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Active immunisation against pregnenolone reduces testicular steroidogenesis and GnRH synthesis in rabbits

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ABSTRACT

To investigate the effects of active immunisation against pregnenolone on reproductive traits in rabbits, 16 early pubertal male rabbits (4 mo old) were randomly and equally allocated into two groups, control or immunised against pregnenolone–hemisuccinate–BSA in Freund's adjuvant (with a booster 4 wk later). Blood samples (for antibody titres and hormone concentrations) were collected at 2 or 4 wk-intervals after immunisation until rabbits were killed, 24 wk after the primary immunisation. Compared to controls, rabbits immunised against pregnenolone had increased serum antibody titres ($P < 0.01$) and decreased serum concentrations of both testosterone and LH ($P < 0.01$ for each). At 24 wk after the primary immunisation, testes were severely atrophied, spermatogenesis was arrested and steroidogenesis was suppressed, as evidenced by lesser amounts of testicular cholesterol side-chain cleavage cytochrome P-450 and 17α-hydroxylase cytochrome P-450 mRNA ($P < 0.05$). Furthermore, the amounts of mRNA for GnRH in the arcuate nucleus (Arc), of the hypothalamus and GnRH receptor and LH-β in the pituitary and genes in sex-hormone negative feedback loops (androgen receptor, oestrogen alpha receptor, kisspeptin encoded gene and kisspeptin receptor) in the Arc were decreased in pregnenolone-immunised rabbits compared to controls ($P < 0.05$). It was concluded that immunisation against pregnenolone directly blocked testicular steroidogenesis, which reduced synthesis of hypothalamic GnRH and subsequently synthesis of pituitary LH by abolishing the permissive action of sex steroids on hypothalamic GnRH neurons, thereby disrupting spermatogenesis. This was apparently the first report that active immunisation against pregnenolone was a means of immunological castration

1. Introduction

Fertility control is an important aspect of management in domestic, farm and recreational animals. Traditional surgical procedures of sterilisation are generally irreversible and have some adverse effects, including surgical trauma, production setbacks, and even death (Moya et al., 2008; Fredriksen et al., 2009). Consequently, immunological castration has been developed as an alternative (D'Occhio, 1993). The endocrine control of reproduction in mammals involves hormone signaling among the hypothalamus, pituitary gland and gonads, all of which can be potentially targeted by immunological methods. Gonadotropin-releasing hormone (GnRH), which is produced and released from the hypothalamic neutrons, initially enhances the hormone cascade within the reproductive axis (Conn et al., 1984); therefore, it appears to be the optimal immunological target. In the past four decades, immunocastration-based research has mainly focused on anti-GnRH immunisation (Fagerstone et al., 2010).

Sex steroids, which directly stimulate reproductive function, are potential immunological targets for suppressing reproduction. Sex steroids targeted include

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testosterone, oestradiol, androstenedione, and progesterone (D'Occhio, 1993). Active immunisation against those steroids suppressed fertility in male and female mammals (D'Occhio, 1993). However, the immunological consequences are unexpectedly divergent. Responses ranged from anti-fertility (immunological castration), through no effects to pro-fertility have been reported, with various steroid antigens and species, or for the same steroid antigen and same animal species (D'Occhio, 1993). That the targeted steroids are either at the intermediate or terminal site of steroidogenic pathways may reduce the efficacy for immunocastration. Moreover, the activities of those steroids can be (at least in part) replaced by the substitutable steroids with similar biological roles. For example, testosterone can be replaced (partly) by its metabolite dihydrotestosterone (Grino et al., 1990; Wersinger et al., 1999), and oestradiol can be derived from aromatisation of testosterone in specific tissues (Smith et al., 2005). Therefore, it is likely that the biological role of these steroids can be resumed by the substitutes (at least in part), if neutralised by immunisation treatment.

Pregnenolone, which is synthesised from cholesterol, is the precursor of all sex steroid hormones (Miller, 2013). A previous study reported that immunisation of adult buffalo using pregnenolone–hemisuccinate–HSA conjugate reduced the circulating testosterone and LH concentrations (Kamonpatana et al., 1988), suggesting the possibility that pregnenolone could be immunologically targeted for suppressing reproduction. However, further evaluations in other animal species are largely limited. Moreover, its effects on other more specific reproductive parameters have not been studied. The objective of the present study was to determine the potential of pregnenolone–hemisuccinate–BSA conjugate as an antigen for immuno-castration, with special consideration to testicular steroidogenesis and spermatogenesis in rabbits. The recently identified gonadal feedback system (Kisspeptin-GPR54) which mediates the feedback actions of steroids on hypothalamic GnRH neurons was also studied (García-Galiano et al., 2012), to evaluate the feedback effects of immunisation treatment on the hypothalamic-pituitary axis.

2. Materials and methods

2.1. Materials

Pregnenolone–hemisuccinate was purchased from Steraloids Inc. (Wilton, NH, USA). Bovine serum albumin (BSA), N-ethyl-N′-(3′-dimethyl-aminopropyl)-carbodiimide (EDC), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FICA) and tetrahydrofuran were all from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and buffer salts were analytical grade.

2.2. Antigen preparation

Pregnenolone–hemisuccinate was covalently linked to BSA via N-ethyl-N′-(3′-dimethyl-aminopropyl)-carbodiimide (EDC). In brief, 22 mg of pregnenolone–hemisuccinate was dissolved in 3 mL of tetrahydrofuran; 700 μL of milliQ-water containing 13.6 mg of EDC was added to the solution and the mixture was kept at 4 °C for 15 min under continuous stirring. Subsequently, 6.8 mg of EDC was added and the mixture was kept at 4 °C for 20 min with continuous stirring, followed by the addition of 3 mL of PBS (pH 8.0, 0.05 M) containing 22 mg of BSA under continuous stirring. Subsequently, this reaction mixture was stored at 4 °C for 24 h under continuous stirring. After 24 h of incubation, 6.8 mg of EDC was added again and the reaction mixture was stored at 4 °C for 4 h under continuous stirring. Finally, the mixture was extensively dialysed (MW cut off 10,000) against milliQ-water. The dialysate was lyophilised and stored at −20 °C.

2.3. Rabbits

Male New Zealand white rabbits raised under laboratory conditions, begin pubertal development at approximately 4 mo of age and reach sexual maturity by 6 mo of age (Macari and Machado, 1978). In present study, sixteen early pubertal male New Zealand white rabbits (2.23 ± 0.16 kg) approximately 4 mo of age from the Sichuan Agricultural University farm were used. Rabbits were maintained in individual cages in a temperature controlled environment (22 ± 1 °C) with a 12L:12D photoperiod, and were provided with a standard rabbit pellet diet and water *ad libitum*.

2.4. Immunisation

After 2 wk of acclimatisation, rabbits were randomly allocated to a treatment group ($n = 8$) and a control group ($n = 8$). The treatment rabbits were actively immunised against pregnenolone–hemisuccinate–BSA conjugate in Freund's complete adjuvant (FCA), with a booster injection given 4 wk later. Freund's incomplete adjuvant was used *in lieu* of FCA for the booster injection. For each immunisation, 1 mg antigen conjugate was dissolved in 1 mL 0.9% NaCl and emulsified with 1 mL adjuvant, and was injected into each rabbit at multiple sc sites on the dorsal aspect of the neck. Control rabbits received placebo injections containing all components except pregnenolone–hemisuccinate–BSA conjugate. All rabbits were killed at 24 wk after the primary treatment administration (approximately 10 mo of age). All procedures involving rabbits were approved by the Sichuan Agricultural University Animal Care and Use Committee.

2.5. Measurements and samples collection

Blood samples for determination of anti-pregnenolone antibody titres and hormone concentrations were collected by puncturing an ear vein on the day of the primary immunisation (0 wpi, weeks post primary immunisation) and at Weeks 2, 4, 6, 8, 12, 16, 20, and 24 thereafter. To minimise diurnal influences on serum hormone concentrations, blood samples were collected between 09:00 and 11:00 h. Blood samples were centrifuged at 2000 × *g* for 15 min at 4 °C and sera were stored at −20 °C pending analysis.