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Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty

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ABSTRACT

This study evaluated the effects of antiandrogen exposure during the prepubertal period on reproductive development and reproductive competence in adults. Male rats were divided into two groups: flutamide, receiving 25 mg/kg/day of flutamide by oral gavage and control, receiving vehicle daily. Dosing continued from PND 21 to 44, and animals were killed on PND 50 or PND 75–80. The epididymis, prostate, vas deferens and seminal vesicle weights were lower in Flutamide group on PND 50, while on PND 80 only seminal vesicle weight was reduced. Fertility assessed by IUI revealed a decrease in the fertility potential in the flutamide-treated adults. Flutamide accelerated sperm transit time through the epididymis, impairing sperm motility and storage. A quantitative analysis of the cauda sperm membrane proteome revealed a few significant changes in protein expression. Thus, exposure to flutamide during the prepubertal period compromises the function of the epididymis along with epididymal sperm quality at adulthood.

1. Introduction

The epididymis, a highly convoluted duct that connects the efferent ducts to the vas deferens, plays a crucial role in the acquisition of progressive sperm motility and ultimate fertilizing ability [1,2]. A significant facet of this maturation process involves reorganization of the molecular architecture of the sperm plasma membrane. Membrane proteins are shed, acquired, and modified as lipid composition changes [3]. Some of these changes are directly pivotal to maturation of the sperm, but others are protective in nature [4]. The mature sperm remain protected during storage in the most distal regions of the epididymis until they are ejaculated or voided in the urine.

Specific functions occur within different regions of the epididymis with specialized microenvironments created along the interior of the epididymal duct [5]. These regional differences are established during the period of postnatal development of the epididymis, from PND 15 to 44 [6]. During this period the epididymis undergoes the requisite morphological and functional changes that resulting this regional differentiation of the duct; distinct gene and protein expression profiles can be found [7]. This differentiation of the epididymis is driven largely by the increase in circulating androgen that occurs around the time of puberty [8]. Studies are needed to elucidate damage to sperm maturation arising from disruption of the differentiation process during development.

The present study was undertaken to interfere with the prepubertal period of differentiation in order to understand the possible adverse effects on sperm quantity and quality at adulthood. We elected to attempt to alter the differentiation of the epididymis with flutamide, a potent non-steroidal antiandrogen [9] that competes for the androgen receptor and is capable of reducing the physiological action of testosterone on target tissues when endogenous tissue levels of androgen are submaximal [10,11]. Between PND 21 and 44 androgen levels in the epididymis are on the rise, but far

Abbreviations: PND, postnatal day; IUI, in utero insemination; GD, gestational day.

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less than adult levels [8]. The choice of an anti-androgen agent was due to extensive environmental exposure of the general population to this group of endocrine disruptors, which include agricultural pesticides, industrial chemicals, pharmaceuticals, and others, many times occurring as complex mixtures of anti-androgens that need not have a common active metabolite to produce cumulative adverse effects [12–15]. Based on the notion that developmental exposure to endocrine disruptive chemicals may have adverse consequences on reproductive competence in men, we believe that this experimental model is currently relevant to toxicology as well as reproductive biology.

2. Material and methods

2.1. Animals

Immature Wistar male rats (21 days old) and Wistar female rats (45 days old) were supplied by the Central Biotherium of UNESP – Univ Estadual Paulista. During the experiment, animals were allocated individually in polypropylene cages, with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (±23 °C) and lighting conditions (12 L/12 D photoperiod). Rat chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP) and filtered tap water were provided ad libitum. Experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Biosciences Institute/UNESP Ethics Committee for Animal Research (protocol number 21/08).

2.2. Experimental design

Male rats were randomly divided into two experimental groups: control group (C, $n = 30$) and flutamide group (F, $n = 30$). The F group rats received daily oral gavage doses of flutamide (Sigma Aldrich–25 mg/kg, at a volume of 3 ml/kg) diluted in corn oil, while C group received only the vehicle (corn oil), at the same volume. Animals were treated from PND 21 to PND 44 corresponding to the prepubertal development of the epididymis. Rats were weighed daily during treatment and on alternate days after treatment. The dose of flutamide chosen in this study is effectively antiandrogenic, as described previously [16]. No clinical signs of toxicity were observed in any of the experiments herein described. The study was conducted in two steps; Experiment 1 and Experiment 2, and described as follows.

2.3. Experiment 1

In this experiment, male ($n = 40$) and female ($n = 30$) Wistar rats were used. The age at acquisition of preputial separation was evaluated starting on PND 30 as an indicator of the onset of puberty. The observation criterion adopted was the age when the prepuce first separates from the glans penis, not considering the complete retraction of the prepuce [17]. On PND 50, 20 male rats ($n = 10$/group) were killed for an evaluation of reproductive organ weights and serum hormone levels. The other 20 male rats ($n = 10$/group) were evaluated at 70 days of age for sexual behavior and reproductive competence following natural mating with naïve females. A 10-day post-mating interval was allowed to permit recovery of sperm reserves; subsequently, at the age of 80 days these male were killed for evaluation of reproductive organ weights, sexual hormone levels, sperm motility and morphology, and the sperm membrane proteome.

2.3.1. Evaluation of sexual behavior and natural mating

At 70 days of age, male rats from each experimental group ($n = 10$/group) were placed individually in polycarbonate crystal boxes, measuring 44 cm × 31 cm × 16 cm, 5 min before introduction of one sexually receptive, adult female rat (70 days old). Sexual receptivity was determined by vaginal smear and by lordosis exhibition in the presence of a sexually experienced, vasectomized male of proven sterility. These females were synchronized to achieve estrous with a single subcutaneous injection of 80 μg of luteinizing releasing hormone (LHRH) agonist (Sigma Chemical Co., St Louis, MO) approximately 115 h prior to the sexual behavior evaluation.

Paired animals were observed in the dark period of the cycle in a separate room under dim red light and all sexual behavior tests were performed 2–4 h after the beginning of the dark period. The following parameters were observed for 40 min: latency to the first mount, intromission, and 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 [18–22]. Males that did not mount in the initial 10 min were considered sexually inactive. After the sexual behavior evaluation was complete, paired animals were kept together for an additional 4 h. After this period, males and females were separated and vaginal smears were collected to confirm that mating occurred.

2.3.2. Fertility evaluation

Twenty days later (GD20), naturally inseminated females were killed by decapitation. After collection of the uterus and ovaries the numbers of corpora lutea, implants, reabsorptions, live and dead fetuses were determined. From these results the following parameters were calculated: fertility potential (efficiency of implantation): implantation sites/corpora lutea × 100; rate of preimplantation loss: [number of corpora lutea − number of implantations/number of corpora lutea] × 100; and rate of postimplantation loss: [number of implantations − number of live fetuses]/number of implantations × 100.

2.3.3. Euthanasia, body weight and reproductive organ weights of male rats

At 50 or 80 days of age 10 male rats per experimental group were killed by decapitation. The right testis, epididymis and vas deferens, ventral prostate and seminal vesicle (without the coagulating gland) were removed and their wet weights (absolute and relative to body weight) were recorded.

2.3.4. Serum testosterone, FSH and LH levels

After decapitation, blood was collected (between 9:00 and 11:30 AM) and serum was obtained by centrifugation (1236 × *g*, for 20 min at 4 °C). The concentrations of testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined by the technique of double antibody radioimmunoassay. Testosterone assay was performed using a TESTOSTERONE MAIA® kit (Biochem Immuno System). The LH and FSH assays were done using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK, USA). All samples were assayed in duplicate and in the same assay to avoid inter-assay errors. The intra-assay error was 3.4% for LH, 2.8% for FSH and 4% for testosterone.

2.3.5. Sperm motility and morphology

Immediately after euthanasia of 80-day-old animals, sperm were obtained from the left vas deferens duct and diluted in 2 ml of modified HTF medium (Human Tubular Fluid, IrvineScientific®), pre-warmed at 34 °C. A 10 μl aliquot was placed in a Makler chamber (Irvine, Israel) and analyzed under a phase-contrast microscope (Leica DMLS) at 200× magnification. One hundred sperm were evaluated per animal and classified for motility into: type A: mobile, with progressive trajectory; type B: mobile, with non-progressive trajectory; type C: immotile [23].

With the aid of a syringe and needle, sperm were recovered from the right vas deferens by flushing with 1.0 ml of saline formol. To analyze the 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) [24]. 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) [25]. Sperm were also classified as to the presence or absence of the cytoplasmic droplet.

2.3.6. Quantitative evaluation of the sperm membrane proteome

Sperm were obtained from the proximal cauda epididymis [26] by nicking the duct with a number 11 scalpel and allowing sperm to disperse into 2 ml of Sperm Isolation Buffer (95 ml/l 10× HBSS, 0.35 g/l $NaHCO_3$, 4.2 g/l HEPES, 0.9 g/l glucose, 10 ml/l Na pyruvate, 25 mg/l STI, pH 7.4). One ml was transferred to a microcentrifuge tube and washed twice by centrifugation (2000 rpm, 5 min, 4 °C) in the same buffer, with freshly-added 0.2 mM phenylmethylsulphonyl fluoride (PMSF, Sigma, St. Louis, MO). After the final wash, sperm were extracted for 1 h at room temperature with 1 ml of 80 mM n-octyl-B-glucopyranoside in 10 mM Tris, pH 7.2 containing freshly added PMSF. Following a final centrifugation (2000 rpm, 5 min, 4 °C), the supernatant was removed and frozen (−70 °C).

Prior to 2-D gel electrophoresis, samples were thawed, and each extract was concentrated with 1 mM Tris buffer, pH 7.2, by three centrifugations (3,000 rpm, 30 min, 4 °C) in Ultrafree-4 centrifugation filter units (Millipore, Bedford, MA). Protein concentration was determined by the Bradford method, using Bradford Reagent (Sigma Aldrich®). The absorbance was read by spectrophotometry (SPECTRO 22RS, Digital Spectrophotometer – Quimis) and protein concentrations determined by interpolation from standard curve using seven different albumin concentrations (μg/ml): 0, 60, 80, 100, 200, 400 and 600 ($R^2 = 0.99$). Sample volumes containing 30 μg of protein were lyophilized, and protein was solubilized for 30 min at room temperature in 45 μl of sample buffer consisting of 5.7 g of urea, 4 ml of 10% NP-40, 0.5 ml of ampholytes (3–10 only; Serva, Heidelberg), and 0.1 g of dithiothreitol per 10 ml. Isoelectric focusing (750 V, 3.5 h) was carried out in capillary tube gels consisting of 6.24 g of urea, 1.5 ml of acrylamide solution (30% acrylamide, 1.2% bisacrylamide), 2.25 ml of 10% NP-40, and 0.65 ml of ampholytes (3–10 only) per 10 ml. Molecular weight separation was carried out in mini 14% acrylamide gels (200 V, 1 h). Gels were fixed in 40% ethanol (v/v) and 10% acetic acid (v/v) in ultrapure water, fluorescence stained using Krypton Protein Stain (Pierce Biotechnology) and, finally, immersed in destaining solution (5% acetic acid in ultrapure water) for 5 min.

A Fluoro Image Analyser (FLA-5100; Fujifilm) was used to scan gels and capture high resolution images with a 532 nm laser light source. Progenesis Same Spots Software (2.0) was used for background correction, spot matching, and spot area quantification. Spots corresponding to proteins whose expression was significantly altered (upregulated or downregulated) were punched from 2D gels using an automated Ettan spot picker. Gel punches were destained twice in an ammonium