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# Hormonal and testicular changes in rats submitted to congenital hypothyroidism in early life

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ABSTRACT

The goal of this study was to evaluate the influence of hypothyroidism induced by MMI, during gestation (G) or gestation plus lactation (GL) on testis and its relation with leptin in rats. Six to eight pups were killed at 90 days of age. For statistical analysis One-way ANOVA followed by the Holm-Sìdak post hoc test was used. Hypothyroidism resulted in a significant reduction in LH, FSH and testosterone and an increase in leptin serum levels ($p < 0.04$). There was a significant decrease in StAR, AR, FSHR, LHR, pSTAT3 and SOCS3 ($p < 0.04$) protein expression and in the fertility parameters ($p < 0.04$). We can conclude that hypothyroidism is associated with reduction of steroidogenesis and spermatogenesis leading to a low fertility potential in these animals. This outcome could be a consequence of low pituitary stimulus and testicular response and probably are not related with leptin hormone since its signaling pathway is down-regulated in the testis.



## 1. Introduction

It is well known that the reproductive system is mainly regulated by the hypothlamic-pituitary-gonad axis. The testis function depends on stimulation by the follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are upregulated by hypothalamic Gonadotropin-releasing hormone (GnRH) (Salisbury et al., 2008; Weiss et al., 1995). In addition to FSH and LH other hormones are also important to control the reproductive system. Evidences support a key role for both leptin and thyroid hormones (TH) in reproduction (Ramos and Zamoner, 2014).

Leptin plays a main role in body weight homeostasis (Bouret and Simerly, 2004) and has recently emerged as a relevant neuroendocrine mediator in different cell types, including testicular cells (Tena-Sempere and Barreiro, 2002). Leptin acts through leptin receptors (ObRs), which are presented in several ObR isoforms, as a result of alternative splicing, conveying different biological activities and are involved in mediating the actions of leptin in the brain and peripheral organs (Héritier and Aubert, 1997).

This hormone appears to act by inhibiting testicular steroidogenesis (Luukkaa et al., 1998) and stimulating GnRH secretion (Louis et al., 2011). A model of leptin action at different levels on hypothalamic-pituitary-testicular axis have been proposed in the past few years. A primary effect on hypothalamus is able to stimulate GnRH release and so on LH and FSH through pituitary stimulation. The stimulatory or inhibitory effect may vary accordingly to metabolic status and can impact testis in a direct way as well. Leptin action on the gonad can be responsible for testosterone (T) secretion inhibition, on the other hand testosterone can inhibit leptin secretion acting directly on white adipose tissue (WAT) (Tena-Sempere and Barreiro, 2002).

Recently we have shown that both leptin and its different receptor isoforms (OBR) are able to regulate its own gene expression both in vivo and in vitro in the testis (Alves-Pereira et al., 2012) and prostate (Colli et al., 2011). Leptin can also have an impact on testis weight and volume, the diameter of the seminiferous tubules and changes on spermatogonia, spermatocytes, sperm and Leydig cell number (Yuan et al., 2014).

The thyroid hormones effects on male reproductive system is related to proliferation and differentiation of Sertoli and Leydig cells during development, spermatogenesis and steroidogenesis and its disorders are correlated with sexual dysfunction (Holsberger and Cooke, 2005; Mendis-Handagama and Siril Ariyaratne, 2005). Thyroidectomy in adult rats leds to a decreased secretion of testosterone and 17b-hydroxy steroid dehydrogenase (HSD) activity (Chiao et al., 1999). Despite the important roles that

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THs play in the reproduction by acting in the testis, there is no consensus about how TH controls GnRH and gonandotropin synthesis and secretion (Kumar et al., 2014).

Systemic administration of leptin increases serum TSH concentration in rats, what could be a result from leptin action on hypothalamus or an outcome from an autocrine–paracrine effect exerted by locally produced leptin (Ortiga-Carvalho et al., 2002; Seoane et al., 2000).

Supporting the link between TH and leptin, it has been shown that TSH can stimulate leptin secretion by human adipose tissue in vitro (Menendez et al., 2003) and hypothyroidism is capable to reduce the expression of members of the OBR signaling pathway in the hypothalamus and pituitary of rats. As expected, hypothyroid rats are resistant to the acute anorectic action of leptin (Calvino et al., 2012) and hypothyroid mice exhibit decreased circulating leptin levels (Groba et al., 2013).

Knowing that alterations on endocrine status during the neonatal period of life may affect the susceptibility to chronic diseases in adulthood (Gombar and Ramos, 2013; Lucas, 1998), in this study we used the congenital-neonatal hypothyroidism (CH) as a model to study the influence of hypothyroidism on the adult rat testicular function and its relation with leptin. The aim of this study was to evaluate wheather adult rats submitted to congenital-neonatal hypothyroidism present changes in fertility potential, leptin, testosterone, FSH and LH serum levels, OBRa and OBRb gene expression, leptin receptor, pSTAT3, SOCS3, AR, FSHR, StAR and LHR protein expression.

## 2. Material and methods

### 2.1. Animals

All experimental procedures were developed at the Laboratory of morphometry, metabolism and cardiovascular disease of Anatomy Department in the State University of Rio de Janeiro. The study was approved by an Institutional Committee for Ethics in Animal Experimentation and were conducted in accordance with The Guide for the Care and Use of Laboratory Animals. Male and female Wistar rats were obtained from the Criation Center of Laboratory animals at the FioCruzFoundation (Cecal/Fiocruz).

### 2.2. Experimental design

Administration of the antithyroid drugs during the fetal-neonatal period of life has been extensively employed to induce a transitory congenital-neonatal hypothyroidism (CH) (Cooke et al., 1991; Aruldhas et al., 2010). In this study nine pregnant rats were divided in 3 groups according to the time they received the drug.

- Group G: free access to drinking water containing 0.03%(w/v) methimazole (MMI) from gestacional day 9.5 until parturition (D0).
- Group GL: free access to drinking water containing 0.03% (w/v) MMI from gestacional day 9.5 until day 8 (D8) after parturition.
- Group C: free access to drinking water containing no MMI during all the experimental window.

We choose to start the MMI treatment from 9.5 days of pregnancy because it is just before testis differentiation period and also because it has been shown that exposure of dams to MMI prior to day 9 of pregnancy resulted in fetal loss (Kala et al., 2002).

From day 18 of gestation, pregnant rats were observed for delivery status and it was considered as day 0 of post natal life (D0). All rats presented spontaneous vaginal delivery. Litters with less than eight pups or litters that were cannibalized in part or totally were discarted. The number of pups per litter was adjusted to six in order to maintain the lactation performance (Fischbeck and Rasmussen, 1987) and, preferentially, male offspring. Pups were not weighed at birth to prevent maternal rejection (Holemans et al., 2003). From day 3 after parturition until weaning all litters were weighted and had their linear growth evaluated daily. After weaning these parameters were evaluated in intervals of 3 days until sacrifice.

The hypothyroid state of dams and pups were confirmed by evaluation of thyroid hormones and/or TSH in the serum of all animals at D0 and D8.

After weaning all male pups (n = 6–8) had free access to pelleted rodent chow (Nuvital; Curitiba, PR, Brazil) and tap water and were maintained in groups of up to three/cage until 90 days of age when they were killed by CO2 inhalation. During the experimental period, food intake, weight rate, body mass and linear growth were evaluated. The percentages of weight rate (WR) were calculated by the following formula: WR = [(W (g) (new) − W (g) (old)/W (g) (old)]*100. Food consumption was estimated by subtracting the amount of food left on the grid from initial food weight.

At sacrifice, blood samples were rapidly obtained by cardiac puncture and the serum obtained by centrifugation (120 g for 15 min) was stored at −20 °C until analyzes of TSH, FSH, LH, free 3,5,3'-triiodothronine (FT3), free thyroxine (FT4), testosterone and leptin levels could be performed. The right testis was removed, weighted and immediately frozen at −80 °C for further analysis of protein and gene expression by Western blot and Real Time PCR, respectively. Left testis was removed and processed to aquire fertility parameters.

### 2.3. Hormone determinations

All measurements were performed in a unique assay. Serum leptin levels was determined by ELISA (Rat/Mouse LeptinELISA Cat. #EZML-82K, Millipore, Missouri, USA). Sensitivity limit was 0.08 pg/ml and intra and inter-assay variation were 1.88% and 3.93%, respectively. Serum FT3, FT4 and testosterone were measured by chemiluminescence (ICN Pharmaceuticals, Inc., CA, USA). Sensitivity limits were 0.260 pg/mL for FT3, 0.023 ng/dL for FT4 and 0.069 nmol/L for testosterone. Intra and inter-assay variation was 5.0% and 8.2% for FT3 and 3.5% and 5.4% for FT4, respectively. Serum TSH, LH and FSH were measured by multiplex kit (Rat Pituitary Magnetic Bead Panel Cat #RPTMAG-86K, Millipore, Missouri, USA). Sensitivity limit was 0.87 pg/ml for TSH, 7.62 pg/ml for FSH and 3.28 pg/ml for LH. The intra and inter-assay variation were 2.8% and 12.8%, respectively.

### 2.4. Western blot

Testis was homogenized in ice-cold lysis buffer (50 mM TRIS, 150 mMNaCl, 0,1%SDS, NP-40%, EDTA 1 mM, Na3VO4 1 mM, NaF 1 mM and 1 mM PMSF, pH 7.8). Protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). After denaturation, the samples were subjected to 10% SDS–PAGE, subsequently transferred to polyvinylidene membranes (PVDF Hybond-P, Amersham Pharmacia Biotech), blocked with 5% BSA in Tris-buffered saline (TBS; 20 mM Tris–HCl, 500 mMNaCl, pH 7.6) and incubated with primary antibodies overnight. The primary antibodies used were obtained from Santa Cruz Biotechnology, Inc., anti-OBR(1:500; sc8391), anti-βactin (1:500; sc81178), anti-AR (1:500; sc816), anti-SOCS3 (1:500; sc7009), anti-STAT3(1:500; sc483), anti-pSTAT3 (1:500; sc7993), anti FSHR (1:500; sc7798), anti-LHR (1:500; sc25828), and from Abcam (Abcam, Cambridge, UK), anti-StAR (1:500; ab58013). Subsequently, the membranes were incubated with the appropriate secondary antibody, further