Three-Dimensional Visualization of Electroacupuncture Activates Brown Adipose Tissue via Sympathetic Innervation in PCOS Rats

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Research Article

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Running title:

Electroacupuncture activates sympathetic nerves in brown adipose tissue in PCOS

Abstract:

Background: Low-frequency electroacupuncture (EA) has been shown to ameliorate obesity and reproductive dysfunctions in patients with polycystic ovary syndrome (PCOS), and further explorations in PCOS-like rats showed that EA could affect white adipose tissue (WAT). However, the function and neuromodulation of brown adipose tissue (BAT) in PCOS and after EA treatment have remained unknown. The present study focused on the role of BAT in PCOS-like rats and its relationship with EA, and it characterized the three-dimensional (3D) innervation of BAT associated with activation molecules.

Methods: Twenty-one-day-old female rats were implanted with dihydrotestosterone or fed high fat diet respectively to establish PCOS-like and obesity models, and then EA treatment at “Guilai” (ST 29) and “Sanyinjiao” (SP 6) was carried out for 4 weeks. In present study, morphology, 3D imaging, molecular biology and other experimental techniques were used to study the sympathetic nerve and activity of BAT.

Results: First, dihydrotestosterone-induced PCOS-like rats showed both obvious weight gain and reproductive dysfunction, similar to what is seen in high-fat diet-induced obesity rats except for the absence of reproductive dysfunction. We found that the body weight gain was mainly caused by an increase in WAT, but surprisingly we also observed an abnormal decrease in BAT. Because both the lipid metabolism and reproductive disorders could be improved with bilateral EA at “Guilai” (ST 29) and “Sanyinjiao” (SP 6), especially the restoration of BAT, we further investigated the neuromodulation and inflammation in BAT and identified the sympathetic marker

43 tyrosine hydroxylase as one of the key factors of sympathetic nerves. We used tissue
44 clearing and 3D high-resolution imaging technology to show that crooked or
45 dispersed sympathetic nerves, but not the twisted vasculature, were reconstructed and
46 associated with the activation of BAT and are likely to be the functional target for EA
47 treatment.

48 **Conclusion:** Taken together, the results of our study highlight the significant role of
49 BAT and its sympathetic innervations in PCOS and in EA therapy.

50 **Keywords:**

51 polycystic ovary syndrome, electroacupuncture, brown adipose tissue, sympathetic
52 nerve, mitochondrial brown fat uncoupling protein 1

Introduction

Polycystic ovary syndrome (PCOS) is a complicated and heterogeneous reproductive-endocrine-metabolic disorder in women and is closely associated with obesity, insulin resistance, type 2 diabetes, liver damage, etc. Recent studies suggest that adipose tissues, including white adipose tissue (WAT), brown adipose tissue (BAT), and perigonadal fat, play important roles in the occurrence, development, and prognosis of PCOS^[1, 2]. About half of all women with PCOS suffer from central obesity and overweight^[3], and the incidence rates of infertility, cardiovascular disease, diabetes, and non-alcohol fatty liver in PCOS patients with obesity are significantly higher than in lean PCOS patients. PCOS also has a significant impact on the metabolism of the offspring^[4-6].

The function of mammalian WAT is to store and release energy, while the function of BAT is to consume energy and produce heat in response to different stimuli. The thermogenic activity of BAT primarily relies on the function of mitochondrial brown fat uncoupling protein 1 (UCP-1), which is located on the inner membrane of mitochondria. Clinical trials have demonstrated that BAT activity is weakened in PCOS patients, probably induced by central obesity^[7]. BAT transplantation can improve whole body energy metabolism in PCOS rodent models and can rescue the reduction of BAT activity^{[8][9]}. BAT is innervated by the sympathetic nervous system and can be activated by norepinephrine, which originates from sympathetic nerves and binds to adrenergic receptors expressed in BAT^{[10][11]}.

Acupuncture, a form of traditional Chinese therapy, has a long history of treating

gynecological and metabolic disorders^[12-14]. In clinical practice, electroacupuncture (EA) has proven to be effective in weight management in PCOS patients^[15], and it is suggested that EA can directly affect adipose tissue and stimulate the browning of WAT through UCP1^{[16][17]}. However, due to the limitations of traditional techniques, the mechanism through which acupuncture improves endocrine and metabolic disorders has remained unclear. Advanced three-dimensional (3D) tissue clearing techniques might provide a new way for addressing these questions. Here we applied a novel tissue-clearing method to adipose tissue in order to investigate the role of sympathetic nerves in BAT in DHT-induced PCOS-like rats and to study the mechanism of action of EA.

Materials and methods

Animals and experimental design

Twenty-one-day-old female Wistar rats (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were randomly divided into control, obesity, obesity+EA, PCOS, and PCOS+EA groups (n = 7–8 per group). All rats were housed in 12-hour light/dark conditions with constant temperature ($22 \pm 2^{\circ}\text{C}$) and humidity (45%–55%) and with free access to food and water. At postnatal day 21, the obesity model was established by feeding the rats a 60 kcal % fat diet (#D12492, Research Diets, Inc. New Brunswick, USA) for 12 weeks. Rats in the PCOS and PCOS+EA groups were implanted with a sustained-release tube containing 15 mg DHT (Sigma-Aldrich, A8380, USA) to be evenly released over 90 days. The obesity+EA and PCOS+EA rats

were given EA treatments from week 9 to week 12 after modeling. The experimental procedures were approved by the local ethics committee of Shanghai Medical College, Fudan University (No. 20130227-024).

EA treatments

Bilateral “Sanyinjiao ”(SP6) and bilateral “Guilai ” (ST29) were the acupoints used in the EA treatment. During the EA treatments, the needles were inserted to a depth of 0.5–0.8 cm in the posterior part of the medial tibia for SP6 and in the bilateral part below the umbilical for ST 29 and attached to an electrical stimulator (HANS-LH202, Huayang Co., Ltd., China) at 2 Hz and 1–2 mA. All of the needles (Suzhou Medical Appliance Factory, China) were sterile, and the acupuncture manipulation was performed under the guidance of a professional. The EA lasted for 30 minutes per day from Monday to Friday (9–11 a.m.) for 4 weeks. In this process the rats were anesthetized briefly by isoflurane (2% in a 1:1 mixture of oxygen and air, RWD Life Science Co., Shenzhen, China) and were suspended on a platform and were conscious during the EA treatment. At the end of the experiment, all rats were sacrificed by deep anesthesia to collect the tissues and serum. The control and obesity groups were sacrificed in the diestrus stage of the estrous cycle.

Estrous cycle

Vaginal smears were carried out to detect the estrous stage from week 8 after DHT implantation until the end of the experiment. Smears were obtained daily at 4–5 p.m. to determine the cyclicity stage by analyzing the predominant cell type under a microscope. The estrous cycle consists of four stages with specific major cell types:

proestrus, including round nucleated epithelial cells; estrus, including cornified squamous epithelial cells; metestrus, including cornified squamous epithelial cells and leukocytes; and diestrus, including nucleated epithelial cells and leukocytes. The representative smears were stained with hematoxylin and eosin (H&E).

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

The OGTT was performed three days prior to sacrifice. All rats were fasted for at least 12 h while water was accessible, and the second drop of blood from the tail tip was analyzed with a glucometer (ACCU-CHECK Performa, Roche, USA). The tail of the animal was properly covered by gauze after the measurement. The blood glucose level was measured before being given the oral dose of D-glucose (3 g/kg body weight, 50% concentration) and then at 30, 60, 90, and 120 min after the dose.

The ITT was performed one day before sacrifice. The operation was the same as for OGTT, and insulin (0.75 U/Kg) was injected after testing the fasting glucose level and then at 30, 60, 90, 120, and 180 min after the injection. It was necessary to prepare an oral glucose solution in case of hypoglycemia.

Micro computerized tomography (micro CT) of BAT

Eight weeks after modeling and at the end of the EA treatment, micro CT (Quantum; PerkinElmer, Hopkinton, MA, USA) was carried out to acquire images of BAT and WAT. The rats were anesthetized continuously by isoflurane (2% in a 1:1 mixture of oxygen and air, RWD Life Science Co., Shenzhen, China) with a mask in the CT machine. The X-ray parameters were 114 μ A and 70 kVp. The scanning process was managed by a computer, and the image field was 72 mm $\times$ 40 mm with 144 voxels.

Analyze 12.0 software (Analyze Direct, KS, USA) was used to acquire the CT images and for image segmentation, and the target area module and volume editing tools in the software were used to quantify the BAT volume.

Endocrine hormone and metabolic index profile

Serum samples were obtained from the abdominal aorta prior to sacrificing the rats and stored at -80°C pending enzyme linked immunosorbent assay (ELISA) of 17β -estradiol (E2), Testosterone (T), Dihydrotestosterone (DHT), Progesterone (P4), sex hormone binding globulin (SHBG), alanine transaminase (ALT), aspartate transaminase (AST), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), free fatty acids (FFA), C-reactive protein (CRP), aldosterone (ALD), chemerin, and leptin. Endogenous hormone and cholesterol levels were measured using radioimmunoassay kits and colorimetric kits (Table S3) following the manufacturer's protocols. The results were recorded by a microplate reader (SpectraMax Paradigm, Molecular Devices, USA). Duplicates were made for each sample.

Quantitative real-time PCR analysis

Total RNA of adipose tissue was isolated by Trizol reagent (9109Q, Takara Bio, Inc. Japan) according to the manufacturer's instructions, and single-stranded cDNA was synthesized from each sample (2 μg) with PrimeScript RT Master Mix (#RR036A, Takara Bio, Inc., Japan). Quantitative real-time RT-PCR (qRT-PCR) was performed with an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). The PCR parameters were set according to the manufacturer's protocols,

and amplifications were performed with a SYBR Premix Ex Taq kit (#RR420A, Takara Bio, Inc., Japan). For each sample, duplicate reactions were performed in 96-well plates, and all primers were checked to ensure the uniformity of the target gene. Primer quality was further demonstrated by the dissociation curve in the qRT-PCR test prior to use. The primer sequences for Uncoupling Protein 1 (*Ucp1*), PR/SET Domain 16 (*Prdm16*), Interleukin-1 β (*IL-1 β*), Interleukin-6 (*IL-6*), and β -actin are listed in Table 1. Relative gene expression was determined with the $2^{-\Delta\Delta CT}$ formula.

Western blot analysis

The protein content of the fat tissues was extracted with a kit (BB31226, Bestbio, China), and a BCA kit (23327, Thermo Scientific, Rockford, USA) was used to determine the protein concentration. Equal amounts (20 μ g) of protein for each treatment group were resolved using an SDS-PAGE Gel Quick Preparation kit (Beyotime Biotechnology Co, China) and transferred onto PVDF membranes. The membranes were probed with primary antibodies in 0.01 M Tris-buffered saline supplemented with Tween-20 containing 5% bovine serum albumin followed by HRP-conjugated secondary antibody. When necessary, the PVDF membranes were stripped by Western blot stripping buffer (Thermo Scientific, Rockford, USA) for 30 minutes at room temperature, washed twice in TBST, and then re-probed. The protein bands were detected with an ImageQuant LAS4000 mini-gel imaging system (GE Healthcare Life Sciences, Pittsburgh, USA). All specific protein band densities were normalized to β -tubulin as the loading control and analyzed with Image-Pro Plus 6.0

(Media Cybernetics, USA).

Tissue-clearing and three-dimensional (3D) imaging analysis

This method followed the advanced iDISCO protocol^[18]. The rats were heavily anesthetized with 20% urethane and fixed with an intracardiac perfusion of 4% PFA in PBS. All samples were postfixed overnight at 4°C and then washed with 1× PBS for 1 h at room temperature three times. The samples were then dehydrated at room temperature in 20%, 40%, 60%, 80%, for 1 h and then incubated in 100% methanol for 3 h. Samples were then bleached with 5% H₂O₂ in 20% DMSO/methanol (1 vol 30% H₂O₂/1 vol DMSO/4 vol methanol, ice cold) at 4°C overnight. After bleaching, the samples were rehydrated in 100%, 80%, 60%, 40%, and 20% methanol and then in PBS for 1 h each and then in PTx.2 (0.2% Triton X100 in PBS) twice.

Pretreated samples were incubated in permeabilization solution and blocking solution at 37°C for 2 days at most. The samples were then immunolabeled with primary antibody in PBS, 0.2% Triton X-100, 20% DMSO, and 0.3 M glycine at 37°C for the indicated time and then washed in PBS and 0.2% Tween-20 with 10 mg/ml heparin for 1 day. Samples were incubated in secondary antibody dilutions in PBS, 0.2% Triton X-100, 10 % DMSO, and 6% donkey serum at 37°C for the indicated time (Table 2) and washed in PBS and 0.2% Tween-20 with 10 mg/ml heparin for 1 day and then dehydrated in 20%, 40%, 60%, 80%, and 100% methanol for 1 h each. Finally, samples were incubated in 66% dichloromethane (Sigma 270997-12X100ML) and 33% methanol for 3 h and in 100% dichloromethane twice for 15 min to wash out the methanol. The samples were immersed in dibenzyl ether (Sigma 108014-1KG)

until clear and then stored in dibenzyl ether at room temperature.

Cleared adipose tissues were imaged on a light-sheet microscope (LS-18, Nuohai Life Science, Co., Ltd., Shanghai, China) with a 1× objective and 6 mm working distance. The 3D images were analyzed and reconstructed by Imaris (v. 9.0, Bitplane, Zurich, Switzerland). The Imaris Spot algorithm was used to semi-manually determine the identity of *UCPI*, while the Surface algorithm and Filament algorithm were used to reconstruct the sympathetic nerves.

Statistical analysis

Data are presented as the mean ± standard error of the mean. The results for three groups were analyzed by one-way ANOVA and two-way ANOVA with Tukey's test (Prism 8; GraphPad Software, San Diego, CA, USA), and p-values <0.05 were considered statistically significant.

Results

Low-frequency EA reduced body weight while restoring BAT in both PCOS-like and obese rats

To investigate the possible positive impact of EA on adipose tissue metabolism in PCOS-like rats, we first monitored the body weight change together with the distribution of adipose tissue and muscle. We found that the body weight began to increase starting from the third week after modeling in both PCOS-like and obese rats, while low-frequency EA treatment could help with the weight control (Figure 1A). Inguinal and mesenteric fat were found to be increased significantly, with a similar

therapeutic effect was seen for EA (Figure 1B). Periovarian fat also increased, but EA did not alter this trend. The muscle mass remained unchanged (Figure 1C), indicating that EA might function through effects on adipose tissue. Using micro CT, BAT was classified *in vivo* from neighboring tissue, and its volume was quantitated, which revealed the restoration of BAT by EA in both PCOS-like and obese rats (Figure 1D and D'). The observed changes in BAT were also consistent with the gross morphological and weight changes observed after sacrifice (Figure 1E and E'). Taken together, both obese and PCOS-like rats showed adipose tissue disorder, especially the reduction of BAT, and EA may improve these disorders by restoring BAT.

Reproductive and metabolic dysfunction occurred in PCOS-like rats, while only the latter occurred in obese rats

After observing the change in adipose tissue, we further investigated the serum profiles of lipid-related metabolism and liver function. As shown in Table 1, abnormal glucose metabolism was seen in both the PCOS-like and obese rats, as reflected as an increased fasting insulin level and insulin resistance. As for the detection of dyslipidemia in both PCOS-like and obese rats, representative indices such as TG, TC, LDL, ApoE, Chemerin, and FFA were increased, while HDL was decreased. Moreover, there was also a certain degree of liver dysfunction, as shown by the elevation of ALT, AST, CRP, and ALD. Interestingly, all these abnormal changes were reverted or improved with EA.

We next evaluated reproductive capacity by means of ovarian histology, cycle monitoring, and hormone detection. Reproductive disorders were only found in the

PCOS-like rats, with significantly decreased numbers of corpora lutea and abnormal estrous cycles (Figure 2A, B) as well as increased levels of T and P4 and decreased levels of SHBG (Figure 2C). Moreover, EA could improve the reproductive parameters in PCOS-like rats, thus indicating the tandem positive effects of EA on both reproductive and metabolic dysfunction in PCOS-like rats.

BAT-related neuromodulation and inflammation were associated with the therapeutic effect of low-frequency EA

To further investigate the mechanism behind changes in BAT, we performed Western blot and qPCR experiments to explore changes in BAT-related neuromodulation and inflammation. Our results showed that UCP1, an important BAT activation marker, together with the sympathetic marker TH, rebounded in scapular BAT and inguinal and mesenteric adipose tissue after EA treatment (Figure 3A). Meanwhile, the level of IL-6 was up-regulated in WAT, which was also decreased in the EA groups (Figure 3B-C). qPCR further confirmed all of these changes. (Figure S1). All of the above highlighted the potentially important role of sympathetic modulation on BAT through the complex network of UCP1 and immunoactivity.

EA increased sympathetic innervation to activate BAT through UCP1

To obtain a detailed view of the connections between the sympathetic innervation and its contact with the BAT-activated marker UCP1, we took advantage of tissue clearing methods and 3D visualization technology (Video S1). The numbers of sympathetic nerves and UCP1-positive cells were decreased in obese and PCOS-like rats, and these were restored after EA, which was similar to our protein-expression results

(Figure 4A, B). Moreover, we found that UCP1 tended to accumulate at the ends of crooked sympathetic nerves (Figure 4C). Thus, we enlarged the images and found three kinds of sympathetic nerves based on their morphological characteristics, including vascular twisted, crooked, and dispersed nerves, which could also be quantitatively classified (data not shown). Compared with the vascular twisted sympathetic nerves, the crooked and dispersed nerves were more likely to be remodeled after EA. To further define the exact relationship between UCP1 and these different kinds of sympathetic nerves, we calculated the colocalization ratio and found that EA mainly increased UCP1 with crooked or dispersed sympathetic nerves (Figure 4D), indicating that EA might act by increasing local sympathetic innervation and thus activating UCP1 in BAT.

Discussion

In the present study, we established a diet-induced obesity model and a DHT-induced PCOS-like model and observed that both simple obesity and obesity caused by hyperandrogenism were characterized by massive increases in visceral fat, especially in PCOS-like rats. We measured a series of parameters of serum lipid metabolism, including TG, TC, LDL, HDL, ApoE, FFA, chemerin, and leptin, and the results were indicative of dyslipidemia in both the PCOS and obesity models, which confirmed the phenotypic observations. In addition, DHT-induced PCOS-like rats exhibited anovulatory disorders and irregular estrous cycles, while obese rats did not show any abnormal reproductive functions. We repeated this experiment several times and

found that the weight and volume of scapular BAT in PCOS-like rats decreased to almost half of that in the control group, and this decrease was reversed after EA treatment. Similar changes occurred in diet-induced obese rats. This indicated that low-frequency EA is effective in treating obesity and its complications. In this study, we chose bilateral “Sanyinjiao (SP6)”, which is located in the posterior part of the medial tibia, and bilateral “Guilai (ST29)”, which is located in the bilateral part below the umbilical for ST 29, as the acupoints in the treatment. Previous studies have shown that EA treatment at these regions could reduce the weight of inguinal^[17] and visceral WAT^[20]. The acupoints are located in the lower abdomen and lower limbs, but the mechanism through which EA’s effect on scapular BAT is exerted far from the selected acupoints is not fully understood. We hypothesize that EA works through the extensive nervous system. Because the role of BAT in the obesity phenotype and how low-frequency EA affects BAT remain unclear, we performed a series of further experiments.

Obesity occurs when energy intake exceeds energy expenditure, and obesity increases the risk of developing diseases like dyslipidemia, insulin resistance, and hypertension. Adipose tissues play an important role in regulating systemic energy levels, especially BAT, which converts chemical energy into heat. UCP1-expressing and mitochondrial-rich adipocytes enable BAT depots to function in thermogenesis in response to cold exposure^[21]. Long-term cold exposure can stimulate the proliferation and differentiation of mature BAT precursors, increase the volume of BAT, and increase heat production. Moreover, cold stimulation can reactivate the inactivated

mature brown adipocytes^[22]. It has been reported that BAT transplantation into recipient mice increases glucose tolerance, increases insulin sensitivity, decreases body weight, and decreases fat mass, and the metabolic effects of transplantation are further improved when increasing the quantity of BAT that is transplanted ^[23]. Thus, based on the changes of BAT seen in our studies, EA might be another stimulus to active BAT like cold stimulation, but the mechanism behind this remains unclear.

Recent studies have shown that women with PCOS have lower BAT activity compared to controls, and BAT thermogenesis and the β -adrenoceptor-stimulated increase in UCP1 expression are negatively associated with androgen levels in PCOS ^[24, 25]. Similarly, transplantation of BAT into PCOS-like rats reverses anovulation, hyperandrogenism, and polycystic ovaries and significantly stabilizes menstruation and normalizes systemic insulin sensitivity^[8]. Our results are in line with these previous studies and highlight that endogenous BAT activity is closely related to the development of PCOS phenotypes and that BAT activation might provide a therapeutic option for the treatment of PCOS.

As a vital component of traditional Chinese medicine, acupuncture has a history of more than 2000 years. Acupuncture harmonizes the “Yin” and “Yang” and dredges the channels of “Qi” and “Blood” for treating diseases. Due to its safety and few side effects, acupuncture has been well-received in treating various diseases and symptoms, especially chronic metabolic illnesses ^[26, 27], and acupuncture has been reported to be a relatively effective and safe method for improving glucose metabolism and insulin sensitivity in patients with PCOS^[28]. EA effectively ameliorated abdominal obesity

and metabolic disorders in HFD-induced abdominally obese rats^[29], and clinical trials have indicated that EA can decrease the levels of ALT, AST, and TG in female patients suffering from nonalcoholic fatty liver disease, suggesting that this approach might be an important treatment for improving liver functions in these patients^[30]. Aging is characterized by an increase in adiposity and a decline in BAT activity and UCP1 expression, and EA has also been shown to significantly decrease fat mass and increase muscle mass in the elderly^[31]. In the present study, EA ameliorated excess visceral fat, liver damage, and impaired glucose tolerance in obese and PCOS-like rats, which confirmed the results of previous studies.

Adipose tissue is also a secretory organ, and adipose tissue dysfunction is responsible for the metabolic alterations and the systemic inflammatory state^[32] seen in obesity, which is characterized by a state of chronic inflammation in adipose tissue mediated by the secretion of a range of inflammatory cytokines. In parallel, there is increasing evidence that pro-inflammatory signals like TNF- α , IL-1 β , and IL-6 represent important components of the thermogenic potential of BAT and may lead to their altered capacity for energy expenditure and glucose uptake in obesity^[33]. In our present study, IL-1 β and IL-6 were detected at higher concentrations in WAT, which indicated inflammatory conditions in both the PCOS-like and the obese rats. The inflammatory effects in BAT will be further explored in the future.

Tissue clearing of adipose tissue overcomes the limitations of conventional adipose tissue histology based on tissue sections and captures holistic filamentous structures such as nerve fibers and blood vessels, and studies using tissue-clearing

methods have shown that abundant sympathetic fibers in WAT are crucial for the cold-induced beiging process^[34]. At the same time, other researchers have shown that the density of sympathetic nerve fibers is regulated by PRDM16 in adipocytes, suggesting that adipocytes can interact with neurons through a process monitored by factors secreted by adipocytes that transmit signals to nerve endings^[35]. It has been confirmed that BAT is also innervated by the sympathetic nervous system and by sensory nerves, and BAT sympathetic denervation eliminates thermogenesis. The best-known activator of BAT heat production is norepinephrine that is released from sympathetic nerves and binds to adrenergic receptors expressed on brown adipocytes. However, to the best of our knowledge the morphology of sympathetic nerve fibers in BAT has not been previously revealed, so we used a tissue-clearing method based on iDISCO^[18] to make the BAT transparent and observe the sympathetic nerves and the expression of UCP1 in PCOS-like rats. We found that the expression of UCP1 and tyrosine hydroxylase (TH) was decreased in the PCOS and obesity models and that EA treatment could rescue this pathological state, indicating that EA can upregulate sympathetic nerve and BAT activity and thus improve the endocrine disorders in PCOS.

Obesity and PCOS are characterized by sympathetic nerve overactivity, which is reflected as metabolic alterations^[36]. Meanwhile, a positive relationship between sympathetic nerve activity and T levels has been demonstrated previously^[37]. In our previous study, we found that EA might rescue PCOS pathophysiology by lowering the DHT level, improving angiogenesis, and regulating ovarian innervation^[38]. In

DHT-induced PCOS-like rats, low-frequency EA reduced the expression of genes encoding markers of sympathetic activity in adipose tissue and had beneficial effects on ovarian morphology, reflecting modulation of sympathetic outflow to the adipose tissue and ovaries^[39]. Therefore, EA might be useful for regulating the peripheral sympathetic nervous system. In conclusion, BAT plays a significant role in PCOS obesity and reproductive endocrinology, which may be one of the mechanisms through which EA regulates PCOS. The mechanism of EA in modulating the dysfunction of scapular BAT and its downstream pathways remain to be explored.

Conclusion

In this study, First, we found that the weight gaining in PCOS-like rats was mainly caused by the increase of WAT, and the abnormal decrease of BAT. As both the lipid metabolism and reproductive disorders could be improved with EA on bilateral “Guilai” (ST 29) and “Sanyinjiao” (SP 9), especially the restore of BAT, we further investigated the neuromodulation and inflammation in BAT, and found sympathetic maker tyrosine hydroxylase (TH) as one of the key factors. Taking advantage of tissue transparency and 3D high resolution imaging technology, crooked or dispersed sympathetic nerves were indicated to be reconstructed and associated with the activation of BAT, which also functioned as potential target for EA treatment. Therefore, our study highlighted the significant role of BAT and its sympathetic innervations in PCOS and EA therapeutic mechanism.

Abbreviations

405 PCOS: Polycystic ovary syndrome

406 EA: Electroacupuncture

407 DHT: Dihydrotestosterone

408 WAT: White adipose tissue

409 BAT: Brown adipose tissue

410 3D: Three-dimensional

411 UCP-1: Uncoupling protein 1

412 H&E: Hematoxylin and eosin

413 OGTT: Oral glucose tolerance test

414 ITT: Insulin tolerance test

415 Micro CT: Micro computerized tomography

416 E2: 17 β -estradiol

417 T: Testosterone

418 DHT: Dihydrotestosterone

419 P4: Progesterone

420 SHBG: Sex hormone binding globulin

421 ALT: Alanine transaminase

422 AST: aspartate transaminase

423 TG: Triglyceride

424 TC: Total cholesterol

425 LDL: Low-density lipoprotein

426 HDL: High-density lipoprotein

427 FFA: Free fatty acids

428 CRP: C-reactive protein

429 ALD: Aldosterone

430 Prdm16: PR/SET Domain 16

431 IL-1 β : Interleukin-1 β

432 IL-6: Interleukin-6

433

434 **Ethics declarations**

435 **Ethics approval and consent to participate**

436 All of the animal experiments were performed according to the Guide for the Care
437 and Use of Laboratory Animals of the Chinese National Institutes of Health. The
438 current study was approved by local ethics committee of Shanghai Medical College,
439 Fudan University.

440 **Consent for publication**

441 Not applicable.

442 **Competing interests**

443 The authors indicate no potential conflicts of interest.

444

445 **Availability of data and materials**

446 The datasets used or analyzed during the current study are available from the
447 corresponding author on reasonable request.

448

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Authors' contributions

Y.F, H.G., and X.T conceived the experiments, designed the project and protocols, and developed the collaborations. Y.W., W.H., K.L., X.X., J.S., W.L. Z.P., Y.C., R.Z., B.Z., Z.W., Z.W., C.H., and Y.W. performed the experiments. H.G. and X.T. analyzed the results. H.G. W.H., and Y.F. wrote the manuscript. X.L. and A.H. provided scientific oversight and guidance and edited the manuscript. H.G., X.T., and Y.F. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

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Figure S1. qPCR demonstrating that EA restored the activity and innervation of scapular BAT and reduced the inflammation in PCOS. (A) *Ucp1* and *Prdm16* mRNA in scapular BAT. (B) *Ucp1*, *Prdm16*, *Chemerin*, and *IL-6* mRNA in the inguinal fat pad. (C) *IL-1 β* and *IL-6* mRNA in the mesenteric fat (n = 6).

Video S1 | BAT of the control group stained using antibodies against UCP1 (red) or TH (green) for the identification of BAT activity and sympathetic nerves, respectively.

Reference

- [1] Messinis I E, Messini C I, Anifandis G, et al. Polycystic ovaries and obesity[J]. Best Practice & Research Clinical Obstetrics & Gynaecology, 2015,29(4):479-488.
- [2] Du L, Wang Y, Li C, et al. Rat BAT xenotransplantation recovers the fertility and metabolic health of PCOS mice[J]. Journal of Endocrinology, 2021,248(2):249-264.
- [3] Patel S. Polycystic ovary syndrome (PCOS), an inflammatory, systemic, lifestyle endocrinopathy[J]. The Journal of Steroid Biochemistry and Molecular Biology, 2018,182:27-36.
- [4] Xie Y, Xiao L, Li S. Effects of Metformin on Reproductive, Endocrine, and Metabolic Characteristics of Female Offspring in a Rat Model of Letrozole-Induced Polycystic Ovarian Syndrome With Insulin Resistance[J]. Frontiers in Endocrinology, 2021,12.
- [5] Battaglia C, Mancini F, Cianciosi A, et al. Cardiovascular risk in normal weight, eumenorrheic, nonhirsute daughters of patients with polycystic ovary syndrome: a pilot study[J]. Fertility and Sterility, 2009,92(1):240-249.
- [6] Kent S C, Gnatuk C L, Kunselman A R, et al. Hyperandrogenism and Hyperinsulinism in Children of Women with Polycystic Ovary Syndrome: A Controlled Study[J]. The Journal of Clinical Endocrinology & Metabolism, 2008,93(5):1662-1669.
- [7] Flávia R O, Mamede M, Bizzi M F, et al. Brown adipose tissue activity is reduced in women with polycystic ovary syndrome[J]. European Journal of Endocrinology, 2019,181(5):473-480.
- [8] Liu X, Zheng Z, Zhu X, et al. Brown adipose tissue transplantation improves whole-body energy metabolism[J]. Cell Research, 2013,23(6):851-854.
- [9] Yuan X, Hu T, Zhao H, et al. Brown adipose tissue transplantation ameliorates polycystic ovary

- syndrome[J]. *Proceedings of the National Academy of Sciences*, 2016,113(10):2708-2713.
- [10] Wang W, Seale P. Control of brown and beige fat development[J]. *Nature Reviews Molecular Cell Biology*, 2016,17(11):691-702.
- [11] Li L, Li B, Li M, et al. Switching on the furnace: Regulation of heat production in brown adipose tissue[J]. *Molecular Aspects of Medicine*, 2019,68:60-73.
- [12] Li J, Ng E H Y, Stener-Victorin E, et al. Acupuncture treatment for insulin sensitivity of women with polycystic ovary syndrome and insulin resistance: a study protocol for a randomized controlled trial[J]. *Trials*, 2017,18(1).
- [13] Johansson J, Redman L, Veldhuis P P, et al. Acupuncture for ovulation induction in polycystic ovary syndrome: a randomized controlled trial[J]. *American Journal of Physiology-Endocrinology and Metabolism*, 2013,304(9):E934-E943.
- [14] Zheng Y, Wang X, Lai M, et al. Effectiveness of Abdominal Acupuncture for Patients with Obesity-Type Polycystic Ovary Syndrome: A Randomized Controlled Trial[J]. *The Journal of Alternative and Complementary Medicine*, 2013,19(9):740-745.
- [15] Ee C, Smith C, Costello M, et al. Feasibility and acceptability of a proposed trial of acupuncture as an adjunct to lifestyle interventions for weight loss in Polycystic Ovary Syndrome: a qualitative study[J]. *BMC Complementary and Alternative Medicine*, 2018,18(1).
- [16] Kokosar M, Benrick A, Perfilyev A, et al. A Single Bout of Electroacupuncture Remodels Epigenetic and Transcriptional Changes in Adipose Tissue in Polycystic Ovary Syndrome[J]. *Scientific Reports*, 2018,8(1).
- [17] Shen W, Wang Y, Lu S, et al. Acupuncture promotes white adipose tissue browning by inducing UCP1 expression on DIO mice[J]. *BMC complementary and alternative medicine*, 2014,14(1):501.
- [18] Renier N, Wu Z, Simon D J, et al. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging[J]. *Cell*, 2014,159(4):896-910.
- [19] Choowanthanakorn M, Lu K, Yang J, et al. Targeting TRPV1 for Body Weight Control using TRPV1^{-/-} Mice and Electroacupuncture[J]. *Scientific Reports*, 2015,5(1).
- [20] Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential[J]. *Nature Medicine*, 2013,19(10):1252-1263.
- [21] Rosenwald M, Perdikari A, Röllicke T, et al. Bi-directional interconversion of brite and white adipocytes[J]. *Nature Cell Biology*, 2013,15(6):659-667.
- [22] Stanford K I, Middelbeek R J W, Townsend K L, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity[J]. *Journal of Clinical Investigation*, 2013,123(1):215-223.
- [23] Shorakae S, Jona E, de Courten B, et al. Brown adipose tissue thermogenesis in polycystic ovary syndrome[J]. *Clinical Endocrinology*, 2018.
- [24] Lerner A, Kewada D, Ahmed A, et al. Androgen Reduces Mitochondrial Respiration in Mouse Brown Adipocytes: A Model for Disordered Energy Balance in Polycystic Ovary Syndrome[J]. *International Journal of Molecular Sciences*, 2021,22(1):243.
- [25] Liu C, Zheng S, Wu W, et al. Effects of acupuncture on the hypothalamus-pituitary-adrenal axis in chronic insomnia patients: a study protocol for a randomized controlled trial[J]. *Trials*, 2019,20(1).
- [26] CABÝOGLU M T, ERGENE N, TAN U. THE MECHANISM OF ACUPUNCTURE AND CLINICAL APPLICATIONS[J]. *International journal of neuroscience*, 2006,116(2):115-125.
- [27] Zheng R, Qing P, Han M, et al. The Effect of Acupuncture on Glucose Metabolism and Lipid

- Profiles in Patients with PCOS: A Systematic Review and Meta-Analysis of Randomized Controlled Trials[J]. Evidence-Based Complementary and Alternative Medicine, 2021,2021:1-11.
- [28] Wang H, Wang Q, Liang C, et al. Acupuncture Regulating Gut Microbiota in Abdominal Obese Rats Induced by High-Fat Diet[J]. Evidence-Based Complementary and Alternative Medicine, 2019,2019:1-12.
- [29] Draz R S, Serry Z M H, Rahmy A F, et al. Electroacupuncture Versus Aerobic Interval Training on Liver Functions in Patients with Nonalcoholic Fatty Liver[J]. The Journal of Alternative and Complementary Medicine, 2020,26(1):51-57.
- [30] Zhou X, Xing B, He G, et al. The Effects of Electrical Acupuncture and Essential Amino Acid Supplementation on Sarcopenic Obesity in Male Older Adults: A Randomized Control Study[J]. Obesity Facts, 2018,11(4):327-334.
- [31] Zoico E, Rubele S, De Caro A, et al. Brown and Beige Adipose Tissue and Aging[J]. Frontiers in Endocrinology, 2019,10.
- [32] Omran F, Christian M. Inflammatory Signaling and Brown Fat Activity[J]. Frontiers in Endocrinology, 2020,11.
- [33] Jiang H, Ding X, Cao Y, et al. Dense Intra-adipose Sympathetic Arborizations Are Essential for Cold-Induced Beiging of Mouse White Adipose Tissue[J]. Cell Metabolism, 2017,26(4):686-692.
- [34] Chi J, Wu Z, Choi C H J, et al. Three-Dimensional Adipose Tissue Imaging Reveals Regional Variation in Beige Fat Biogenesis and PRDM16-Dependent Sympathetic Neurite Density[J]. Cell Metabolism, 2018,27(1):226-236.
- [35] Grassi G, Biffi A, Seravalle G, et al. Sympathetic Neural Overdrive in the Obese and Overweight State[J]. Hypertension, 2019,74(2):349-358.
- [36] Sverrisdóttir Y B, Mogren T, Kataoka J, et al. Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth?[J]. American Journal of Physiology-Endocrinology and Metabolism, 2008,294(3):E576-E581.
- [37] Tong X, Liu Y, Xu X, et al. Ovarian Innervation Coupling With Vascularity: The Role of Electro-Acupuncture in Follicular Maturation in a Rat Model of Polycystic Ovary Syndrome[J]. Frontiers in Physiology, 2020,11.
- [38] Mannerås L, Cajander S, Lönn M, et al. Acupuncture and exercise restore adipose tissue expression of sympathetic markers and improve ovarian morphology in rats with dihydrotestosterone-induced PCOS[J]. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2009,296(4):R1124-R1131.

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