneally; (2) the EA + OHSS (EAO) group, which underwent the same hormonal stimulation protocol as the OHSS group in addition to be anesthetized (4% chloral hydrate, 0.6 ml/100 g) for EA stimulation for 15 min/day from D22 to D31.

All rats were sacrificed on D31, 48 h post-hCG injection, and the ovaries were removed, cleaned using saline solution, and dried out. The left ovaries were then weighed and stored at −80 °C for subsequent assays. Consequently, contralateral ovaries were fixed in 4% paraformaldehyde for 24 h and transferred into 70% alcohol for further dewatering and disposal procedures.

All animal studies (including the rat euthanasia procedure) were done in compliance with the regulations and guidelines of Nanjing Jinling Hospital and conducted according to the AAALAC and the IACUC guidelines (reference number 20140729).

2.2. Electroacupuncture stimulation

The EA stimulation protocol was preformed according the protocol described [11]. Briefly, all animals were anesthetized with 4% chloral hydrate (0.6 ml/100 g). EA stimulation was then applied to the acupuncture points of unilateral Sanyinjiao (SP6, the same side was selected) and Guanyuan (CV4) using an EA stimulator instrument (Model KDZ-I; Yangzhou Kaida Medical Equipment Co., LTD, Yangzhou, China). Two 0.3 mm stainless steel acupuncture needles (Shanghai Taicheng Technology Development Co., LTD, Shanghai, China) were inserted at a depth of 2 mm into the above-mentioned acupuncture points and connected with the output terminal. The stimulation with 2/15 Hz frequencies was generated at an intensity of muscle twitch threshold and lasted 15 min every day from D22 to D31. The rats in OHSS group were also anesthetized (4% chloral hydrate, 0.6 ml/100 g) during the rats in EAO group undergoing EA stimulation, which was to eliminate the influence of anesthesia.

2.3. Hormone assays

Blood samples were collected from orbital puncture immediately after the animals were euthanized. Blood samples were centrifuged at 3000*g* for 10 min after 10 min's standing on ice. The serum was collected and stored at −80 °C until use. Serum levels of E2, P and T were determined by chemiluminescent immunoassay system (Backman Coulter, Inc., Brea CA, USA). The detection limits of E2, P and T were 65.8–714.4 pmol/l, 0.6–3.8 nmol/l and 0.34–2.55 nmol/l, respectively.

2.4. Ovarian tissue histology

Fixed right ovary samples were dehydrated and embedded in paraffin by manual manipulation. The whole ovary was serially sectioned using 5 mm thickness, mounted on glass slide (using every consecutive six section), and then stained with hematoxylin and eosin for morphological observation, according to the method described [21]. Follicles were classified as either preantral follicles (PFs) or antral follicles (AFs) according to the presence, or absence, of either an antrum or preovulatory follicles (POFs) with cumulus-oocyte complex. An atretic follicle (Atret. F) was determined in case pycnotic or deformed nuclei were observed; in the smallest follicles, the criterion for atresia was a degenerate oocyte, precocious antrum formation, or both. CL was counted from the largest three sections of ovaries (three sections per ovary, five ovaries per group), finally average of the three values was calculated as the number of CL for one ovary, as previously described [22,23]. The ovarian follicle at different stages from the same three sections was counted only for nuclear staining and avoided repeat count. The total number of follicles including PFs, AFs, POFs, CLs and Atret follicles, the number of CLs expressed as percentage of CL/Total number (Follicles and CLs).

To assess structural luteolysis (structural regression of the CL), we used the technique described in previous studies [21,24]. We measured the diameters of the CL that were located mainly in the cortex of the ovary.

2.5. Serum and ovarian PGE2 and PGF2α assays

Ovaries were homogenized in ice-cold phosphate buffered saline (0.01 mol/l, pH 7.0–7.2) with ultrasonic cell crusher (Sonice, USA). Supernatants were obtained by centrifugation at 12000*g* for 10 min. Protein concentration was determined by BCA (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA). PGE2 and PGF2α concentrations were measured by commercially available ELISA kits (Rat PGE2 and PGF2α ELISA Kit, Cat. #: CK-E30446 and Cat. #: CK-E30588, respectively, Suzhou) using serum and supernatant of ovarian lysate. Assay was performed according to the manufacturer's instruction. The detection sensitivity range of the kits was 1.0 pg/ml and showed no cross-reactivity with a series of soluble molecules with a good reproducibility. The coefficient of variation was < 15%.

2.6. Immunohistochemistry

Sections of ovary from each group were deparaffinized in xylene and rehydrated through a series of graded alcohol washes, and washed in phosphate-buffered saline (PBS). The tissue sections were treated with 3% hydrogen peroxide (H_2O_2) solution to block endogenous peroxidase activity. Sections with 1× sodium citrate antigen repair solution were boiled in microwave at high heat for 1 min, medium heat for 2 min and low heat for 7 min and cooled down to room temperature natural. Washed in PBS then punched with solution (0.5% (*v*/v) Tween-20 and 0.5% (v/v) Triton-100 in PBS) for 15 min at room temperature. Nonspecific binding sites were blocked by 1% BSA in PBS for 1 h at room temperature, and subsequently incubated with rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA 1:100) overnight at 4 °C. After incubation with primary antibody, the sections were washed with PBST and treated with the anti-rabbit IgG secondary antibody. Protein expression was visualized with diaminobenzidine (DAB, Dako Cytomation, Carpinteria, CA) staining. The reaction was stopped with distilled water, stained with hematoxylin and dehydrated before mounting. The images were digitized by fluorescence microscopy on an IX73 microscope (Olympus Corporation, Shinjuku, Tokyo, JPN).