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Pharmacology, Biochemistry and Behavior 71 (2002) 301–305

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Reproductive changes in male rats treated perinatally with an aromatase inhibitor

Daniela C.C. Gerardin, Oduvaldo C.M. Pereira*

Department of Pharmacology, Institute of Biosciences, São Paulo State University 18618-000 Botucatu, São Paulo, Brazil

Received 11 February 2001; received in revised form 14 June 2001; accepted 11 September 2001

Abstract

The effects of maternal exposure to aromatase inhibitor during the perinatal period of sexual brain differentiation were studied. The fertility was assessed in adult, male rat offspring of aromatase inhibitor-treated dams. The following results were obtained: (1) Sexual maturation, body weight, and wet weights of testis, pituitary, seminal vesicle, ventral prostate, and levatori ani muscle were unchanged at adult life. (2) Fifty percent of the animals were able to mate with normal females, which became pregnant but exhibited an increased number of preimplantation loss. (3) There was a decrease in the number of spermatozoa found in the testes and in the daily sperm production. (4) Of those, 25% of the male rats treated with aromatase inhibitor did not present male sexual behavior, showing female behavior when pretreated with estrogen. These results indicate that perinatal exposure to aromatase inhibitor during the critical period of male brain sexual differentiation has a long-term effect on the reproductive physiology and behavior of male rats.

Keywords: Aromatase inhibitor; Brain sexual differentiation; Fertility; Reproduction; Sperm production; Rat

1. Introduction

The process of sexual differentiation in rats begins in the last phase of gestation and continues into the first week to 10 days of postnatal life. It is also well established that sexual behavioral dimorphism is determined at least partially by exposure to androgen during the critical period of sexual differentiation of the neural tissue (Maclusky and Naftolin, 1981). Exposure to testosterone or its metabolites during this period is critical for masculinization and defeminization of sexual behavior, for the establishment of gonadotropin secretion patterns, and also for various morphological indices. In the absence of testosterone or its metabolites, sexually dimorphic structures and functions are feminized (Rhees et al., 1997). However, it is not androgen "per se" that is responsible for masculinizing the brain (Roselli and Klosterman, 1998), it is necessary to have the conversion of androgen to estrogen. Then, the conversion of testosterone to estradiol via cytochrome *P*450 aromatase is an important step in the sexual differentiation process. Specifically in rats, this activity is expressed in several sites of the brain. In general, the sites of aromatase expression in various brain regions include areas involved in regulating neuroendocrine function and reproductive behavior (Lephart, 1996). The manipulation of steroid hormones during prenatal and early postnatal development may alter the capacity to express the intrinsic reproductive behavior in adult rats (Parsons et al., 1984). Thus, sexual differentiation of the hypothalamus of male and female rats involves complex phenomena and an important participation of estrogen, as well as androgens (Dohler, 1991). In addition, the reproductive function is impaired by exposure to estrogen in the perinatal life of rats, but the mechanisms involved in this effect are distinct for males and females (Pereira et al., 1997). On the basis of these considerations, the aim of the present study was to investigate the effects of aromatase inhibitor during the prenatal period of brain sexual differentiation on the later fertility of male rats.

2. Method

2.1. Animals

After acclimatization in standard conditions (temperature at 25 ± 1 °C, humidity 55 ± 5%, and light from

* Corresponding author. Tel.: +55-14-6802-6253; fax: +55-14-6821-3744.

E-mail address: pereira@ibb.unesp.br (O.C.M. Pereira).

0091-3057/01/$ – see front matter
PII: S0091-3057(01)00667-0

Table 1
Body weight and wet weight of organs from male rats

Weight	Control	Aromatase inhibitor
Body (g)	386.31 ± 10.9	341.74 ± 15.32
Testes (g)	1.59 ± 0.03	1.55 ± 0.03
Pituitary (mg)	7.82 ± 0.48	6.58 ± 0.57
Seminal vesicle (mg)	179.1 ± 14.74	157.17 ± 7.43
Ventral prostate (mg)	385.08 ± 28.38	324.79 ± 21.41
Levatori ani muscle (mg)	286.43 ± 21.94	298.22 ± 21.54

Values expressed as means ± S.E. of body weight and wet weight of organs from male rats of control rats and rats treated with aromatase inhibitor. Ten animals/group. There was no significant difference between the groups ($P > .05$, by Student's t-test).

06:00 to 18:00 h), virgin female Wistar rats (200 ± 10 g) were mated within their own colony. Daily inspections of the vaginal smear were then carried out and the first day of pregnancy was considered as the morning in which spermatozoa were found. These pregnant females were separated into two groups according to treatment administered to their pups, as described below. The animals used in this study were maintained in accordance with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation.

2.2. Experimental groups

2.2.1. Control rats

On the 22nd day of pregnancy, the female rats were anaesthetized with ethyl ether, killed, and laparotomized. Their pups were immediately removed and then fostered to recipient dams on the first day of lactation.

2.2.2. Treated with aromatase inhibitor

On the morning of the 21st and 22nd days of pregnancy, the pregnant rats received 1 mg/kg/day of aromatase inhibitor (Letrozol, Novartis Pharma, Basiléia Swiss) by oral gavage (Tobin and Canny, 1998) dissolved in corn oil. This dose has been previously demonstrated to lead to a significant decrease in the concentration of estrogen in the serum of female rats (Bhatnagar et al., 1993). Six hours after the second administration, they were anaesthetized with ethyl ether, killed, and laparotomized. Their male pups were immediately removed and then fostered to recipient dams on the first day of lactation.

Each recipient dam always received eight male newborns. These pups were maintained with their recipient dams until the 21st day of lactation. At this age, they were housed in collective cages.

2.3. Testicular descent

Since the age of 15 days, the male rats of each group were examined daily for determination of testicular descent.

2.4. Body weight, weight of organs, and volume of testes

At 90 days of age, some rats (10/group) were weighed, anaesthetized under ethyl ether inhalation, and killed. The testes, pituitary, ventral prostate, seminal vesicle, and levatori ani muscle were removed and their wet weight were determined. The volume of the testes (in cm^3) were determined ($V=(4/3)\pi ab^2$, in which a = half-bigger axis and b = half-minor axis).

2.5. Fertility test

After the males reached maturity, each one of them was individually housed in a large cage with five regularly cycling females of the same age. Vaginal smears were daily examined for the presence of spermatozoa (indicating Day 1 of pregnancy). On the 21st day of pregnancy, all females were anaesthetized under ethyl ether and killed. After removing the uterine horns, the rate of pregnancy, the number of implantation sites, alive fetuses, dead fetuses, reabsorbtion sites, and corpora lutea were recorded. The proportions of females with pre- and postimplantation loss were quantified, and the mean rates were calculated. The preimplantation loss was calculated as the number of corpora lutea minus implantation sites × 100/number of corpora lutea, and the postimplantation loss as the number of implantation sites minus alive fetus × 100/number of implantation sites.

2.6. Sexual behavior

For the analysis of the onset of sexual behavior, at the age of 75 days, both the control group and the group treated with aromatase inhibitor male rats were anaesthetized with ethyl ether and bilateral castration performed on each one of them. Fifteen days after the castration, these animals received testosterone propionate for male sexual behavior testing and 15 days after, these same rats received estradiol benzoate for female sexual behavior testing, respectively.

Table 2
Total amount of corpora lutea, alive and dead fetuses, and resorption and implantation sites observed in pregnant control female rats mated with control and treated with aromatase inhibitor male rats

	Total amount in control female rats (5 females/male)					
Male rats	Pregnant females	Corpora lutea	Alive fetuses	Dead fetuses	Reabsorbtion sites	Implantation sites
Control ($n=8$)	37 (92%)	463	413	0	18	431
Aromatase inhibitor ($n=10$)	34 (68%)	440	289	0	11	300

2.6.1. Male sexual behavior

At 24 h before the test, the male rats were treated with testosterone propionate (1 mg/animal, ip) and observed under red-light illumination during the dark phase of their cycle. The females utilized for the behavioral test were previously treated with estradiol benzoate (20 μg/kg, ip) for inducing estrus 24 h before the test (Arteche et al., 1997). The animals were placed into the observation cage, and they were observed for 15 min for male sexual behavior (recognizance the receptive female and mount) (Almeida et al., 2000).

2.6.2. Female sexual behavior

At 24 h before the test, the male rats were treated with estradiol benzoate (20 μg/kg, ip). These animals were placed into the observation cage with male rats with sexual experience. The animals were observed for 15 min for female sexual behavior (receptive behavior and mount acceptance).

2.7. Quantity of spermatozoa

Homogenization-resistant testicular spermatids in the testes and sperm in the caput/corpus epididymidis and cauda epididymidis were enumerated as described previously (Robb et al., 1978). Daily sperm production (i.e., DSP) was derived by dividing the total number of homogenization-resistant spermatids per testis by 6.1 days, the number of days of a seminiferous cycle in which these spermatids are present. Transit time through the caput/corpus epididymidis was calculated by dividing the number of sperm within each of these regions by the DSP.

2.8. Statistical analysis

Student's *t* test, chi-square test, and Fisher test were employed, and the results were considered significant at $P < .05$.

3. Results

Perinatal exposure to aromatase inhibitor did not modify the time (day) of testicular descent (control group = 20.8 ± 0.61, $n = 10$, treated group = 22.4 ± 0.54, $n = 10$). Furthermore, no differences were observed in the volume of testes (control group = 13.58 ± 0.38 cm^3, $n = 10$; treated group = 13.19 ± 0.75 cm^3, $n = 10$), or body weight, and testis, pituitary, seminal vesicle, ventral prostate, and levatori ani muscle wet weight between the control and treated groups (Table 1).

There was a reduction of 24% in the pregnancy rate of normal females mated with treated males when compared with control males (Table 2). The data from maternal autopsy carried out on Day 21 of pregnancy were used to estimate fertility rates, which showed that the females mated with males treated with aromatase inhibitor exhibited a significant increase in the preimplantation loss (Fig. 1).

Fifty percent of the male rats treated with aromatase inhibitor did not present normal male sexual behavior, while 25% of these males showed female sexual behavior (receptive behavior and acceptance of the mount) (Fig. 2).

The mean amount of spermatozoa in the testes and epididymis and the daily sperm production of control and treated with aromatase inhibitor rats are expressed in Figs. 3

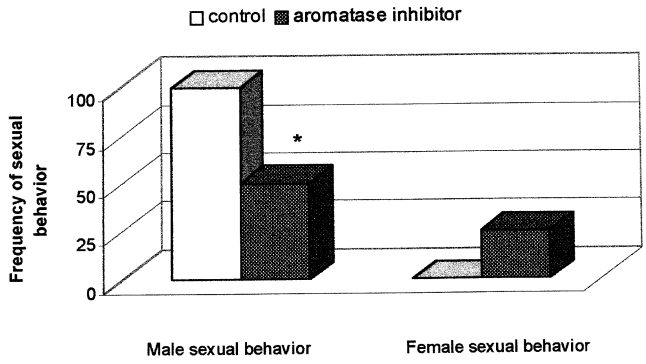

Fig. 2. Frequency of sexual behavior of control male rats and male rats treated with aromatase inhibitor that showed male sexual behavior after received testosterone propionate ($n = 10$ animals/group) and that showed female sexual behavior after received estradiol benzoate ($n = 8$ animals/group). (* $P < .05$ by Fisher test.)

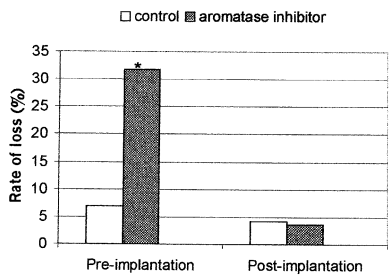

Fig. 1. Rates of pre-implantation and post-implantation loss in normal females mated with control and treated with aromatase inhibitor adult rats. The rates of pre- and postimplantation loss were determined on Day 21 of pregnancy. (* $P < .05$ by chi-square test.)

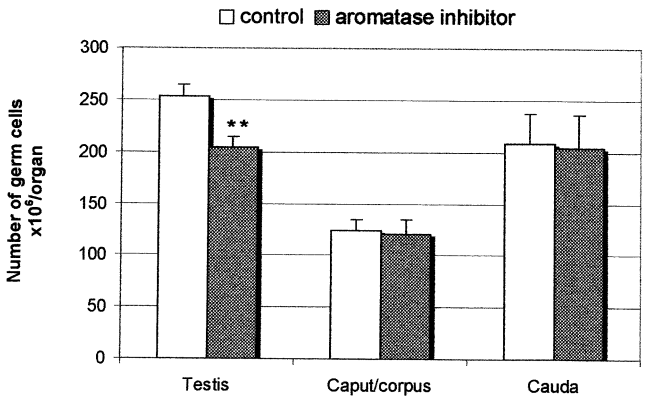

Fig. 3. Mean amount of spermatids in the testes and sperm in the caput/corpus and cauda of epididymidis of adult male rats from control group and group treated with aromatase inhibitor in prenatal period. Values represent the means ± S.E. of 10 animals/group. (** $P < .01$ by Student's *t*-test.)

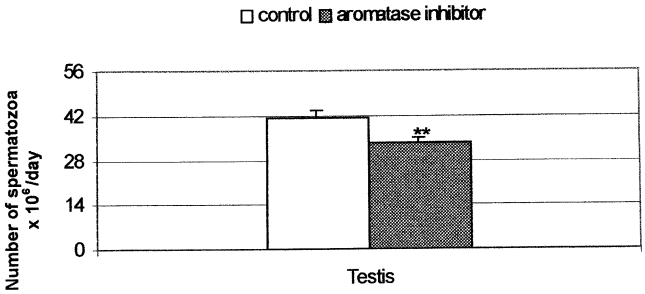

Fig. 4. Daily sperm production of adult male rats from control group and group treated with aromatase inhibitor in prenatal period. Values represent the means ± S.E. of 10 animals/group. (** $P < .01$ by Student's t-test).

and 4, respectively. Aromatase-inhibitor-exposed animals exhibited a decrease in the number of spermatozoa found in the testes, in the daily sperm production and, there was no significant increase ($P > .05$) in the transit time between the groups (caput/corpus: control group = 2.96 ± 0.13, $n = 10$, treated group = 3.55 ± 0.32, $n = 10$; cauda: control group = 4.85 ± 0.54, $n = 10$, treated group = 5.85 ± 0.78, $n = 10$).

4. Discussion

Hormonal treatment during the period of hypothalamic sexual differentiation has shown that development of reproductive functions of rats is impaired (Aguilar et al., 1987; Barraclough, 1966; Bellido et al., 1985; Dohler, 1991; Dohler et al., 1984; McEwen, 1978).

The male organs for which weights may be useful for reproductive risk assessment include the testis, epididymidis, pituitary gland, seminal vesicles, and prostate (Zenick et al., 1994); changes in the wet weight of these organs are used as a parameter for indicating an alteration in the sexual-hormone level. These organs are androgen dependent and may reflect changes in the animal's androgen status or testicular function. In the present study, the treatment with aromatase inhibitor in the perinatal period showed no significant difference in the wet weight of the pituitary, testes, ventral prostate, seminal vesicle, or levatori ani muscle. In spite of this, changes were observed in the fertility of these males.

In neonatal male rats, the presence of androgen is necessary for the perfect sexual hypothalamic differentiation for later normal masculine behavior patterns and masculine pattern of gonadotropins secretion. However, the testosterone needs to be aromatized to estrogen (Dohler, 1991; Dohler et al., 1984; DonCarlos et al., 1995; McEwen, 1978; Rhoda et al., 1984) and this estrogen will be involved in the sexual differentiation of the male rat brain (Barraclough, 1966). On the other hand, it was observed that the enhancement of estrogen at birth interfered with the later level of testosterone, damaging the reproductive function (Pereira et al., 1997). Thus, the physiological level of testosterone is important to sexual differentiation of the newborn male hypothalamus.

The conversion of testosterone to estradiol via cytochrome *P*450 aromatase represents an important step in the sexual differentiation process (Lephart, 1996); and the aromatization of testosterone is a necessary event in the masculinization of the rodent brain (Naftolin et al., 1975). Normal female rats that mated with male rats treated with aromatase inhibitor during the perinatal period showed an increase in the number of preimplantation losses. As we had found spermatozoa in the vaginal smear, these results suggest that the damage to the fertility of these rats can be related to a variation in the morphology and/or motility of the spermatozoa or to an alteration in the fluid of sexual glands. There were reductions in the number of spermatozoa in the testes and in daily sperm production. It is probable that a lack of production of tonic gonadotropin secretion may have occurred, induced by noncomplete defeminization of central nervous system, which is responsible for the hormonal levels in the adult male reproductive system. Therefore, it was suggested that the inhibition of *P*450 aromatase during the critical period of sexual differentiation of the hypothalamus may have altered the tonic liberation of gonadotropin and androgen levels in adult animals.

The presence of estrogen receptors in the testes and epididymidis strongly suggests that these tissues are estrogen targets and that estrogen may have a regulatory role in these regions. The estrogen-binding cells are reportedly responsible for resorptive activity (Janulis et al., 1998). Approximately 89–96% of luminal fluid is absorbed in the efferent ductules and initial segment of the caput epididymidis. Thus, an imbalance in the estrogen receptors of the epididymidis cells, as a consequence of the treatment with an aromatase inhibitor, could have led to a disruption in the absorbtion of the fluid in this organ.

Despite the decreased number of spermatozoa in the testes, the number of spermatozoa of the epididymidis isolated from rats treated with aromatase inhibitor in perinatal period was similar to that observed in the control group, and there was a no significant increase on the transit time of spermatozoa in the epididymidis, suggesting that there was an increase in the fluid reabsorbtion or a decrease in the contractility of the epididymidis.

In the course of their differentiation, certain cells of the brain express genes for steroid hormone receptors, which enable them to respond to hormones that regulate particular aspects of brain development, as well as activate behavioral and neuroendocrine functions in adult life. In the rat, manipulation of steroid hormones during prenatal and early postnatal development altered the capacity to express feminine reproductive behavior in adulthood (Parsons et al., 1984). Thus, the manipulation of steroid hormones in the perinatal period may result to changes in other sexually dimorphic neuroendocrine events, such as the regulation of gonadotropin secretion. Neonatal castrated males treated with estrogen are defeminized and masculinized as adults

(Booth, 1977). On the other hand, blocking the aromatization of testosterone in developing males inhibits defeminization and masculinization (Parsons et al., 1984). The animals treated with aromatase inhibitor at the hypothalamus sexual differentiation period showed alteration in sexual behavior testing. Fifty percent of these animals did not show male sexual behavior, while 25% of these males showed female sexual behavior (receptive behavior and acceptance of mount). It was also demonstrated that the differentiation of the male rat hypothalamus is not exclusively estrogen dependent and that, during differentiation of the brain, estrogen is supportive to the primary actions of androgens (Dohler, 1991). On the basis of these observations, our results showed the importance of controlling the use of hormones and drugs in hormone-sensitive periods, which could affect perfect sexual hypothalamic differentiation and alter later physiological and behavioral components in adult life.

Acknowledgments

Financial support of the CAPES is gratefully acknowledged. We are also grateful to Dr. Wilma G. Kempinas for the valuable support.

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