Effect of electro-acupuncture on skeletal muscle insulin sensitivity, on adipose tissue cellularity and on central nervous system in adult female rats with dihydrotestosterone induced polycystic ovary syndrome

General aim
To elucidate the mechanisms of the effect of low-frequency EA treatments, given 5 days per week which is a higher dose than given before, on insulin resistance in rats with DHT-induced PCOS. This will be done by phenotypic measurements; body composition including adipose tissue mass and adipocyte size distribution curves, DEXA, insulin clamp test and glucose uptake in muscle and adipose tissue; and by analyses of molecular mechanisms in skeletal muscles, adipose tissue and liver involved in low-frequency EA-induced increase in insulin sensitivity e.g. effects on key insulin signalling proteins, AMP-kinase activity and muscle lipid oxidation, hepatic steatosis, lipid profile; and to elucidate the effect of this treatment on ovarian function and the central nervous system.

The specific part for the thesis project (30hp) will be the phenotyping work which includes e.g. acupuncture treatment, body composition (DEXA), blood sampling and analysis and metabolic measurements (food intake, euglycemic hyperinsulinemic clamp test).

Background
Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in women of reproductive age affecting approximately 10 % (1). Excess of androgens is the cardinal feature of the syndrome as well as anovulation and disordered gonadotropin secretion (1). PCOS is also associated with obesity, insulin resistance which precedes the development of type 2 diabetes and reduced health related quality of life.

Animal models of PCOS
Animal PCOS models are needed when making the transition from scientific concepts to attaining an understanding of a human disease. There are several different animal models that have been used to study polycystic ovaries (PCO) and the similarity with the human PCOS. Recently, our group developed a new rat PCOS model that incorporates ovarian and metabolic characteristics of the syndrome. After continuous exposure to DHT, a nonaromatizable androgen, from prepuberty until adult age, the rats have typical PCO with an increased number of apoptotic follicles (2). Moreover, the rats develop obesity accompanied by enlarged adipocyte size and insulin resistance, indicating that high levels of androgens induce alterations in body composition and reduced insulin sensitivity in this PCOS model (2).

Efficacy and mechanisms of low-frequency EA
Diet, exercise, smoking, stress, and other factors adversely affect reproductive and metabolic outcomes in PCOS (3). Lifestyle-modification programs, which include exercise and diet, have been shown to assist women to lose weight and improve reproductive functioning and insulin sensitivity (4). Combining weight loss and exercise also assists with maintenance of a healthy lifestyle and enhance psychological well-being.

Many women with PCOS require prolonged treatment. Current pharmacological approaches are effective but have adverse effects. Therefore, non-pharmacological treatment strategies need to be evaluated. Clearly, acupuncture can affect PCOS via modulation of endogenous regulatory systems, including the sympathetic nervous system, the endocrine and the
neuroendocrine system (5). Experimental observations in rat models of steroid-induced polycystic ovaries (PCO) and clinical data from studies in women with PCOS suggest that low-frequency electro-acupuncture (EA) exert long-lasting beneficial effects on reproductive function (6-8). Recently, our own group demonstrated that low-frequency EA and exercise ameliorate insulin resistance in rats with DHT induced PCOS (9). This effect seems to involve regulation of adipose tissue metabolism and production since EA and exercise each partly restore divergent adipose tissue gene expression associated with insulin resistance, obesity, inflammation and sympathetic activity. In contrast to exercise, EA improves insulin sensitivity and modulates adipose tissue gene expression without influencing adipose tissue mass and cellularity (9). One reason might be the dose of EA. However, the major weakness of this study was the lack of mechanism for the effects of low-frequency EA. The effect of EA most likely involves a direct effect on skeletal muscle insulin sensitivity with secondary effects on adipose tissue. An interesting observation was the increase in soleus muscle mass, at least when expressed per total body weight, which might be relevant. However, it would be of interest to look further at the molecular mechanisms involved in muscle-contraction-induced (i.e. EA-induced) increase in insulin sensitivity in this animal model e.g. effects on key insulin signalling proteins (IRS-1 and 2, GLUT 4), AMP-kinase activity and muscle lipid oxidation. There is a large recent literature on muscle-contraction induced glucose transport. Muscle contractions during low-frequency EA and physical exercise most likely stimulate glucose uptake via an insulin-independent pathway. This is especially interesting in insulin resistant states because the contractile-induced mechanisms are still functional. The mechanism by which muscle contraction activates glucose transport has been attributed to roles for cytosolic calcium, AMP-activated protein kinase activity and nitric oxide (10). Chronic low-frequency electric stimulation and exercise has also been shown to affect insulin signal transduction, via changes in protein expression of key genes in the insulin-signalling pathway in skeletal muscle of both rats (11, 12) and humans (13). Further, exercise may enhance insulin sensitivity by affecting the oxidative capacity of the skeletal muscle (10), muscle fibre type distribution, and muscular capillarization (14). Whether these mechanisms are involved in the observed improved insulin sensitivity after low-frequency EA and exercise in our PCOS model is a challenge for future studies.

Further mechanisms involved in the metabolic disturbances may be triglyceride (TG), cholesterol and free fatty acid (FFA) concentrations and hepatic triglyceride and lipid content and the regulatory effects of ovary and central nervous system.

Specific research questions:
The effect of low-frequency EA five days per week on
1. Body composition (fat distribution and muscle mass) – DEXA
2. Metabolic disturbances (body weight, food intake, insulin sensitivity and muscle and adipose tissue glucose uptake, lipid profile and adipokines)
3. Adipocyte size distribution
4. Molecular mechanisms in skeletal muscles, adipose tissue and liver, hepatic steatosis
5. Molecular mechanisms in the ovary and central nervous system
6. CNS – food regulatory systems
in female rats with PCOS induced by continuously pre-pubertal administration of DHT.

Material and methods
Pre-pubertal, 21 days of age, female Wistar rats (Charles River) are implanted subcutaneously in the neck with a 90-day continuous-release pellets (Innovative Research of America,
Sarasota, FL) containing 7.5 mg DHT (daily dose, 83 μg), and a microchip (AVID, Norco, CA) with an identification number during lightly anesthetized with isoflurane (2% in 1:1 mixture of oxygen and air; Isova vet®, Schering-Plough AB, Stockholm, Sweden). The DHT dose has been selected after our first dose-response study (2) in order to mimic an excessive androgen release from pre-pubertal age.

Rats are randomly divided into two experimental groups and treatment starts 7 weeks after implantation of the pellet:

1. **PCOS - Electro-acupuncture (n=12):** Acupuncture needles will be placed in abdominal and in the hind-limb muscles and connected to an electrical stimulator with at 2 Hz stimulation and an intensity of 2-3mA during 25 minutes per treatment, five days / week, in total 20-25 treatments.

2. **PCOS (n=12) :** Will be handled in the same way as rats in the electro-acupuncture group without any stimulation and exercise, 25 minutes, in total 20-25 times.

3. **Control group (n=12):** Will be handled in the same way as rats in the electro-acupuncture without any stimulation and exercise, 25 minutes, in total 20-25 times.

All rats, both treated and untreated, will be conscious during treatment.

The following examinations and measurements will be done in all study groups.

**Metabolic effects are going to be study by:**

- Continuous measurements of weight to the end of the study at 14-15 weeks of age.
- Daily registration of food intake during the treatment period.
- Tail blood samples are taken after 4 weeks treatment for measurement of circulating levels of leptin, adiponectin and resistin, and lipid profile.
- Body composition will be measured by Dual-emission X-ray absorptiometry (DEXA) that is a convenient and non-invasive method based on the attenuation of x-rays. Tissues absorb x-rays to a varying degree and it is thereby possible to distinguish between soft tissue and bone. At week 13 of age total body composition will be determined by using whole body DEXA (QDR-1000/W, Hologic Inc., Waltham, MA, USA). Rats are anesthetized by inhalation of Isoflurane (Abbott Scandinavia AB, Solna, Sweden; 2% in 1:1 mixture of oxygen and air) prior to scanning. Bone mineral content (BMC), bone mineral density (BMD; BMC/body area), fat tissue mass, lean tissue mass, and total mass (BMC + lean mass + fat mass) are obtained for each rat.
- In the end of the study insulin sensitivity will be measured by Euglycemic hyperinsulinemic clamp and at the same time the glucose uptake in muscle will be investigated by $^{3}H$-2-DOG tracer technique. Insulin at a fixed rate and glucose are infused intravenously. Arterial blood samples are taken regularly for continuous analysis of the glucose concentration. The glucose concentration in blood is kept at an euglycemic level (6mM) by adjusting the glucose infusion. The glucose infusion rate at steady state is a measure of the rat’s insulin sensitivity.
- After completion of the clamp the rats are killed and tissues are dissected out
  - Adipose tissue; mesenteric, parametrial, retroperitoneal, inguinal and intrascapular
  - Muscles; soleus, tibialis and extensor digitorum longus muscles
  - Heart and Liver; hepatic steatosis Oil red O.
- Dissected tissue is placed formaldehyde buffer or in All protect, Qiagen immediately frozen in liquid nitrogen and stored in -80 until protein- and mRNA-analysis.
  - Computerized determination of adipocyte size: Part of mesenteric adipose tissue will be used for mean cell size and distribution, determined by computerized image analysis. Fat cell suspension are placed between a siliconized glass slide and a cover slip and transferred to the microscope stage. Twelve random visual fields are
photographed with a camera. Relevant surface areas are measured automatically, and diameters of the corresponding circles calculated.

Reproductive and CNS effects

- Vaginal smear is done continuously from w 5 (rats are 42 days old) to study if and when the rats start to cycle regularly.
- One ovary are dissected for morphology and one ovary and uterus are dissected for gene and protein expression and distribution, placed in all protect (Qiagen) immediately frozen in liquid nitrogen and stored in -80 until protein- and mRNA-analysis.
- Brainparts are dissected and immediately frozen in liquid nitrogen and stored in -80 until protein- and mRNA-analysis.

Ethical permission:
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