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Long-term adverse effects on reproductive function in male rats exposed prenatally to the glucocorticoid betamethasone

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ABSTRACT

Betamethasone is the drug of choice for antenatal treatment, promoting fetal lung maturation, decreasing the incidence of respiratory distress syndrome and neonatal mortality. Previous studies reported that prenatal treatment with this drug reduced testosterone levels, sperm quality and fertility in adult rats. We aimed to further evaluate the reproductive consequences of prenatal betamethasone exposure in male rats. Pregnant Wistar rats (n = 13/group) were separated into two groups: control (vehicle) and betamethasone- treated (0.1 mg/kg IM) and rats were injected on gestational days 12, 13, 18 and 19. Body weight, sexual behavior, reproductive organ weights, serum hormone levels, accessory glands contractility, sperm parameters, and fertility after *in utero* artificial insemination were evaluated. Our results showed that prenatal betamethasone exposure provoked a significant reduction in body weight at PND 01 and, at adulthood, decrease in FSH levels, sperm motility and production. Furthermore, seminal vesicle weight was decreased while testicular and ventral prostate weights were increased. Serum LH levels and the percentage of abnormal sperm were significantly increased. Although sexual behavior was not altered, a significant reduction in fertility in the adult rats exposed prenatally to betamethasone was noted. We concluded that prenatal betamethasone exposure leads to long-term reproductive impairment in male rats. These results may have important implications for humans, considering the use of this glucocorticoid in pregnant women.

1. Introduction

Fetal lung maturation is important before birth to avoid the respiratory distress syndrome, a serious complication of preterm birth and the primary cause of early neonatal death (Jobe and Soll, 2004; Roberts and Dalziel, 2007). The treatment used for women at risk of preterm birth to promote accelerating fetal lung maturation is denominated antenatal corticosteroid therapy, and the drug of choice is the glucocorticoid betamethasone (Drake et al., 2011).

Glucocorticoids play a key role in intrauterine programming, inducing permanent changes in physiological systems (Fowden and Forhead, 2004; Moisiadis and Matthews, 2014). (Fowden and Forhead, 2004; Moisiadis and Matthews, 2014). An important target of fetal programing is the reproductive system, once effects throughout the sexual development to adulthood can be observed.

The exposure to betamethasone *in utero* can promote changes in brain development (Yawno et al., 2014), renal function (Bi et al., 2014) and neuroendocrine alterations in different species (Matthews, 2000) as well as suppression of maternal and fetal adrenal (de Souza et al., 2001). Furthermore, the betamethasone was responsible for several alterations on male reproductive system, such as testis development in sheep (Pedrana et al., 2008; Pedrana et al., 2013) and sperm quality, fertility and testosterone levels when rats were exposed *in utero* at gestational days 12,13, 18 and 19 (Piffer et al., 2009a,b).

It is known that glucocorticoids, at high concentrations, can reduce fetal testosterone, which is fundamental for sexual differentiation (Corbier et al., 1992; Hardy et al., 2005; Page et al., 2001; Ward and Weisz, 1984). Thus, the present study

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investigated the long-term reproductive effects on male rats whose mothers were exposed to betamethasone during critical days of sexual development, with emphasis on sperm quality and fertility.

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 of the University of São Paulo State (protocol number 451-CEEA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All surgical procedures were performed under ketamine-xylazine anesthesia and euthanasia performed by decapitation following CO_2 asphyxiation.

2.2. Experimental design

Two nulliparous female rats were mated with one male, during the dark cycle of the photoperiod. The detection of sperm in the vaginal smear of rats in estrus was considered as gestational day 1 (GD1). Pregnant and lactating rats were maintained in individual cages.

Pregnant female rats were randomly allocated into two experimental groups: control (treated with saline – vehicle, n = 11) and treated (0.1 mg/kg; Betamethasone 21-phosphate disodium – Sigma-Aldrich, St Louis, MO, diluted in vehicle, n = 13). This dose was selected based on the dose used in clinic for maternal treatment and modified to rodents (Piffer et al., 2009a,b). Rats received an intramuscular injection of vehicle or betamethasone on days 12, 13, 18 and 19 of pregnancy. The injection protocol was based on the maternal corticosteroid therapy adapted by de Souza et al. (2001) which takes into account differences in the sexual differentiation in male fetuses (Pereira and Piffer, 2005; Pereira et al., 2003; Piffer et al., 2009a,b).

After birth, on post-natal day (PND) 1, the litters were cutoff for 8 animals per dam, 4 males and 4 females at random. Then, the male offspring was weighted. At PND 21, rats were housed in separate cages (n = 4 males per cage). For the experiments herein presented two male rats from each litter were used, divided into two studies.

2.3. Study 1: sexual behavior, organ weights, hormones, sperm quality and fertility

2.3.1. Evaluation of male sexual behavior

At PND 90, one male per litter was held placed in a cage alone for 5 min prior to the introduction of a sexually receptive female (70 days old). Paired animals were observed during the dark cycle of the photoperiod. Males that did not mount the female in the first 10 min were considered sexually inactive. Male sexual behavior was evaluated for 40 min and included the following parameters: latency to the first mount; latency to the first intromission; latency to the first ejaculation; number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and total number of ejaculations.

2.3.2. Organ weights

Thirty days later, these same animals tested for sexual behavior were weighed and euthanized by CO_2 asfixiation followed by decapitation. Then, blood was collected (9:00 and 11:30 AM) from the ruptured vessels and the right testis (intact and without the albuginea and fluid), epididymis, seminal vesicle (full and empty, without the coagulating gland), and ventral prostate were dissected out and weighed. The sexual glands were discarded. 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 posterior sperm counts. The right epididymal cauda was used for sperm collection for intrauterine artificial insemination and the remainder tissue was frozen for sperm counts, as well as the epididymal caput/corpus. The left testis and epididymis were kept frozen for future studies.

2.3.3. Fertility assessment

Eight animals per group were used for fertility assessment using *in utero* artificial insemination (Kempinas et al., 1998a; Klinefelter et al., 1994). In brief, females in LHRH-induced 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, first site where fertile sperm is encountered in the rat, by nicking the duct and collecting the sperm in 1–2 ml of modified human tubular fluid (HTF) medium (Irvine Scientific). After a 10-fold dilution, sperm were counted and each uterine horn was injected with a volume containing 5×10^6 sperm. One female was inseminated per male and when insemination was completed, the abdominal musculature was sutured. All surgery was performed under ketamine-xylazine anesthesia, and all efforts were made to minimize suffering.

Twenty days later, the females were euthanized by decapitation to enable fertility evaluation. After collection of the uterus and ovaries the numbers of corpora lutea, implants and reabsorptions were recorded and the following endpoints determined: fertility potential (efficiency of implantation): implantation sites/corpora lutea × 100; rate of pre-implantation loss: (number of corpora lutea − number of implantations/number of corpora lutea) × 100; rate 4of post-implantation loss: (number of implantations − number of live fetuses)/number of implantations × 100.

2.3.4. Sperm motility

Sperm motility was evaluated in the same sperm sample used for artificial insemination. For this, an aliquot of 10 μl of sperm suspensions was immediately transferred to a Makler chamber maintained at 34 °C. Using a phase-contrast microscope (400× magnification), 100 sperm were counted and classified as Type A (mobile with progressive movement) Type B (mobile without progressive movement) and Type C (immobile).

2.3.5. Sperm morphology

An aliquot of 100 μl of the sperm suspension was added to 900 μl of formol saline. To analyze sperm morphologically, smears were prepared on histological slides that were left to dry for 90 min and 200 spermatozoa per animal were analyzed in a phase-contrast microscope (400× magnification). Morphological abnormalities were classified into two general categories: head morphology (without curvature, without characteristic curvature, pin head or isolated form, *i.e.*, no tail attached) and tail morphology (broken or rolled into a spiral) (Filler, 1993). Sperm were also classified as to the presence or absence of the cytoplasmic droplet.