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Prenatal and neonatal exposure to flutamide affects function of Leydig cells in adult boar

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Abstract

In this study, flutamide, an androgen receptor antagonist, was used as a tool to better understand the role of androgen receptor signaling and androgen signaling disruption during fetal and neonatal periods on porcine Leydig cell development and function. Flutamide, 50 mg kg^{-1} d^{-1} was administered into pregnant gilts during gestational days 20 to 28 and days 80 to 88 and into male piglets on postnatal days 2 to 10 (PD2). Leydig cells of flutamide-exposed boars, especially those of PD2 males, displayed morphologic alterations, increased size, and occupied increased area ($P < 0.001$) of the testes when compared with the control. Despite this, testosterone concentrations were reduced significantly in comparison with those of controls ($P < 0.05$, $P < 0.001$). Reduced testosterone production in response to flutamide exposure appeared to be related to changes in testosterone metabolism, as shown by increased aromatase mRNA ($P < 0.05$, $P < 0.01$), protein expression ($P < 0.01$, $P < 0.001$), and elevated estradiol concentrations ($P < 0.001$). Moreover, impaired Leydig cell responsiveness to LH was indicated by the decreased expression of LH receptor ($P < 0.05$, $P < 0.001$). No significant effect of flutamide was found on LH and FSH concentrations. Taken together, our data indicate that flutamide when administered during prenatal or neonatal period have a long-term effect on Leydig cell structure and function, leading to androgen–estrogen imbalance. Leydig cell failure was most evident in adult boars neonatally exposed to flutamide, suggesting that androgen action during neonatal development is of pivotal importance for the differentiation and function of porcine adult Leydig cell population.

Keywords: Leydig cell; Aromatase; Luteinizing hormone receptor; Testis; Flutamide; Boar

1. Introduction

It is well established that development of the male reproductive system is androgen dependent, and exposure to antiandrogens during fetal development has been linked to a wide range of reproductive system abnormalities, which could affect testicular function later in life [1,2]. Flutamide is a synthetic nonsteroidal chemical that inhibits the action of androgens at the receptor level. This chemical is commonly used as a model antiandrogen in studies on the effects of androgen disruption on male reproductive functions in rodents [3]. It is worth noting, however, that androgens might act not only by an androgen receptor–mediated action, which can be inhibited by flutamide, but also by androgen receptor–independent mechanisms, such as by effects on testosterone-metabolizing enzymes, intracellular calcium, and membrane fluidity [4]. The latter effects are probably not affected by flutamide. Therefore, in fact flutamide exposure could serve as a model for investigating the role of androgen receptor signaling.

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In the studies by the group of Bilinska spermatogenic impairment concomitantly with altered intratesticular concentrations of steroid hormones was observed in adult boars either prenatally or neonatally exposed to flutamide [5,6]. Because it is widely accepted that the androgen–estrogen balance is essential for normal sexual development and reproduction in mammals [7], herein we focused on potential factors affecting testicular hormonal milieu in these pigs.

In the testis, the maintenance of androgen–estrogen balance is controlled by numerous endocrine and paracrine factors. However, the leading role has been attributed to action of aromatase, a microsomal enzymatic complex that ensures androgen conversion to estrogens (for review see Carreau et al [8]). All data available indicate that aromatase expression in adult boar testis is restricted to Leydig cells [9,10], similar to what has been observed in ram and stallion testes [11,12], whereas in rodents it is also detected in the seminiferous epithelium [13,14]. Nevertheless, compared with males of most other species, the boar testes secrete unusually high amounts of estrogens, wherein plasma estrogen concentrations even exceed those detected in females [15].

Luteinizing hormone (LH) is the main factor controlling Leydig cell testosterone secretion under physiological conditions. It is well documented that LH regulates testicular aromatase expression and activity in rodents and pigs [16,17]. Luteinizing hormone elicits response within target cells by binding to specific G protein–coupled receptor, LH receptor (LHR) [18]. It is noteworthy that besides LH, androgens modulate aromatase expression and estradiol production in the testis [19].

In the present study, to determine the role of androgen receptor signaling and androgen signaling disruption during fetal and neonatal periods in porcine Leydig cell development and function, we focused on the expression of aromatase and LHR in testes of flutamide-exposed pigs. Moreover, to evaluate the delayed effect of androgen disruption during the different developmental periods on the hypothalamic–pituitary–gonadal axis, we measured plasma concentrations of gonadotropins, testosterone, and estradiol in adult boars.

2. Materials and methods

2.1. Animals and experimental design

Porcine testes used in the present investigation derived from the same experimental animals that were examined in our previous studies [5,6]. Briefly, sexually mature gilts (n = 8; Large White × Polish Landrace) that exhibited two estrous cycles of normal duration were mated to a boar at the onset of estrus and again 12 and 24 h later. Pregnant pigs were randomly divided into four groups. The animals of the first group served as a control and were given a vehicle only (corn oil). Gilts of the second and third groups were injected with flutamide (Sigma-Aldrich, St. Louis, MO) from days 20 to 28 (GD20), or from days 80 to 88 (GD80) of gestation, respectively. Gilts of the last group were not injected during pregnancy, but their newborn male offspring were treated with flutamide on days 2 to 10 after birth (PD2). It should be mentioned, that after birth male and female offspring were separated, and the testes and ovaries served also as a material for the studies by Hejmej et al [5], Kopera et al [6], and Durlej et al [20], respectively.

Flutamide was suspended in corn oil and administered subcutaneously at a dose of 50 mg/kg BW five times every second day. This dose level was chosen on the basis of the previously published data, to effectively antagonize androgen action without producing toxic effect in the sows and offspring [21]. The first in utero exposure to flutamide included the period of sexual differentiation of the gonads in embryonic pigs [22], whereas the second flutamide exposure was based on previous studies reported changes in the appearance of steroid hormone receptor and in the expression of steroidogenic enzymes in the pig reproductive tract during this critical window of time [23]. The neonatal flutamide exposure was chosen on the basis of numerous evidences that neonatal period is critical for fertility at adulthood [24].

Male offspring from control pigs (n = 3), from pigs treated with flutamide during pregnancy (GD20, n = 3, and GD80, n = 3) and from piglets injected with flutamide postnatally (PD2; n = 3) were maintained under identical conditions, with ad libitum feeding and water until 270 d of age when they were slaughtered. All surgical procedures were performed by a veterinarian and followed approved guidelines for the ethical treatment of animals in accordance with the Polish legal requirements under the license given by the Local Ethics Committee at the Jagiellonian University (number 4/2008).

2.2. Tissue preparation

Both testes of each individual of control and flutamide-treated groups were removed and cut into small fragments. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80°C for RNA isolation and protein extraction. Other tissue fragments were fixed by immersion in either Bouin's solution for routine histology (hematoxylin and eosin staining) or in 4% formaldehyde freshly prepared from paraformalde-