Reproductive Toxicology 71 (2017) 108–117

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Betamethasone causes intergenerational reproductive impairment in male rats

Cibele dos Santos Borges[a,*], Taina Louise Pacheco[a], Katiussia Pinho da Silva[b], Fábio Henrique Fernandes[c], Mary Gregory[d], André Sampaio Pupo[b], Daisy Maria F. Salvadori[c], Daniel G. Cyr[d], Wilma De G. Kempinas[a]

[a] Department of Morphology, Institute of Biosciences of Botucatu, UNESP – São Paulo State University, Distrito de Rubião Junior s/nº, Botucatu, SP 18618-689, Brazil
[b] Department of Pharmacology, Institute of Biosciences of Botucatu, UNESP – São Paulo State University, Distrito de Rubião Junior s/nº, Botucatu, SP 18618-689, Brazil
[c] Department of Pathology, Botucatu Medical School, UNESP – São Paulo State University, Distrito de Rubião Junior s/n°, 18618-689 Botucatu, SP, Brazil
[d] Laboratory for Reproductive Toxicology, INRS-Institut Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Québec H7 V 1B7, Canada

ARTICLE INFO

Article history:
Received 19 October 2016
Received in revised form 27 March 2017
Accepted 26 April 2017
Available online 10 May 2017

Keywords:
Betamethasone intergenerational effects
Puberty onset
Sperm quality
Fertility
Seminal vesicle contractility

ABSTRACT

Prenatal betamethasone (BM) exposure in rats negatively impacts sperm quality and male fertility. Studies have shown that BM can cause multi-generational effects on the pituitary-adrenal-axis of rats. The objective of this study was to assess the reproductive development and fertility of male rats (F2) whose fathers (F1) were exposed to BM (0.1 mg/kg) on gestational days 12, 13, 18 and 19. In F2 rats, there was a significant reduction in body weights of the BM-treated group at PND 1 as well as delayed onset of puberty, and decreases in FSH levels, Leydig cell volume, sperm number and motility, seminal vesicle contractility and ejaculated volume. Furthermore, increased serum LH levels, sperm DNA damage and abnormal morphology were observed, resulting in reduced fertility. In conclusion, prenatal BM-treatment leads to intergenerational long-term reproductive impairment in male rats, raising concern regarding the widespread use of BM in preterm births.

1. Introduction

Increased levels of glucocorticoids are necessary in the later stages of pregnancy to promote the maturation of several organs, including the lungs [1]. For pregnant women at risk of preterm birth, prenatal synthetic glucocorticoids, especially betamethasone (BM), is administered to decrease the incidence of neonatal mortality due to respiratory distress syndrome [2,3].

Thus, glucocorticoids play an important role in promoting fetal lung maturation and reducing neonatal death, and because of this, have a fundamental role in determining fetal programming [4,5]. Animal studies have demonstrated that fetal exposure to elevated doses of synthetic glucocorticoids at the end of the gestational period results in behavioral, endocrine, and metabolic abnormalities [6].

Our laboratory has previously demonstrated that, *in utero*, BM-treatment during two critical periods of development (days 12 and 13, corresponding to the period of germ cell migration and proliferation, and days 18 and 19, when testosterone levels increase) resulted in several important alterations to male sexual development, sperm quality and fertility. Furthermore, the treatment altered the normal pattern of Sertoli and germ cell organization in the testis of the rat, suggesting that reproductive programming may be dramatically altered by BM-treatment [7–10].

In the past several years, studies have attempted to demonstrate the influence of intra-uterine treatment of glucocorticoids using a multi-generational approach [5,6]. Drake et al. [11,12] observed changes in body weights, as well as in the expression of growth factors and hepatic proteins of rat fetuses exposed to dexamethasone during intrauterine development in both the first and second generation. Furthermore, prenatal exposure to BM resulted in trans-generational changes to the hypothalamic-pituitary-adrenal axis, resulting in altered ACTH (adrenocorticotropic hormone) secretion and changes in both glucocorticoid and mineralocorticoid receptors in the adrenal gland [13]. Thus, knowing the effects on the male reproductive tract in first generation [7,8], the present study investigated the possible inter-generational reproductive impact of BM on sperm quality and fertility of second generation males

* Corresponding author.
E-mail address: cibelesantosborges@gmail.com (C.d.S. Borges).

http://dx.doi.org/10.1016/j.reprotox.2017.04.012
0890-6238/

whose fathers were exposed at critical stages of *in utero* sexual differentiation.

2. Materials and methods

2.1. Animals

Male (90 days old/300–350 g) and female (90 days old/225–230 g) *Wistar* rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas, and maintained under controlled conditions (25 °C, 30% air humidity, 12/12-h light/dark cycle) with food and water available *ad libitum*.

The experimental procedures used in this study were approved by the local Ethics Committee for the Use of Experimental Animals under protocol number 451-CEEA in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

2.2. Experimental design

One nulliparous female rat was mated with a male during the dark cycle of the photoperiod (Fig. 1). The detection of sperm in the vaginal smear of the female rat in estrus was considered as gestational day (GD) 1. Pregnant and lactating rats were maintained in individual cages.

Pregnant female rats were randomly allocated into two experimental groups: control (saline vehicle, n = 11) and BM-treated (0.1 mg/kg; Betamethasone 21-phosphate disodium; Sigma-Aldrich, St Louis, MO, diluted in vehicle, n = 13). Rats received an intramuscular injection of vehicle (0.1 ml/kg) or BM on days 12, 13, 18 and 19 of pregnancy. Days 12 and 13 correspond to the period of germ cell migration and proliferation while days 18 and 19 are when gestational testosterone levels increase. After birth, several analyses were performed on pups, as described previously by Borges et al. [7,8]. One male per litter was kept until post-natal day (PND) 90 (n = 11/group), and was mated with a non-treated female to generate the second generation. The detection of sperm in vaginal smears was considered as GD 1. Dam gestation body weight and body weight gain were monitored. After birth, on PND 1, the number of pups per litter (F2) and sex ratio was calculated and, the pups were randomly selected in order to have 8 pups (4 male and 4 female littermates) per lactating female in order to maintain a similar pattern of food distribution between pups. Rats were weaned at PND 21 and housed in separate cages (n = 4 males per cage).

2.3. Study 1: initial development and sperm quality

2.3.1. Anogenital distance and external examination at puberty

Rat pups (F2) were weighed and the anogenital distance was measured at PND 1 in four male rats per litter (n = 11 per group). The onset of puberty was determined by manual retraction of the prepuce in the same pups (four males/litter) [14].

2.3.2. Organ weights

One male rat per litter on PND 45 (n = 10/group) and PND 110 (n = 10/group) were weighed and euthanized. Blood was then collected (9:00 and 11:30 AM) and the right testis, seminal vesicle (full and empty, without the coagulating gland), and ventral prostate were dissected and weighed. The left testes were fixed for histology and immunohistochemistry. For animals at PND 110, the parenchyma of right testis was obtained by cutting out the albuginea and removing the testicular fluid by centrifugation (3000 rpm) for 30 min at 4 °C and frozen at −20 °C for determining sperm counts. The right cauda epididymidis was used for sperm collection for intrauterine artificial insemination and sperm quality parameters. The remaining tissue was frozen and used for measuring sperm counts, as was the caput/corpus epididymidis.

2.3.3. Serum hormonal levels

Blood samples from animals described above, on PND 45 and 110 (n = 10/group) were allowed to coagulate and the serum obtained by centrifugation (2400 rpm) for 20 min at 4 °C. Serum samples were subsequently stored at −20 °C. Serum testosterone, FSH and LH levels were determined by radioimmunoassay. Testosterone levels (serum and intratesticular) were measured using the Coat-A-Count® assay (Diagnostics Products Corporation, Los Angeles, USA), while LH and FSH levels were measured using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK). Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for testosterone. Using the hormone values, Leydig cell function was determined by measuring intratesticular testosterone levels (ITT)/LH levels (IT ratio) as described previously [15].

2.3.4. Fertility assessment *by* in utero *artificial insemination*

Rats (F2) euthanized on PND 110 (n = 10/group) were used for fertility assessment using *in utero* artificial insemination as described by Borges et al. [8]. Briefly, females in proestrus were paired with sexually experienced vasectomized males for 1 h. Receptive females were selected for the insemination procedure. Sperm were released from the right proximal epididymal cauda and a volume containing 5×10^6 sperm was injected into each uterine horn. One female was inseminated per male.

Twenty days later, the females were euthanized to evaluate fertility. After collection of the uterus and ovaries, the number of corpora lutea, implanted fetuses, and reabsorption sites were recorded.

2.3.5. Sperm motility

Sperm motility was evaluated in the same sperm sample used for artificial insemination [7]. An aliquot of 10 μl of sperm suspension was immediately transferred to a Makler chamber and

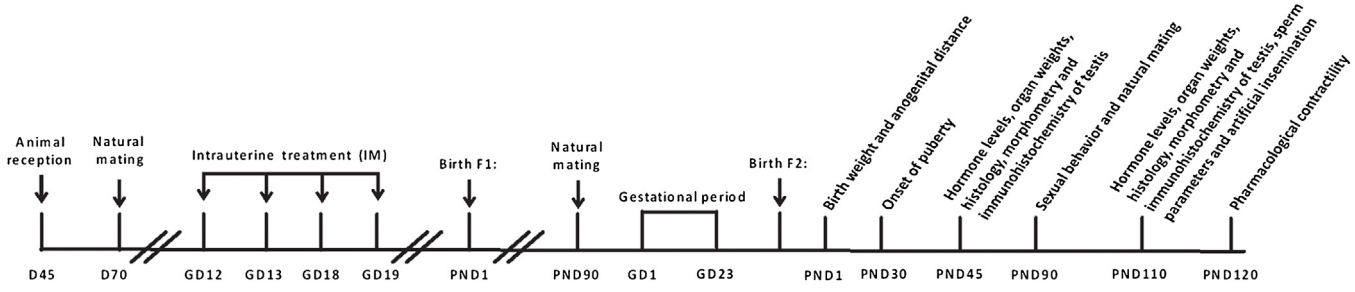

Fig. 1. Experimental design of animals whose fathers were exposed to vehicle (controls) or BM during the intrauterine development.