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Original article

Manipulation of pre and postnatal androgen environments and anogenital distance in rats

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ABSTRACT

We examined the anogenital distance (AGD) plasticity in rats through the manipulation of the androgen environment *in utero* and during puberty. Dams were treated from gestation days 13–20 with vehicle, flutamide (20 mg/kg/day), di-(2-ethylhexyl) phthalate (DEHP, 750 mg/kg/day), or testosterone (1.0 mg/kg/day). After weaning, male pups were randomly assigned to one of four postnatal groups, which received the same treatments given prenatally. Sixteen treatment groups were established based on the combination of pre- and postnatal exposures. The postnatal treatments were conducted from postnatal days 23–53. *In utero* flutamide and DEHP exposure significantly shortened male AGD, although this effect was more pronounced in flutamide-exposed rats. Postnatal flutamide, DEHP, and testosterone induced slight but significant reductions in male AGD. Our study indicates that AGD is a stable anatomical landmark that reflects the androgen action *in utero*, although it can also be slightly responsive to changes in the androgen environment following pubertal exposure.

1. Introduction

Trends in male reproductive health indicate a possible increase in the incidence of reproductive disturbances in the last decades, such as low sperm count, genital malformations (hypospadias and cryptorchidism), and testicular germ cell cancer (Toppari et al., 1996; Paulozzi, 1999; Mendiola et al., 2011; Dean et al., 2012; Nordkap et al., 2012). According to Skakkebaek et al. (2001) these reproductive disorders are interrelated and constitute a common syndrome with prenatal origin known as Testicular Dysgenesis Syndrome (TDS). Genetic and environmental factors, including the increasing exposure to endocrine disrupting chemicals (EDCs) have been described as possible etiological factors of the human TDS. However, certain TDS disorders (*e.g.* low sperm counts) and other consequences of *in utero* exposures to EDCs are not manifested until late in life, which can represent a particular challenge in the study of the impact of EDCs on human health.

The anogenital distance (AGD), defined as the measured distance between the anus and the genitals, is a sexually dimorphic anatomical marker in humans and rodents that reflects the androgenic action during the formation of the reproductive system *in utero*. It has been demonstrated that the AGD and the size of male reproductive organs are determined by androgens during a specific time frame, the masculinization programming window (MPW), which occurs during gestation days 15–18 in laboratory rats and in humans between weeks 8–14 gestation (Welsh et al., 2008).

Postnatally, androgen exposure is needed for achievement of the maximum growth of androgen-dependent structures, but the degree of postnatal plasticity of the AGD is largely unknown. In humans, neonatal and adult male AGD is negatively associated with the incidence and severity of a number of reproductive alterations, including TDS components such as cryptorchidism, hypospadias and low sperm counts (Bay et al., 2006; Mendiola et al., 2011). These results corroborate experimental data with laboratory animals and indicate that neonatal AGD can be used as a

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marker of the prenatal androgenic environment (Gray et al., 2001; Welsh et al., 2008; van den Driesche et al., 2011, 2012). However, the animal literature has focused mainly on the relationship between neonatal AGD and reproductive tissues weights and malformations measured in adulthood. In order to validate the use of AGD as a stable and permanent marker of the prenatal androgen environment it is necessary to establish the impact of postnatal androgen exposure and blockage on this endpoint.

Many industrial chemicals and pharmaceuticals can impair the androgen action during the MPW and cause adverse reproductive effects in laboratory rats and in humans. In rats, administration of the potent androgen receptor antagonist flutamide during this window can shorten (feminize) the AGD and induce several reproductive abnormalities, including cryptorchidism and hypospadias (McIntyre et al., 2001). Certain phthalates, industrial chemicals used as plasticizers of polyvinyl chloride (PVC) plastics and as additive in many other products, can inhibit the testosterone production by the fetal testis and induce similar anti-androgenic effects, although the spectrum and severity of reproductive abnormalities induced by phthalates may be variable depending on rat strain susceptibility (Wilson et al., 2007).

The usefulness of AGD as a long-life marker of the androgen action during the MPW depends on the postnatal plasticity of the perineum to hormonal stimuli. Few studies have explored the effects of postnatal androgen manipulation on AGD. van den Driesche et al. (2011) demonstrated that early postnatal exposure to flutamide or di-butyl phthalate (postnatal days 1–15 *via* maternal milk) can impair penis growth, but have little or no effects on AGD. More recently, Mitchell et al. (2015) demonstrated slight but significant AGD changes in adult male rats treated with the estrogenic compound diethylstilbestrol (DES). However, in the study by Mitchell et al. (2015), the hormonal manipulation was restricted to adult life. This study aims to investigate the effects of pre- and postnatal androgen manipulation on male rat AGD. Rats were exposed to vehicle, flutamide, di-2(ethylhexyl) phthalate, and testosterone, *in utero* and later during puberty.

2. Material and methods

2.1. Animals, doses, and treatment

The study was conducted at the Laboratory of Endocrine and Reproductive Physiology of the Federal University of Paraná (UFPR). Wistar rats (*Rattus norvegicus)* were obtained from the animal facilities of UFPR after approval by the Ethics Committee on Animal Use (CEUA). All animals received food and water *ad libitum* and were maintained at controlled conditions of light (12 h light/dark cycle) and temperature (21 ± 2 °C). We selected Wistar rats as this is the standard strain used in our laboratory.

Adult female rats were mated for 3 h during the dark phase of the light/dark cycle to obtain the progenitors. The detection of sperm on the vaginal smear was considered as day zero of gestation. Mating procedures were repeated until sufficient number of progenitors was obtained for the experiment (N = 8–16/group). However, due to the large number of animals in the postnatal period, the study was divided into two blocks.

We used canola oil as vehicle (CAS 120962-03-0), Flutamide (CAS 13311-84-7 −99.5%), Di(2-ethylhexil) phthalate (CAS 117-81-7 −99.5%) and Testosterone Propionate (CAS 57-85-2 −98.1%). Canola oil and DEHP were obtained from Sigma-Aldrich (St. Louis, MO, USA), Flutamide and Testosterone were obtained from Dermoformulas laboratory (Curitiba, Brazil).

The treatment was divided in two periods (*in utero* and pubertal phase). Wistar rat dams were treated once a day from gestation day (GD) 13–20, which includes the masculinization programming window, with canola oil (control group), flutamide (20 mg/kg/day – flutamide group), DEHP (750 mg/kg/day – DEHP group), or testosterone (1.0 mg/kg/day – testosterone group). All substances were given by oral route with exception of testosterone, which was administered by subcutaneous injections. Groups receiving testing substances by oral route were also treated subcutaneously with vehicle, while animals receiving subcutaneous injections of testosterone received oral vehicle. The administration volumes for oral and subcutaneous routes were 5 ml/kg and 1.0 ml/kg, respectively. After birth, on postnatal day four (PND 4), pups were individually marked with black ink tattoo on their limbs. After weaning, male pups within each litter were randomly assigned to one of four postnatal groups using a split-litter-design (Beck et al., 2006; Ricceri et al., 2006). Each of the four groups was given one of the prenatal treatments – vehicle, flutamide, DEHP, and testosterone – at the same doses and routes. The postnatal treatments were conducted once a day during pubertal development from PND 23 to 53. Thus, sixteen treatment groups were established based on the combination of pre- and postnatal exposures. The treatment groups are shown schematically in Fig. 1. Although the split-litter design allows a uniform distribution of pups among groups, some littermates may be assigned to the same postnatal treatments (litters with more than 4 males).

The dose of flutamide was selected based on the data of McIntyre et al. (2001) which showed that doses ranging from 6.25 mg/kg/day to 50 mg/kg/day resulted in significant changes in the AGD as well as other reproductive tract abnormalities in male rats exposed *in utero*. The DEHP dose was based on the study by Parks et al. (2000), which demonstrated that *in utero* exposure to 750 mg/kg/day lowered testicular testosterone concentrations and reduced AGD, with no evidence of severe maternal or fetal toxicity. Wolf et al. (2002) indicated that exposure to doses of 0.5 mg/kg and 1.0 mg/kg of testosterone propionate (TP) during pregnancy do not induce delayed parturition or affect pup viability.

	Dams (prenatal treatment: GD13-20)	Offspring (postnatal treatment: PND23-53)
TREATMENT GROUP	VEHICLE (N=16)	VEHICLE (N=16)
		FLUTAMIDE (N=13)
		DEHP (N=15)
		TESTOSTERONE (N=16)
	FLUTAMIDE (N=15)	VEHICLE (N=15)
		FLUTAMIDE (N=15)
		DEHP (N=14)
		TESTOSTERONE (N=14)
	DEHP (N=11)	VEHICLE (N=10)
		FLUTAMIDE (N=09)
		DEHP (N=10)
		TESTOSTERONE (N=09)
	TESTOSTERONE (N=10)	VEHICLE (N=07)
		FLUTAMIDE (N=06)
		DEHP (N=06)
		TESTOSTERONE (N=07)

Fig. 1. Pre- and postnatal treatment groups. Dams were treated with vehicle, flutamide (20 mg/kg/day, orally), DEHP (750 mg/kg/day, orally) or testosterone (1.0 mg/kg/day, subcutaneously) from GD 13 to 21. After weaning male pups were split into four different groups, which received one of the prenatal treatments – vehicle, flutamide, DEHP, and testosterone – at the same doses and routes from PND 23–53.