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Carbamazepine-exposure during gestation and lactation affects pubertal onset and spermatic parameters in male pubertal offspring

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ABSTRACT

Carbamazepine (CBZ) is an anti-epileptic drug that acts on Leydig cells, affecting steroidogenesis and causes fetal malformation. The aim of this study was to investigate the effects of CBZ on male sexual maturation and other male parameters. Rat dams were treated with CBZ during pregnancy and breastfeeding. The anogenital distance (AGD) and the anogenital index (AGI) were obtained. Testicular descent and preputial separation were also evaluated. The offspring was euthanized at PND 41 and 63. The accessory glands were weighed and the testes were collected for histopathological, morphometric and sterological analyses. The numerical density of Leydig cells and hormone dosage were obtained. CBZ caused an increase of AGI and a delay of testicular descent and of preputial separation. CBZ also caused a decrease of testosterone level and of sperm count and an increase of abnormal sperm. These results indicate that CBZ delays puberty onset and affects steroidogenesis and sperm quality.

1. Introduction

Antiepileptic drugs (AEDs) are used to treat a variety of neuropsychiatric illnesses commonly encountered in women during their reproductive years, including epilepsy and bipolar disorder. For the past few decades, carbamazepine (CBZ) has been used as an effective treatment of seizures, bipolar disorder and certain types of pain [1]. Despite their widespread use, the impact of maternal exposure on fetal development remains obscure [2]. CBZ is one of the most commonly used antiepileptic drugs in Europe among women of childbearing age [3]. Treatment of active epilepsy is important during pregnancy because seizures can lead to falls, injury and physical stress that can endanger the health of the woman and the fetus [4].

Various clinical and experimental studies involving the effects of CBZ and other AEDs on male reproduction have been carried out. However, they have focused mainly the seminal alterations occurred as a result of drug administration in adult phase. The alterations reported include reduced sperm motility and concentration, as well as sperm morphological alterations [5,6]. Another common side effect related to the CBZ long-term treatment is an increase of sex hormone binding globulin (SHBG) levels in the plasma, resulting in a reduction in the level of free bioactive testosterone [7,8]. Moreover, CBZ can alter steroidogenesis by inhibiting the cytochrome P450 monooxigenase system that is responsible for the biosynthesis of the sexual steroid hormones [9,10]. Postnatal therapy with CBZ can lead to endocrine changes and has a negative late impact on pubertal development and fertility of both boys and young men [11]. CBZ also provokes seminal alterations when used during the adulthood [5,6]. In a previous study, our group evaluated the side effects of CBZ on the spermatogenic process of rats from weaning to peripuberty, puberty, as well as their sexual maturation in adult phase; late seminiferous epithelium damage and alterations of the sex hormone levels were noted in these rats. CBZ, when administered from pre-puberty, can provoke specific side effects on rat testes, resulting in more severe damage in the adult phase [12].

In addition, CBZ is able to cross membranes in the body such as blood–brain [13] and placental barriers [14,15]. It has been shown that it can accumulate in the placental tissue and quickly reach the

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embryo, leading to toxicity in post-implantation rat embryo [16]. In addition, CBZ is also excreted into breast milk [15]. Although around 3000 pregnancies with recorded CBZ exposure have been described in the literature, each individual study on its own is too small to have the statistical power to detect risks for specific congenital malformations compared with other antiepileptic drugs [3,17–19].

As previously mentioned, the effects of post-natal CBZ administration on spermatogenesis have been addressed [5,6,12]. Conversely, experimental data about spermatogenesis and sex hormone levels in rats exposed to the CBZ during the prenatal phase and breastfeeding were not found in the literature. This is an important subject, since several studies showed that CBZ exposure in the first trimester of pregnancy increases the risk for major congenital malformations [3]. It is also important to take into account that epileptic patients need to make continuous use of anticonvulsants and that, in pregnant women, the drug withdrawal involves a balance of risks with loss of seizure control having potential implications not only for the mother and the course of her pregnancy but, probable, her child as well [20,21].

Considering all these data, we decided to scrutinize the effects of CBZ treatment on the sex hormones and LH levels, as well as on testicular, epididymal and spermatic parameters in rats from mothers exposed to CBZ during all pregnancy and breastfeeding. In addition, we hypothesize that, in these circumstances, CBZ could alter the adult Leydig cell population as well as act on the masculinization programming window, delaying the puberty onset in the progenies.

2. Material and methods

2.1. Animals

Female and male Wistar rats were housed in polypropylene cages (40 cm × 30 cm × 15 cm) filled with a layer of white pine shavings, under controlled conditions: hygiene, photoperiod (12 h light/dark cycle), humidity (60%) and temperature (22–23 °C). They had free access to tap water and commercial lab chow (Nuvilab, Nuvital Nutrientes). The females were mated overnight with males (two females per male); every morning, males were separated from the females and vaginal smears of each female were examined for the presence of sperm; sperm presence in vaginal wet smears was defined as the first day of pregnancy [22]. The rat dams were treated with CBZ or propylene glycol during whole gestation and breastfeeding period, as sequentially described. Pregnant rats were observed every morning for signs of toxicity. The pregnant rats were housed individually and observed daily for delivery. The rat dams were daily weighed during whole pregnancy and breastfeeding for analysis of weight gain. From the progenies obtained, six newborn rats (preferentially males) were kept with their dams throughout the breastfeeding period to obtain better and equal feeding for all pups. After weaning (21 days), the rats were maintained in the cages (four per cage) at standard controlled conditions. The rats were submitted to euthanasia at 41 (peripuberty [23]) and 63 (late puberty) days of age [24]. In rats, at 63 days of age, the testes are still growing and the number of step 19 spermatids per testis and per gram of testis and the daily sperm production are not stabilized [25]. The day of birth was considered postnatal day (PND) zero.

The experimental protocol followed the ethical principles adopted by the Brazilian College of Animal Experimentation. The schedule for animal care and treatment was approved by the local Institutional Ethics Committee (Protocol number 0513/11).

2.2. Experimental schedules

A total of 48 male pups from Control and CBZ rat dams were used in this study. Thus, the male progenies were randomly divided into two groups with 24 animals each: CBZ group, whose dams were 20 mg/kg/day CBZ-treated (C-8981, Sigma Chemical Co., St. Louis, MO; 99.5% purity) diluted in propylene glycol (20 mg/mL) and Control (C) group, whose dams were treated with propylene glycol (vehicle of CBZ; 99.8% purity; density 1.034 g/mL; 1 g/kg b.w.), following the same protocol of CBZ group. CBZ and propylene glycol were administered *via* intraperitoneal route (i.p.) during all pregnancy and breastfeeding. The rats of each group were again distributed into two subgroups ($n = 12$) according to the euthanasia ages (PND 41 and 63). The CBZ dose chosen in the current study is the usual anticonvulsive dose used for preventing kindled seizure in Wistar rats [26,27].

2.3. Body weight, anogenital distance and sexual development

At the PND 4, the body weight was obtained and the anogenital distance (AGD) of each male pup was recorded with a digital micrometer caliper. The AGD is defined as the distance between the anterior end of the anus and the posterior end of the genital papilla [28]. The anogenital index (AGI) of male pups was also calculated; it is defined as the ratio between the AGD and the body weight at the moment of examination [AGI (mm/g) = AGD/body weight] [29]. After the obtainment of these morphometric measurements, the neonate male rats were kept with their dams until the weaning after what they were allocated in the cages (4 rats per cage) up to the euthanasia ages: 41 or 63 days. Thus, the following subgroups ($n = 12$ each one) were formed based on the type of their dam treatment and on the euthanasia age: (a) Control subgroups: C41 and C63, (b) CBZ subgroups: CBZ41 and CBZ63.

The weights of the male progenies (subgroups C41, CBZ41, C63 and CBZ 63) were daily obtained, during the whole experiment to evaluate body weight gain. Each subgroup was constituted of rats from different dams.

For sexual development evaluation, the day of the testicular descent was monitored by daily palpation of the scrotum from PND 15 [30]. From PND 33, the preputial separation was also daily investigated through manual retraction of the prepuce [31–33]. A magnifying glass was used for this goal. The preputial separation was classified as: (a) stage 1: start of separation; (b) stage 2: prepuce could be retracted about halfway between the point of initial separation and the base of the phallus, and (c) stage 3: when separation was complete [34].

2.4. Blood collection and hormonal analysis

The peripubertal and late pubertal rats were weighed and submitted to euthanasia through CO_2 inhalation [35]. Clexane (Sanofi Winthrop Industry – France) (1 mL/kg) was administered 10 min before the euthanasia. The blood was collected from the inferior cava vein and the plasma was separated and stored at −20 °C for further hormonal analyses [12]. Luteinizing hormone (LH), testosterone and estradiol plasma levels were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using the kits (Uscn Life Science Inc., Wuhan, P.R. China) according to the manufacturer's instruction. The detection limit for LH was <144.5 pg/mL. The detection limits for testosterone and estradiol were 43.7 pg/mL and 4.38 pg/mL, and the intra-assay variations were 1.8% for estradiol, and 4.5% for testosterone.