

ORIGINAL ARTICLE

Changes in HbA_{1c} and circulating and adipose tissue androgen levels in overweight-obese women with polycystic ovary syndrome in response to electroacupuncture

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Summary

Aim

Insulin sensitivity is ~40% lower in women with polycystic ovary syndrome (PCOS) than in controls. We tested the hypothesis that 5 weeks of electroacupuncture treatment improves glucose regulation and androgen levels in overweight/obese women with PCOS.

Material and Methods

Seventeen women with PCOS, aged 18 to 38 years, with a body mass index (BMI) ≥ 25 kg/m² and diagnosed with PCOS were included in this experimental and feasibility study and subjected to five weeks of electroacupuncture treatments three times/week. The primary outcome was changes in whole-body glucose homeostasis measured by euglycemic hyperinsulinemic clamp before and after the intervention. Secondary outcomes were changes in HbA_{1c}, circulating catecholamines, adipocyte size and adipose tissue expression of sex steroids and nerve growth factor (NGF).

Results

No significant change in glucose homeostasis was observed, but HbA_{1c} decreased by 9.5% ($p=0.004$), circulating testosterone decreased by 22% ($p=0.0007$) and dihydrotestosterone decreased by 12% ($p=0.007$). The two vagal activity markers of plasma serotonin levels and the dopamine metabolite homovanillic acid decreased by 21% ($p=0.027$) and 20% ($p=0.011$), respectively. Adipose tissue concentrations of testosterone decreased by 18% ($p=0.049$), and androstenedione decreased by 13% ($p=0.035$), and mature NGF/proNGF ratio, a marker of sympathetic activity, increased ($p=0.04$). These changes occurred without changes in anthropometrics.

Conclusion

Five weeks of electroacupuncture treatment improves HbA_{1c} and circulating and adipose tissue androgens in women with PCOS. This effect is mediated, at least in part, via modulation of vagal activity and adipose tissue sympathetic activity. Based on these findings, we have recently initiated a randomized controlled study (NTC02647827).

Keywords: Acupuncture, adipose tissue, hyperandrogenism, insulin resistance, sympathetic nervous system.

Introduction

Women with polycystic ovary syndrome (PCOS) have a three to sevenfold increased risk of developing type 2 diabetes, and this is a major health burden (1). Independent of body weight, insulin sensitivity is ~40% lower in women with PCOS than in controls, and insulin resistance has been attributed to defects in insulin signaling in adipocytes and skeletal muscle (2). Further, women with PCOS display both insulin resistance and reduced insulin responsiveness (2). Compensatory hyperinsulinemia fuels ovarian androgen production and secretion by theca cells and reduces sex hormone binding globulin (SHBG), which in turn increases free androgen levels and further exacerbates PCOS symptoms (3). Conversely, female rats that are continuously exposed to dihydrotestosterone (DHT) from puberty exhibit irregular estrous cycles, insulin resistance and obesity (4). Thus, there is a strong association between hyperinsulinemia and hyperandrogenemia that creates a vicious circle. Further, hyperandrogenism is strongly associated with high sympathetic nerve activity in women with PCOS (5), and sympathetic nerve activity increases in response to hyperinsulinemia (6). Whether sympathetic activation is a cause or a consequence of metabolic disturbances is unclear.

Overweight and obesity are closely linked to the development of PCOS. Moreover, women with PCOS have enlarged adipocytes, which might in part explain the insulin resistance in women with PCOS (7). This highlights the role of adipose tissue dysfunction as an important mechanism by which adipose tissue can negatively affect metabolic health.

Women with PCOS require long-term individualized treatment programs. Pharmacological treatments, including the glucose-reducing drug metformin, have limitations related to adverse effects and patient compliance (8). Therefore, there is a need for inexpensive and easily administered treatments with few negative side effects. Lifestyle management, including exercise and dietary changes, is the first line of treatment for improving whole-body glucose homeostasis and preventing type 2 diabetes, and if successful it has the potential to improve most PCOS-related symptoms (8). Recently, it was demonstrated that lifestyle modification with a targeted weight loss of 7% has beneficial metabolic and reproductive effects (9). For those who have difficulties in performing exercise or who are not able to follow a diet, alternative treatments such as acupuncture might be needed.

Acupuncture needles inserted into the muscle and fat tissue and stimulated manually by rotation initiate a specific pattern of afferent activity in A-delta fibers and C-fibers (10). When needles are stimulated by low-frequency (2Hz) electrical stimulation, i.e. electroacupuncture, they

cause muscle contractions that activate specific physiological pathways similar to those from voluntary muscle contractions during exercise (11). Both exercise and low-frequency electroacupuncture have been demonstrated to increase ovulation rates and decrease circulating androgens in women with PCOS (12,13), and this effect is at least in part mediated through a decrease in muscle sympathetic nerve activity in women with PCOS (14). The activation of sensory afferents that innervate the skin, fat and muscle by low-frequency electroacupuncture might locally affect glucose uptake by increasing the glucose transporter 4 (GLUT4) concentration (15) as well as by increasing the microcirculation through the release of a number of neuropeptides (11). It also modulates sympathetic efferent activity at the spinal and supraspinal level (11,16). The dosage of acupuncture is a continuous matter of discussion, and a number of parameters can affect the physiological responses to acupuncture treatment, including the number of needles used, the placement of the needles, the type of stimulation (manual rotation of the needles or electrical stimulation with different frequencies) and the frequency, duration and total number of treatments. In the present study, we decided to give acupuncture 3 times/week because lower treatment frequency (1–2 times/week) did not improve insulin sensitivity in women with PCOS (17). Comparisons between manual stimulation and electrical stimulation of the needles demonstrate that electroacupuncture improves insulin sensitivity and modulates skeletal muscle gene and protein expression more than manual stimulation of the needles in rats (18). In the present study, we combined electrical stimulation with manual stimulation of needles which is a common stimulation paradigm in the clinic. The placement of the needles is another important factor, and if placed in the same innervation area as an organ, they might directly modulate the organ's autonomic activity. In the present study, the needles were placed in the same innervation area as the pancreas and ovaries as well as at distal points in the hands and feet to enhance the effect of local needles (11). Of note, the activation of adrenergic pathways has been shown to stimulate glucose uptake, and this provides new opportunities for the treatment of type 2 diabetes (19). Further, electroacupuncture has the potential to modulate the vagal activity of the immune system, an effect that is mediated via dopamine (20). Whether or not acupuncture has such an effect in overweight or obese women with PCOS is unknown.

In this experimental and feasibility study, we tested the hypothesis that 5 weeks of low-frequency electroacupuncture treatment given three times per week results in clinically improved glucose uptake and improved levels of HbA_{1c} and circulating sex steroids in

women with PCOS. We also hypothesized that improvements in glucose control are related to changes in adipose tissue sex steroid metabolism, to the expression of pro- and mature (m) nerve growth factor (NGF) (markers of sympathetic nerve activity), to changes in plasma noradrenaline and to vagal activity as reflected by dopamine turnover as measured by plasma dopamine, the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA).

Research design and methods

This prospective clinical study was conducted at the Sahlgrenska Academy at the University of Gothenburg, Sweden, in accordance with the Declaration of Helsinki and was approved by the Regional Ethical Review Board of the University of Gothenburg. All participants gave oral and written consent before inclusion. The study was registered at ClinicalTrials.gov (NCT01457209) and is reported according to the CONSORT and STRICTA guidelines (21).

Study participants

Overweight/obese, body mass index (BMI) ($\text{kg}/\text{m}^2 \geq 25$ to < 35) women 18 to 38 years of age with PCOS were recruited by advertisements in local newspapers, in the community and at medical clinics between October 2011 and December 2013 in the Västra Götaland region, Sweden. PCOS was diagnosed as having two out of the following three Rotterdam criteria (22): polycystic ovaries verified by ultrasound, oligo/amenorrhea (> 35 days or < 6 menstrual bleedings in the past year) or amenorrhea (total absence of menstrual bleeding in the past 90 days), and clinical signs of hyperandrogenism defined by a self-reported Ferriman–Gallwey (FG) score ≥ 8 . Women were excluded if they had taken any pharmacological treatments in the previous 3 months, had received acupuncture the last 2 months or had breastfed during the last 6 months prior to the study. Other endocrine disorders, including thyroid disease and prolactin excess, were excluded as well as those with type 1 diabetes and cardiovascular disease. Further exclusion criteria were a history of daily smoking or alcohol consumption.

Study procedure

Baseline measurements started at 7:30 a.m. after an overnight fast and were performed at day 1 to 10 of a spontaneous cycle or independent of cycle stage because the majority of the participants had oligo/amenorrhea. Anthropometric measurements, including body weight

and height, were taken in an upright position with light clothing and no shoes. BMI was calculated as body weight (kg) divided by body height (m) squared. Waist circumference was measured in centimeters at the midpoint between the iliac crest and lower rib margin at the end of expiration while standing and without clothing. Hip circumference was measured in centimeters at the widest point between waist and thighs and waist–hip ratio (WHR) was calculated. Body composition, including fat mass (%) and muscle mass (%), was measured with a Tanita foot-to-foot bioelectrical impedance device (Middlesex, UK), and blood pressure was calculated as the mean arterial blood pressure (MAP) of three measures.

Before the start of the euglycemic hyperinsulinemic clamp, blood samples were taken for later analysis of luteinizing hormone (LH), follicle stimulating hormone (FSH), sex hormone binding globulin (SHBG), testosterone, DHT, dehydroepiandrosterone (DHEA), androstenedione, estrone (E1), estradiol (E2), triglycerides, total cholesterol, apolipoprotein A (ApoA) and B (ApoB), HbA_{1c}, insulin, C-peptide and glucose as well as noradrenaline, dopamine, the dopamine metabolites DOPAC and HVA, serotonin and the serotonin metabolite 5HIAA.

Insulin sensitivity

The clamp examination was performed as described (23). In brief, insulin (Actrapid, 100 IU/mL; Novo Nordisk, Bagsvaerd, Denmark) was infused at 500 mU/mL in isotonic saline containing 2 mL of plasma from the subject to prevent insulin loss. A 10-min primed insulin infusion was followed by a constant infusion ($40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) for 120 min to reach steady state. The blood glucose levels were determined before infusion, every 10 min during the first 90 min of infusion, and every 5 min during the last 30 min of infusion. Euglycemia was maintained by infusing 20% glucose (1.11 mol/L), and the rate was adjusted to maintain a glucose level of 5.5 mmol/L. The mean glucose infusion rate (GIR) was calculated as M/I_{clamp} where M is mg glucose per kg body weight per minute, and I_{clamp} is insulin levels during the last 30 min of the clamp. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) \times fasting insulin (mU/L) / 22.5, and the C-peptide index (CPI) was calculated as fasting C-peptide (nmol/L) / fasting glucose (mmol/L) \times 100.

Adipose tissue biopsy

At baseline, a needle biopsy of subcutaneous abdominal adipose tissue was obtained under local anaesthesia at two-thirds of the distance from the iliac crest to the umbilicus. One part was snap frozen in liquid nitrogen, and one

part was immediately isolated to determine adipocyte size as described (7).

All baseline measurements were repeated within 48h after the last acupuncture treatment day 1 to 10 of a spontaneous cycle or independent of cycle stage.

Intervention

The intervention started within one week after the baseline measurements were taken. We used a Western medicine style of acupuncture, referred to as low-frequency electroacupuncture, with a fixed protocol based on previous studies of acupuncture in women with PCOS (12,13) and on experimental studies (15,24,25). Two therapists educated and experienced in Western medical acupuncture delivered the acupuncture. Treatment was given three times per week over 5 weeks, and every treatment lasted for 30 min. Two sets of needle placements were alternated for every other treatment to avoid soreness (Supplemental Table 1). Needles were inserted to a depth of 15–40 mm with the aim of reaching the muscles and structures in the hands, abdominal muscle, quadriceps muscle and lower legs with innervation corresponding to the ovaries and uterus. All acupuncture points, the number of needles and the stimulation are given in Supplemental Table 1. The needles were sterile stainless steel (Hegu Xeno, Hegu Svenska) with a length of 30 or 50 mm and a diameter of 0.30 mm. When inserted, all needles were rotated manually to evoke needle sensation (*de qi*). Needles placed in the abdominal and quadriceps muscles were connected to an electrical stimulator (CEFAR ACUS 4; Cefar-Compex Scandinavia, Landsbro, Sweden) and stimulated with a low-frequency (2 Hz) electrical signal. The intensity was adjusted to produce local muscle contractions without pain or discomfort and was adjusted every 10th minute, which was when all needles not connected to the electrical stimulator were stimulated by manual rotations to evoke *de qi* (Supplemental Table 1).

Biochemical analyses, adipose tissue extraction and western blot

Plasma glucose was measured with a One Touch Ultra2 (LifeScan). Insulin, HbA1c, total cholesterol, triglycerides, ApoA, ApoB, SHBG, LH and FSH were analysed by an accredited laboratory at the Department of Clinical Chemistry of Sahlgrenska University Hospital. Serum C-peptide was measured with a human diabetes C-peptide magnetic bead set (#171B7003M, Bio-Rad, USA). Testosterone, DHT, DHEA, E1, E2, androstendione and progesterone in serum and adipose tissue were measured by gas

chromatography-tandem mass spectrometry (GC-MS/MS) as described previously (26). For analyses of sex steroids in adipose tissue, biopsies were homogenized in 0.45 mL PBS buffer and frozen at -80°C . Measurement of plasma noradrenaline, dopamine, DOPAC, HVA, serotonin and 5HIAA was performed with a split fraction HPLC-ED system as described (27).

For western blot, adipose tissue samples (1 mg) were homogenized by mechanical dissociation in 10 μL ice cold lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA pH 8.0, 20 mM NaF, 30 mM sodium pyrophosphate ($\text{Na}_4\text{P}_7\text{O}_2$), and protease and phosphatase cocktail inhibitors (cat P8340, P5276 and P0044, Sigma)) without detergent using a polytron. The samples were centrifuged at $3,000\times g$ for 6 min at 4°C to remove the fat cake, and the supernatant was retained as the sample. Five volumes of ice cold supplemented detergent mix (6% NP40, 0.6% SDS, 1.5% NaDOC) was added to each sample followed by incubation and shaking for 45 min on ice. The samples were then centrifuged at 13,000 rpm for 20 min at 4°C . Protein concentration was quantified by the Bradford assay.

Adipose tissue protein concentration of mature mNGF and proNGF were detected and quantified using MAB5260Z clone 27/21 and EP1318Y antibodies (Merck Millipore), respectively, in a homemade sandwich ELISA device.

Western blot analysis for proNGF, p75^{NTR}, phospho-TrkA and GAPDH was performed on six tissue samples from each group. Samples (30 μg of total protein) were separated by 8% or 12% SDS-PAGE and electrophoretically transferred to a PVDF membrane overnight. The membranes were then blocked and incubated with specific antibodies (SantaCruz Biotech, CA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, MA, USA) at room temperature. The blots were developed with an ECL assay (Millipore, MA, USA). The GAPDH bands were used as a control for equal protein loading. The ImageJ software (<http://rsb.info.nih.gov/ij/>) was used for gel densitometry and protein quantification.

Sample size calculation

The sample size for detecting changes in glucose uptake as measured by the euglycemic hyperinsulinemic clamp was determined based on a previous study with a similar design but with exercise instead of acupuncture (28). We expected a $\Delta\text{M}/I_{\text{clamp}}$ change of 1.5 with a standard deviation (SD) of 2 from baseline after 5 weeks of the intervention. With a target power of 80% and the significance

level set to 5%, we required a minimum of 16 subjects to detect a 15% change in M/I_{clamp} .

Statistical analyses

Data are presented as the mean and SD or standard error of the mean (SEM) for western blot densitometries. Fisher's test for pair-wise comparisons was used to analyse changes between measurements at baseline and at follow-up after 5 weeks of acupuncture per-protocol. A p -value < 0.05 was considered significant.

Results

Clinical characteristics, treatment compliance and side-effects

In total, 21 women with PCOS were included and underwent all baseline measurements and started treatment (Supplemental Figure 1). Four dropped out after one to six treatments and did not return for follow-up, leaving 17 women who were included in the analysis. The reasons for dropout were time constraint ($n=3$) and starting a pharmacological treatment ($n=1$). The number of treatments varied from 11 to 19. Few side effects of the acupuncture treatment were reported, and the most common were temporary pain and bruises. Nine women fulfilled all three diagnostic criteria of PCOS, one presented with hyperandrogenism and oligomenorrhea, two presented with hyperandrogenism and PCO morphology, and six presented with oligomenorrhea and PCO morphology. The low number of participants did not allow sub-group analyses. Baseline characteristics of the study participants are presented in Table 1.

Changes after 5 weeks of electroacupuncture

Five weeks of electroacupuncture did not significantly affect whole-body glucose homeostasis as measured by M/I_{clamp} (Figure 1A). HbA_{1c} decreased by 9.5% ($p=0.004$) (Figure 1B), and the fasting-derived markers of insulin resistance (HOMA-IR and C-peptide index) tended to decrease ($p=0.051$ for both) after 5 weeks of treatment (Table 1).

Circulating testosterone decreased by 22% and DHT by 12% after 5 weeks of electroacupuncture treatment ($p=0.0007$ and $p=0.007$, respectively) (Figure 1C–D), with no changes in androstenedione, DHEA, E1, E2 or progesterone (Table 1). Because electroacupuncture has been shown to decrease circulating sex steroids in several studies (12,13), we used the highly sensitive and specific GC-MS/MS method to analyse adipose tissue

concentrations (for the first time) of the same panel of sex steroids as in circulation. Adipose tissue concentrations of testosterone decreased by 18% and androstenedione by 13% after 5 weeks of treatment ($p=0.049$ and $p=0.035$, respectively) (Figure 1E–F), with no changes in DHT, DHEA, E1, E2 or progesterone (Table 1). Although not significant, adipocyte size tended to decrease after 5 weeks of treatment ($p=0.092$) (Table 1). All of these changes occurred without affecting weight or any other anthropometrics.

Dopamine turnover was measured because the effect of electroacupuncture, at least in part, has been shown to be mediated via changes in the autonomic nervous system. Serotonin decreased by 21% and HVA by 20% after 5 weeks of electroacupuncture ($p=0.027$ and $p=0.011$, respectively) (Figure 2A–B). The plasma levels of dopamine, noradrenalin and DOPAC were below the level of detection of the HPLC system.

Further, NGF, a marker of sympathetic activity, was analysed in adipose tissue and we found an increase in the ratio of mNGF to pro-NGF protein after 5 weeks of treatment (Figure 3C). The most expressed forms of pro-NGF were the 50kDa form (Figure 3D), which represents a major glycosylation state, and the 34kDa form, which corresponds to the pro-NGF-A splicing variant, and neither of these changed after 5 weeks of electroacupuncture (Figure 3E–G). The expression of phosphorylated TrkA, which is the receptor of pro-NGF and mNGF, was significantly increased by electroacupuncture indicating the activation of TrkA downstream signaling pathways (Figure 3H–I), but there was no change in the protein expression of the p75^{NTR} receptor (Figure 3H, J).

Discussion

Persons with impaired glucose tolerance and compensatory hyperinsulinemia are at increased risk of developing type 2 diabetes and should be referred to lifestyle management programs, including physical exercise and dietary advice, to improve their insulin sensitivity (29,30). The increasing referrals to different complementary and alternative treatments indicate a shift towards non-drug-based therapies to complement more conventional approaches for improving health (31). To increase our knowledge of the effect and mechanism of action of one such treatment, we investigated the effect of low-frequency electroacupuncture as an alternative to lifestyle management. We focused on overweight and obese women with PCOS and found that 5 weeks of electroacupuncture improved HbA_{1c} even though there was no change in peripheral insulin sensitivity (M/I_{clamp} value). Improved glucose regulation is supported by the non-significant decrease in HOMA-IR and CPI, two

Table 1 Changes from baseline to after 5 weeks of electroacupuncture (EA) treatment (three treatments/week) in women with PCOS

Variable	Baseline (n=17)	After EA (n=17)	Δ (After – Baseline)	p *
<i>Body composition</i>				
Body mass index (kg/m ²)	30.8±4.20	30.8±3.84	-0.012±0.77	0.952
Weight (kg)	85.1±14.6	83.2±17.3	-1.84±6.59	0.314
Waist circumference (cm)	95.5±10.1	95.4±9.23	-0.12±2.39	0.921
Hip circumference (cm)	110.8±8.34	110.3±7.71	-0.47±3.06	0.586
Waist-to-hip ratio	0.86±0.074	0.87±0.08	0.003±0.02	0.751
Fat mass (%)	34.6±9.74	34.4±9.00	-0.19±1.53	0.638
Lean mass (%)	50.5±5.41	50.6±5.21	0.10±1.15	0.723
Adipocyte volume (µm ³)	117.28±9.07	112.34±12.48	-4.93±11.52	0.098
Ferriman Gallwey score	10.4±7.17	11.1±8.34	0.50±3.69	0.492
<i>Metabolic variables</i>				
ApoA1 (g/L)	1.40±0.24	1.39±0.22	-0.01±0.09	0.516
ApoB (g/L)	0.88±0.26	0.83±0.22	-0.04±0.16	0.272
ApoA1/ApoB ratio	0.65±0.18	0.64±0.20	-0.006±0.17	0.877
Cholesterol (mmol/L)	4.69±1.36	4.56±1.21	-0.13±0.70	0.434
Triglycerides (mmol/L)	1.15±0.51	1.10±0.56	-0.05±0.34	0.556
Glucose (mmol/L)	4.91±0.33	4.92±0.30	0.00±0.23	0.811
Insulin (mU/L)	12.4±6.89	11.2±5.57	-0.18±5.03	0.408
HOMA-IR	3.08±1.92	2.46±1.61	-0.62±1.21	0.051
C-peptide (ng/mL)	1.28±0.52	1.15±0.45	-0.13±0.34	0.137
C-peptide index	8.91±4.05	7.65±3.20	-1.26±2.35	0.051
<i>Endocrine variables</i>				
LH (IU/L)	6.81±5.36	7.79±9.25	0.94±8.79	0.798
FSH (IU/L)	3.96±1.39	3.96±1.87	-0.001±2.70	0.981
LH/FSH ratio	1.75±1.25	1.66±1.43	-0.08±1.11	0.831
SHBG (nmol/L)	38.2±14.9	36.9±15.3	-1.23±7.31	0.506
Progesterone (pg/mL)	1436±2877	2486±3776	1049±5376	0.432
DHEA (pg/mL)	6732±3101	8087±5417	1354±4068	0.191
E1 (pg/mL)	66.3±29.0	65.8±29.6	-0.49±28.1	0.943
E2 (pg/mL)	76.8±37.7	71.1±47.6	-5.64±55.5	0.680
<i>Adipose tissue sex steroids</i>				
Progesterone (pg/g)	12,382±24,960	27,152±38,813	14,769±52,528	0.246
DHEA (pg/g)	23,439±10,646	24,252±10,841	813±7080	0.943
E1 (pg/g)	522±273	570±280	47.8±229	0.407
E2 (pg/g)	162±116	707±1960	561±1963	0.084

All values are means±SD.

*: Fisher's test for pair-wise comparisons (baseline vs. after 5 weeks of low-frequency EA).

ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; DHEA, dehydroepiandrosterone; E1, estrone; E2, estradiol; FSH, follicle stimulating hormone; HOMA-IR, homeostasis model assessment of insulin resistance; LH, luteinizing hormone; SHBG, sex hormone binding globulin.

markers believed to primarily reflect hepatic insulin sensitivity (32), and suggests that electroacupuncture improves hepatic insulin sensitivity. These changes occurred together with a decrease in circulating and adipose tissue concentrations of androgens. The decrease in HbA_{1c} is equivalent to un-supervised exercise in persons with impaired glucose tolerance (33). Remarkably, the changes observed in our study occurred with no changes in body weight or waist circumference.

It is well known that physical exercise with muscle contraction is an effective strategy to prevent and treat type 2 diabetes (34). Exercise in insulin-resistant and overweight/obese women with PCOS improves insulin

sensitivity (28,35) and reduces fat content (35), and this effect does not appear to be related to changes in mitochondrial variables (36).

This study is the first to translate the experimental findings in the DHT-induced rat PCOS model (25) into the clinical situation by demonstrating that electroacupuncture has the potential to improve HbA_{1c} in overweight and obese women with PCOS. The decrease in HbA_{1c} by electroacupuncture is in agreement with the decrease observed with short bouts of moderate to vigorous-intensity physical activity in persons at risk for type 2 diabetes (37) and is aligned with physical activity guidelines for at-risk persons (38).

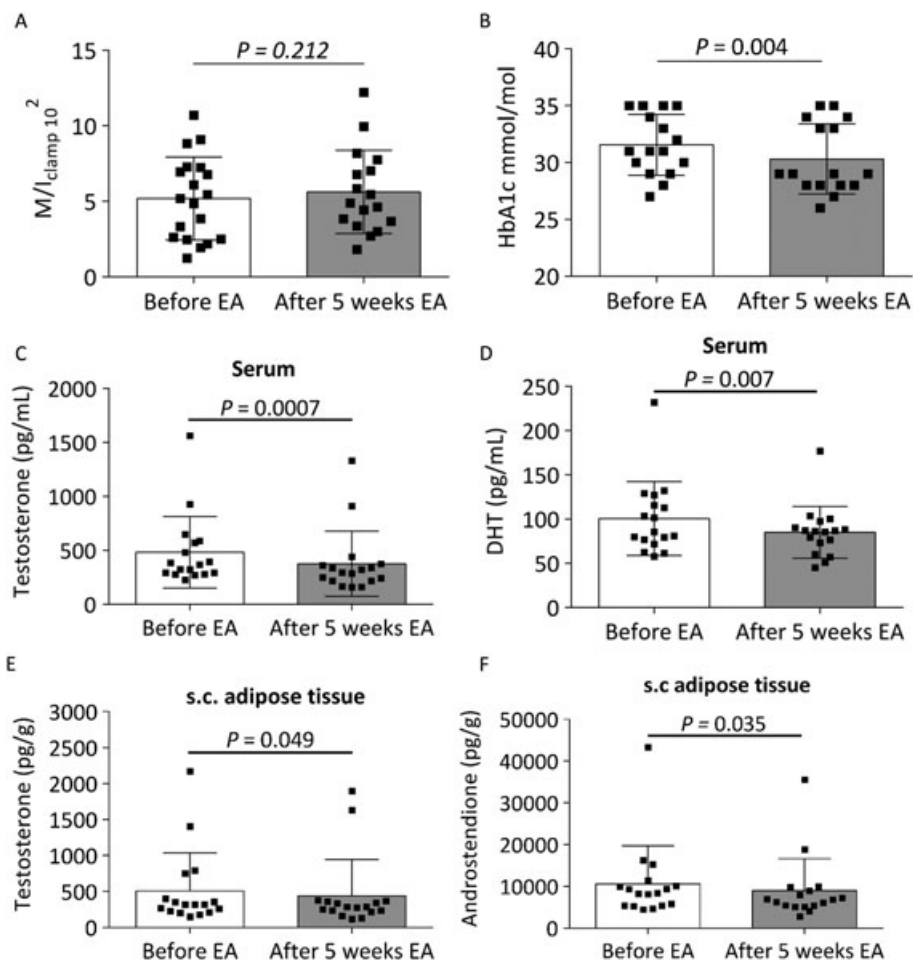


Figure 1 Changes in A) euglycemic hyperinsulinemic clamp (M/I_{clamp}); B) HbA_{1c}; C) circulating testosterone; D) circulating dihydrotestosterone (DHT); E) subcutaneous adipose tissue concentrations of testosterone and F) subcutaneous adipose tissue concentrations of androstenedione after 5 weeks of electroacupuncture (EA).

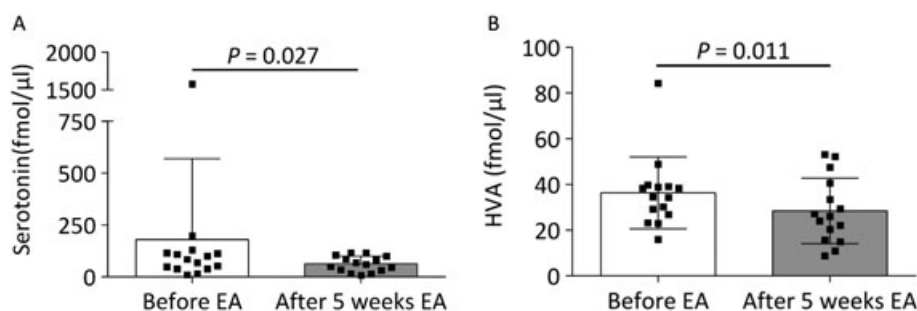


Figure 2 Changes in plasma levels of A) serotonin and B) homovanillic acid (HVA) after 5 weeks of electroacupuncture (EA).

Circulating testosterone and DHT levels were decreased by 5 weeks of electroacupuncture. This is in line with our previous observations in two independent randomized controlled trials in which menstrual frequency and ovulation frequency were also improved by electroacupuncture (12,13). Together with the marked decrease in circulating testosterone and DHT, we also found a tendency for a

decline in adipocyte size. The novelty of the present study is that we measured subcutaneous adipose tissue sex steroid concentrations with the highly sensitive and specific GC-MS/MS method, and we found that androstenedione and testosterone concentrations were significantly lower after 5 weeks of treatment, mimicking the decrease in circulating androgens. The reduction of both circulating

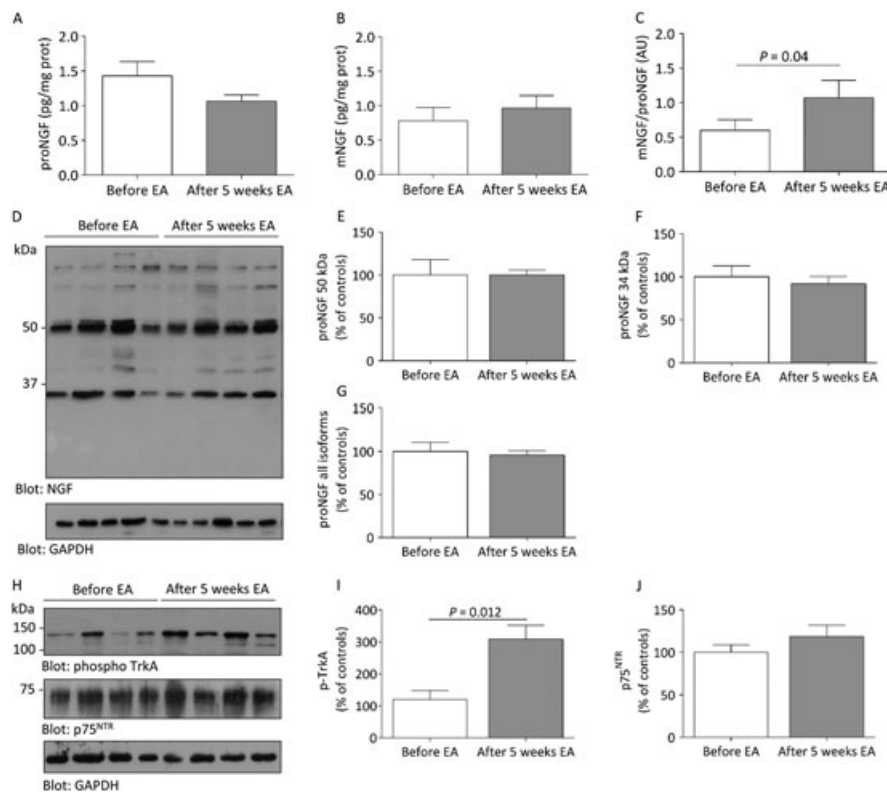


Figure 3 Changes in adipose tissue expression of A) pro nerve growth factor (proNGF), B) mature NGF (mNGF) and C) the mNGF/proNGF ratio after 5 weeks of electroacupuncture (EA) measured by ELISA. D) Representative blot of each proNGF splicing variant from before and after 5 weeks of EA measured by western blot and corresponding densitometry analyses for E) proNGF 50kDa; F) proNGF 34kDa and G) all proNGF variants detectable in adipose tissue, and H) representative western blots and corresponding gel densitometry analyses for adipose tissue expression of the mNGF/proNGF receptors I) phosphorylated-TrkA and J) p75^{NTR}.

and adipose tissue concentrations of androgens by electroacupuncture might indicate decreased production of ovarian testosterone, which in turn decreases subcutaneous adipose tissue concentrations and reflects the modulation of sex steroid-inactivating enzymes in adipose tissue. These assumptions require further investigation. The adipose tissue is known to play a central role in determining whole-body insulin sensitivity, and it has been shown that testosterone induces selective insulin resistance in female subcutaneous adipose tissue (39). Thus, decreased circulating and subcutaneous adipose tissue concentrations might directly modulate glucose regulation as reflected by a decrease in HbA_{1c} and a tendency for decreased HOMA-IR and CPI.

Because the effect of electroacupuncture is at least in part mediated via modulation of sympathetic nerve activity (14,16) and vagal activity (20), we analysed the adipose tissue protein expression of NGF. It has been shown that mNGF is a major regulator and activator of sympathetic drive towards peripheral organs and that it is involved in adipocyte metabolism *in vitro* (40), and that proNGF is able to activate both TrkA and p75^{NTR}-related signaling

pathways (41). In the present study, the adipose tissue mNGF/proNGF ratio was increased after 5 weeks of electroacupuncture, indicating that mNGF activity was stimulated. Consistent with this, the phosphorylation of TrkA, which is the high-affinity receptor for mNGF, was also increased by 5 weeks of electroacupuncture, indicating that the activation of a mNGF/TrkA-mediated downstream signalling pathway might contribute to the electroacupuncture-mediated improvement of glucose regulation and the circulating and adipose tissue concentrations of androgens. Serotonin affects glucose homeostasis and insulin resistance by acting via vagal afferent serotonergic neurons and receptors in peripheral tissue and is increased in type 2 diabetes patients (42), and HVA is responsive to changes in blood glucose levels (43). Thus, it is important to note that both of these decreased indicating that electroacupuncture was able to modulate the vagal system.

Considering that only a single group was studied and the exploratory/experimental nature of this study, the effect of the treatment might not be an actual effect but a statistical artifact. This underlies the importance of performing

controlled studies. Thus, the limitations of this study are the lack of a comparison group and its small sample size. Based on these findings, we have now been able to perform a formal sample size estimation and have initiated a randomized controlled study (Clinicaltrial.gov: NTC02647827).

In conclusion, this study has shown that 5 weeks of electroacupuncture has a significant effect on improving glucose regulation, including HbA_{1c} and circulating and adipose tissue androgen concentrations, an effect that, at least in part, is mediated via activation of adipose tissue sympathetic activity and modulation of the vagal system.

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Conflict of interest

The authors have nothing to disclose.

Author contributions

E.S.-V. and A.B. designed the study, acquired and analysed the data, and wrote the manuscript. M.M., M.K. M.S., V.P. and L.M. acquired the data and contributed to the discussion and reviewed/edited the manuscript. E.J.-H and CO acquired the data and reviewed/edited the manuscript. A.Z. screened all of the study subjects and

reviewed/edited the manuscript. C.-J.B. and K.H. contributed to study design, acquired the data and reviewed/edited the manuscript. M.L. reviewed/edited the manuscript.

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Supporting Information

Supplemental Figure 1: Study flow chart.

Supplemental Table 1: Acupuncture points, stimulation, localization, tissue in which needles were inserted and innervation areas. The two sets were alternated every other treatment