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Initiation of active immunization against testosterone during early puberty alters negative feedback regulation of the hypothalamic-pituitary-testicular axis in rabbits

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ABSTRACT

To investigate the effects of antitestosterone immunization, initiated during early puberty, on hypothalamic-pituitary-testicular feedback in rabbits, 16 early pubertal male rabbits were randomly allocated into 2 groups (n = 8), control or immunized against testosterone-3(O-carboxymethyl)oxime-BSA in Freund adjuvant at 4 mo of age (with a booster immunization 4 wk later). Blood samples (for antibody titers and hormone concentrations) were collected at 2- or 4-wk intervals after immunization. Compared with controls, antitestosterone immunization triggered: a substantial and sustained antibody response ($P < 0.01$); increases in serum concentrations of luteinizing hormone (LH) and testosterone and testis weight and volume ($P < 0.05$); hyperplasia of testicular interstitial tissue with clustered and hypertrophic Leydig cells; and greater ($P < 0.05$) enzyme protein and messenger RNA (mRNA) expression levels for testicular cholesterol side-chain cleavage cytochrome P-450, 17α-hydroxylase cytochrome P-450, and 3β-dydroxysteroid dehydrogenase. Furthermore, immunoneutralization of testosterone upregulated mRNA expressions for genes in sex steroid negative feedback loops, including androgen receptor (*AR*), estrogen receptor alpha (*ER-α*), kisspeptin encoded gene (*kiss-1*) and kisspeptin receptor (G-coupled receptor 54) and gonadotropin-releasing hormone (*GnRH*) in the hypothalamic arcuate nucleus, GnRH receptor and *LH-β* in pituitary, and *AR*, inhibin-α and β_A subunits in testes ($P < 0.05$). However, immunization did not affect mRNA expressions for follicle-stimulating hormone β, *AR*, and *ER-α* in pituitary, or *ER-α* in testes. We concluded that antitestosterone immunization in male rabbits, initiated during early puberty, increased *GnRH* mRNA expression, and in turn LH synthesis by reducing testicular feedback signaling. Reduction of direct steroidal effects on the testis may also have increased testosterone secretion. Consequently, there was an accelerated testicular development during puberty and enhanced testicular function after puberty, which likely conferred prolonged reproductive advantages.

1. Introduction

Active immunization against testosterone, early in life, was reported to confer lifetime reproductive advantages in rams [1] and bulls [2,3]. In that regard, in young rams and bulls actively immunized against testosterone, daily sperm production increased without negative effects on epididymal mass and other androgen-dependent organs. Although testosterone-specific antibodies may neutralize the biological activity of testosterone, as demonstrated in rats by passive immunization against testosterone [4], immunoneutralization of testosterone seemed to occur only in nongonadal tissues and thus had no apparent detrimental effects on testicular functions in early-immunized animals, especially

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after puberty when antibody titers declined [5]. Furthermore, such an early vaccination seemed to cause either immediate stimulation of testicular growth or it enhanced testicular development during and after puberty [2,3].

The profertility effects resulting from antitestosterone immunization, initiated at an early age, were presumably associated with reduced effects of testosterone feedback on gonadotropin secretion [6]. Indeed, increased blood testosterone concentrations and daily sperm production occurred in association with increased blood concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in testosterone-immunized young rams [1] and bulls [3]. However, in another study in prepubertal bulls, blood testosterone concentrations were significantly increased, without increased gonadotropin secretion after anti-testosterone immunization [2]. Furthermore, in testosterone-immunized adult rabbits, serum testosterone concentrations increased before any rise in serum LH [7]. In addition, immunoneutralization of testosterone in prepubertal boars increased (rather than reduced) the negative feedback on gonadotropin secretion; consequently, plasma concentrations of LH and FSH were significantly decreased [8]. Therefore, whether antitestosterone immunization can reduce feedback on the hypothalamic-pituitary axis and confer reproductive advantages, in early-immunized animals, requires further validation. Furthermore, the underlying molecular mechanism should be elucidated.

Based on recent studies, steroid feedback on the hypothalamic gonadotropin-releasing hormone (GnRH) signaling is mediated by the so-called Kisspeptin-GPR54 system [9,10]. Kisspeptin, encoded by *Kiss-1* gene, acts via its receptor G-coupled receptor 54 (GPR54), a G protein–coupled receptor 54 located on GnRH neurons, to elicit a GnRH release, which subsequently triggers a gonadotropin surge [9]. Moreover, the action site of kisspeptins in conveying the negative feedback effects of sex steroids on GnRH physiology is located in the arcuate nucleus (Arc) of the hypothalamus [10,11]. Therefore, it is important to study the Kisspeptin-GPR54 system in the hypothalamic Arc to elucidate the negative feedback effects of antitestosterone immunization.

The present study, using a rabbit model, examined the effects of antitestosterone immunization, initiated during early puberty, on hypothalamic-pituitary-testicular feedback, as well as testicular steroidogenesis and spermatogenesis after puberty.

2. Materials and methods

2.1. Rabbits

All procedures involving rabbits were approved by the Sichuan Agricultural University Animal Care and Use Committee.

In present study, 16 male New Zealand white rabbits (weight, 2.18 ± 0.15 kg) aged approximately 4 mo and from the Sichuan Agricultural University farm, were used. In general, when raised under laboratory conditions, these rabbits reach puberty, sexual maturity, and adulthood at approximately 4, 6 to 7, and 16 mo of age, respectively [12]. They were maintained in individual cages in a temperature-controlled environment (22 ± 1°C) with a 12L:12D photoperiod and had ad libitum access to a standard rabbit pellet diet and water.

2.2. Immunization procedure

After 2 wk of acclimatization, rabbits were randomly allocated to either a treatment group (n = 8) or a control group (n = 8). Treated animals were actively immunized against testosterone-3(O-carboxymethyl)oxime-BSA conjugate (testosterone-3-BSA; Sigma Chemical Co, St. Louis, MO, USA) in Freund complete adjuvant (Sigma Chemical Co), with a booster injection given 4 wk later, using Freund incomplete adjuvant (Sigma Chemical Co) in lieu of Freund complete adjuvant. For each immunization, 1 mg antigen conjugate was dissolved in 1 mL 0.9% NaCl, emulsified with 1 mL adjuvant, and was injected into each rabbit at multiple subcutaneous sites on the dorsal aspect of the neck. Control rabbits received placebo injections containing all components except testosterone-3-BSA. All rabbits were humanely killed 24 wk after the primary vaccination (ie, ~10 mo of age).

2.3. Measurements and sample collection

Blood samples for the determination of antitestosterone antibody titers and hormone concentrations were collected by puncturing an ear vein on the day of the primary vaccination (0 wpv, weeks post primary vaccination) and thereafter at 2, 4, 6, 8, 12, 16, 20, and 24 wpv. To minimize diurnal influences on serum testosterone concentrations, blood samples were collected between 09:00 and 11:00 AM. Samples were centrifuged at 2,000*g* for 15 min at 4°C and sera stored at −20°C pending analysis. Furthermore, rabbits were weighed to obtain body weight at these time points before collection of the blood samples.

The Arc of the hypothalamus and the pituitary gland was removed (postmortem), frozen immediately in liquid nitrogen, and then transported and stored at −80°C for gene expression analyses. Both testes were excised, dissected free of epididymides, weighed as a pair, and then the length and width of each testis were measured with vernier calipers. Testis volume was estimated using a prolate spheroid formula [volume = 4π (width/2)^2 (length/2)/3] and recorded as an average of both testes. For each rabbit, 1 testis was frozen immediately in liquid nitrogen, transported to the laboratory, and stored at −80°C for gene expression analysis and enzyme activity assay, whereas the other testis was fixed in buffered formalin (10%, vol/vol) for histologic examination.

2.4. Antibody titer assays

Circulating antibody titers were determined using an enzyme-linked immunoabsorbant assay (ELISA). The 96-well plates (Thermo Electron Corporation, Waltham, MA, USA) were coated with testosterone-3(O-carboxymethyl) oxime-BSA (Sigma Chemical Co) overnight at 4°C. Plates were washed 3 times with phosphate-buffered saline containing tri-(hydroxymethyl)-aminomethane (PBS-T) and the remaining binding sites were blocked by coating with 300 μL of 5% (wt/vol) skim milk powder (Molico Skim