Molecular and Cellular Endocrinology 442 (2017) 125–133

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

# The impact of postnatal leuprolide acetate treatment on reproductive characteristics in a rodent model of polycystic ovary syndrome

Lady Katerine Serrano Mujica [a], Kalyne Bertolin [a], Alessandra Bridi [a], Werner Giehl Glanzner [a], Vitor Braga Rissi [a], Flávia de los Santos de Camargo [a], Renato Zanella [c], Osmar Damian Prestes [c], Rafael Noal Moresco [d], Alfredo Quites Antoniazzi [a], Paulo Bayard Dias Gonçalves [a], Melissa Orlandin Premaor [b], Fabio Vasconcellos Comim [a, b, *]

[a] Laboratory of Biotechnology and Animal Reproduction - BioRep, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil
[b] Department of Clinical Medicine, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil
[c] Laboratory of Pesticide Residue Analysis-LARP, Chemistry Department, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil
[d] Laboratory of Clinical Biochemistry, Department of Clinical and Toxicological Analysis, Federal University of Santa Maria, Santa Maria, RS, Brazil

ARTICLE INFO

*Article history:*
Received 20 July 2016
Received in revised form
11 December 2016
Accepted 13 December 2016
Available online 14 December 2016

*Keywords:*
Polycystic ovary syndrome
Developmental programming
Leuprolide acetate

ABSTRACT

In this study, a GnRH agonist, leuprolide acetate (LA), was given as a single depot injection before 48 h of life to Wistar female rats allotted to prenatal (E16-18) and postnatal androgenization (day 5 of life) by the use of testosterone propionate, looking for reproductive endpoints. Remarkably, a single injection of LA increased the estrus cycles in the postnatal group (PostN) from 0% to 25% of the estrus cycles in the postnatal LA treated group (PostN L). LA also reduced the serum testosterone levels and cysts and atretic follicles in PostN L in contrast with rats (>100 days) from the PostN group ($p = 0.04$). Prenatally androgenized rats (PreN) exhibited significant modifications in the hypothalamic genes, such as *Gnrh*. To the best of our knowledge, this is the first study to show that blockage of the GnRH axis with leuprolide acetate depot prevented the development of typical features (anovulation, cysts, atretic follicles) in a postnatal testosterone propionate rat model of PCOS.



## 1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most frequent causes of anovulatory infertility, affecting 5–10% of women of reproductive age worldwide. PCOS is an endocrine disorder characterized by hyperandrogenism and ovarian abnormalities related to a disruption in the hypothalamic–pituitary–ovarian axis (Baptiste et al., 2010; Davies et al., 2011; Franks, 1995, 2012; Glintborg, 2016).

Growing evidence in the literature has suggested that changes occurring during gestation or even early after birth could be related to many reproductive conditions that replicate PCOS features in adult life (Barker, 1990; Franks, 2012). Indeed, rhesus monkeys, sheep, and rodents exposed to high levels of androgens or aromatase inhibitors demonstrate biochemical hyperandrogenism or chronic anovulation in adult life (Abbott et al., 2005; Birch et al., 2003; Chinnathambi et al., 2012; Franks, 2012; Manneras et al., 2007; Ortega et al., 2009; Padmanabhan and Veiga-Lopez, 2013; Padmanabhan et al., 2014; Walters et al., 2012; West et al., 2001).

Rats submitted to testosterone propionate treatment developed PCOS features according to the time androgenization begins. Prenatally androgenized rats, exposed to an androgen excess *in uterus*, do not develop a consistent phenotype, and frequently exhibit corpus luteum and normal cyclicity (Tyndall et al., 2012; Tehrani et al., 2014; Wu et al., 2010; Walters et al., 2012; Slob et al., 1983). In contrast, postnatal rodent models of PCOS obtained by the administration of androgens between days 1 and 5 of life are better characterized by disrupted ovulation (Anderson et al., 1992; Huffman and Hendricks, 1981; Lee et al., 1991; Tyndall et al., 2012; Weisz and Lloyd, 1965), development of cysts, decrease or absence of corpus luteum ovulation (Anderson et al., 1992; Pinilla

* Corresponding author. Federal University of Santa Maria (UFSM), Av Roraima 1000, Building 97 – BioRep, Santa Maria,RS, 97105900, Brazil. Tel./fax: +55 55 32208752.
*E-mail address:* fcomim@ufsm.br (F.V. Comim).

http://dx.doi.org/10.1016/j.mce.2016.12.015
0303-7207/

et al., 1993; Huffman and Hendricks, 1981; Lee et al., 1991; Tyndall et al., 2012; Weisz and Lloyd, 1965), and an increase in androgen levels (Lee et al., 1991; Marcondes et al., 2015; Weisz and Lloyd, 1965). Interestingly, in the work of Tyndall et al., female rats submitted to treatment with testosterone propionate from the 15th to the 24th day of life did not exhibit changes in ovarian follicle numbers or ovary function. These results might suggest the existence of a timeline for the development of the PCOS phenotype in rats treated with testosterone propionate.

One important factor associated with the reproductive phenotype in animal models of PCOS is the GnRH axis. Changes in GnRH neurons (number and GABAergic neurotransmission), LH hypersecretion, and modification in the steroid feedback have been reported in different animal models of PCOS (Roland et al., 2010; Padmanabhan and Veiga-Lopez, 2013).

In this study, we investigated whether the blockage of the GnRH axis by the use of an agonist (leuprolide depot) could modify the development of the reproductive features of PCOS in rats androgenized *in uterus* (from the 16th to the 18th embryonic day) or after birth (5th day of life). As shown below, the treatment before 48 h of life with a single injection of leuprolide acetate depot (lasting 3–4 weeks) was able to increase ovulatory rates and reduce the cysts and atretic follicles in a postnatal androgenized rat model.

## 2. Materials and methods

### 2.1. Animals

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Santa Maria (UFSM), Brazil, under protocol number 100/14. Overall, 30 female and 20 male Wistar rats (Rattus norvegicus albinus) aged 70 days were used in this study and were housed at the Laboratory Animal Reproduction (BioRep) of the Federal University of Santa Maria (UFSM). The animals were maintained at a temperature of 22 °C, and at 55–65% humidity under artificial illumination on a light–dark cycle of 12:12 h, with daylight from 7 a.m. to 7 p.m. Food and water were given *ad libitum*.

### 2.2. Synchronization of estrus and treatment protocols

Thirty female rats were submitted to the protocol for the synchronization of estrus. They received an intraperitoneal injection of 10 IU of equine chorionic gonadotropin (eCG; Folligon™, Intervet, Brazil), followed 48 h later by 10 IU of human chorionic gonadotropin (hCG; Pregnyl™, Organon, Brazil), and were placed with a male for 24 h (Agca et al., 2013). The pups were individually identified and kept with mothers from the same group, to avoid possible bias due cross-contamination from fluids (e.g urine). Dams were maintained with their pups until weaning (21 days). After 21 days, they were transferred to polypropylene cages identified by group.

#### 2.2.1. Prenatal androgenization

Prenatal hormone exposure was accomplished by treatment of pregnant dams from embryonic days 16, 17, and 18 by a subcutaneous (s.c.) injection of 2.5 mg testosterone propionate (Androgenol™, Hertape Calier, Brazil) (PreN group), as previously described (Wu et al., 2010), while vehicle control exposures were accomplished by treatment of pregnant dams from embryonic days 16, 17, and 18 with 2.5 mg corn oil s.c. (ControlPreN).

#### 2.2.2. Postnatal androgenization

Postnatal hormone exposures were performed by the treatment of 5-day-old animals through a s.c. injection of 1.25 mg testosterone propionate (PostN), while vehicle control postnatal 5-day-old animals received a 1.25-mg corn oil s.c. (ControlPostN) (Swanson and Werfftenbosch, 1964)

#### 2.2.3. Leuprolide acetate treatment

The treatment with leuprolide acetate was realized in 2-day-old animals through an intramuscular injection (i.m.) of 0.40 mg leuprolide acetate depot (Lectrum™, Sandoz, Brazil) in prenatal androgenized (PreN L) and postnatal androgenized (PostN L) rats (Fig. 1). A dose of leuprolide was based on its use for infants with precocious puberty, that is, 0.3 mg/kg. A vehicle control was given to 2-day-old animals through a 0.40-mg corn oil i.m. injection (Control L).

Groups at the end of the study were prenatal (PreN n = 8), postnatal (PostN n = 7), control prenatal (ControlPreN n = 4), control postnatal (ControlPostN = 4), prenatal-leuprolide (PreN L n = 4), postnatal-leuprolide (PostN L n = 8), and control leuprolide (Control L n = 8). For analysis of gene expression of the ovary and hypothalamus, preN control and postN control were included as a unique control group.

### 2.3. Estrus cycle

Vaginal smears were collected on glass slides to evaluate the animal's estrus cycles from 90 days to 100 days of age. Panótico™ (Laborclin, Brazil) staining was used to analyze the vaginal cytology. Cytology was examined by a blind examiner (K.B.) with experience in this procedure. A normal estrus cycle was defined as exhibiting all phases (proestrus, estrus, metestrus, and diestrus) over a period of 4–5 days, as previously characterized in the literature (Sun et al., 2013; Feng et al., 2012). In proestrus, oval nucleated epithelial cells, occasionally with a small number of keratinocytes, were detected. In estrus, epithelial keratinocytes with irregular shapes were detected; they resembled deciduous leaves or were interconnected into pieces, among which there was a small number of nuclear epithelial cells. In metestrus, irregular epithelial keratinocytes, nucleated epithelial cells, and leukocytes were detected. In diestrus, a large number of leukocytes and a small number of nuclear epithelial cells were detected.

### 2.4. Euthanasia

At 110 days of age, the animals were transferred and then anesthetized with isoflurane plus administration of tramadol chloride (Tramadol™, Pfizer, Brazil) i.m. (20–40 mg/kg). The minimum alveolar concentration (MAC) of isoflurane used for anesthesia of the rats was estimated at 5%, at a laboratory temperature established artificially between 20 °C and 25 °C (or about 31.7 kPa or 238 mm Hg to 39.3 kPa 295 mm Hg). Between 9:00 a.m. and 1:00 p.m., blood samples were collected, and the ovaries were removed before the animals were finally euthanized with cardiac puncture under deep anesthesia in the absence of pedal and corneal reflexes.

### 2.5. Steroid analysis through UHPLC-MS/MS

The identification and quantification of the androstenedione limit of detection (LOD) 0.003 $\mu gL^{-1}$ and limit of quantification (LOQ) 0.01 $\mu gL^{-1}$, and the testosterone detection LOD 0.003 and LOQ 0.01 $\mu gL^{-1}$ were also performed by a blind examiner using ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS), as detailed below. The UHPLC-MS/MS was from Waters (USA), equipped with Acquity UPLC™ liquid chromatography, a Xevo TQ™ MS/MS triple quadrupole detector, an autosampler, a binary pump, and a column temperature controller. A nitrogen generator, model NM30L-MS (Peak Scientific, Scotland), with argon gas 6.0, was used as the collision gas. Data acquisition