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## Plasma LH, FSH, testosterone, and age at puberty in ram lambs actively immunized against an inhibin $\alpha$ -subunit peptide

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### Abstract

Active immunization against inhibin has been shown to advance puberty and increase ovulation rate in ewe lambs; but in ram lambs, effects on puberty and sperm production are equivocal. The objective of the present study was to determine whether active immunization against an inhibin  $\alpha$ -subunit peptide advances the onset of puberty in ram lambs. St. Croix hair sheep ram lambs were assigned to inhibin-immunized ( $n = 7$ ) and control ( $n = 8$ ) treatment groups. Lambs in the inhibin-immunized group were immunized against a synthetic peptide-carrier protein conjugate,  $\alpha$ -(1–25)-human  $\alpha$ -globulin (h $\alpha$ -G), and control lambs were immunized against h $\alpha$ -G. Lambs were immunized at 3, 7, 13, 19, 25, 31, and 37 weeks of age. On the day of immunization a blood sample was collected and lambs were weighed. Another blood sample was collected 1 week following each immunization. At 20 weeks of age additional blood samples were collected at 20 min intervals for 8 h. Beginning at 20 weeks of age and at weekly intervals thereafter, scrotal circumference (SC) was measured and semen was collected using electroejaculation. A subsequent ejaculate was collected 1 week following onset of puberty, which was defined as the week of age when an ejaculate first contained  $\geq 50 \times 10^6$  sperm cells. In control lambs, plasma  $\alpha$ -(1–25)-antibody (Ab) was nondetectable. In inhibin-immunized lambs,  $\alpha$ -(1–25)-Ab titer increased from 7 to 25 weeks of age and then plateaued at a level that varied ( $P < 0.001$ ) among animals. Body weight and SC of control and inhibin-immunized lambs were similar at the onset of puberty. At pubertal onset inhibin-immunized lambs were older than control lambs ( $31.9 \pm 0.5$  vs.  $29.5 \pm 0.7$  weeks of age,  $P < 0.05$ ). Plasma FSH concentrations were similar in control and inhibin-immunized lambs from 3 to 38 weeks of age. Plasma LH levels were lower ( $P < 0.01$ ) in inhibin-immunized than control lambs. During the 8-h blood sampling period at 20 weeks of age, LH and testosterone concentrations were lower ( $P < 0.05$ ) in inhibin-immunized than control ram lambs, and the LH pulse frequency was similar in the two groups of animals. The decreased LH secretion is consistent with the immunoneutralization of a

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putative inhibin  $\alpha$ -subunit-related peptide that stimulates LH secretion in ram lambs. Present findings show that active immunization against an inhibin  $\alpha$ -peptide delays rather than advances puberty in ram lambs.

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*Keywords:* Active immunization; Inhibin; Ram lambs; Puberty; LH; FSH

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## 1. Introduction

Development of a method to advance puberty in ram lambs may prove worthwhile by enabling younger males to be used in the breeding flock. Use of younger males may reduce production costs, accelerate benefits of genetic selection, and allow earlier progeny and libido testing. Benefits of advanced puberty in ram lambs may be accentuated in year-round breeding programs that can take advantage of development of early fertility. Active immunization against inhibin as a method to induce early and enhanced gametogenesis has been shown to advance puberty and to increase ovulation rate in ewe lambs [1,2]. In ram lambs, however, Al-Obaidi et al. [1] reported that active immunization against inhibin did not advance puberty. The authors noted that observations may have been started too late to reveal differences in the first appearance of spermatozoa in ejaculates.

Different types of inhibin immunogens have been employed [3]. These include partially purified (PP) inhibin preparations derived from follicular fluid, recombinant DNA-produced inhibin  $\alpha$ -subunit, and synthetic  $\alpha$ -subunit peptides. Immunogens comprised of PP inhibin or a synthetic  $\alpha$ -subunit peptide have been used successfully to advance puberty in ewe lambs [1,2]. Synthetic  $\alpha$ -subunit peptides afford purity, chemical consistency, and availability compared to the other types of immunogens.

The objective of the present study was to determine whether active immunization against an inhibin  $\alpha$ -subunit peptide advances the onset of puberty in St. Croix hair sheep ram lambs in St. Croix, US Virgin Islands. At this location, most St. Croix ewes express estrus during each month of the year [4].

## 2. Materials and methods

St. Croix ram lambs born from November 12 to 19 were assigned alternately to control ( $n = 8$ ) and inhibin-immunized ( $n = 7$ ) treatment groups. Lambs assigned to the latter group were immunized against a synthetic peptide,  $\alpha$ -(1–25), that matched in sequence the N-terminal 25 amino acids of the  $\alpha$ C segment [5] of the ovine inhibin  $\alpha$ -subunit (residues 233–257 as reported by Forage et al. [6]). The  $\alpha$ -(1–25) peptide was coupled through a glycine–tyrosine C-terminal extension with human  $\alpha$ -globulin (h $\alpha$ -G). The  $\alpha$ -(1–25)-h $\alpha$ -G conjugate was diluted in saline and emulsified in Freund's adjuvant (2:1 adjuvant:saline) for primary (complete) and booster (incomplete) immunizations as described by Meyer et al. [7]. Approximately 0.25 mg conjugate in 2 ml was administered s.c. over four injection sites. Lambs in the control group were immunized against 0.25 mg h $\alpha$ -G. Lambs

were immunized at 3, 7, 13, 19, 25, 31, and 37 weeks of age. On the day of immunization a blood sample was collected and lambs were weighed. Another blood sample was collected 1 week following each immunization. At 20 weeks of age, additional blood samples were collected at 20 min intervals for 8 h. All blood samples were drawn using venipuncture and heparinized vacuum tubes.

At 20 weeks of age and at weekly intervals thereafter, scrotal circumference (SC) was measured using a flexible tape and semen was collected using electroejaculation. Ejaculate volume was recorded and semen was evaluated for sperm concentration using a hemocytometer. Onset of puberty was defined as the week of age when an ejaculate first contained  $\geq 50 \times 10^6$  sperm cells. This number was estimated by Evens et al. [4] to impart fertility to St. Croix rams managed under local conditions. A subsequent ejaculate was collected 1 weeks following onset of puberty. Animal procedures were approved by and followed the guidelines put forth by the UVI-AES Animal Care Committee.

Plasma samples were assayed for  $\alpha$ -(1–25)-antibody (Ab), FSH, and LH as described previously [7]. Titer of  $\alpha$ -(1–25)-Ab was measured by incubating 0.1 ml diluted (1:4000) plasma with  $^{125}\text{I}$ - $\alpha$ -(1–25) peptide (20,000 cpm). The radioiodination procedure has been described [7]. Cold ethanol was used to precipitate Ab bound  $^{125}\text{I}$ - $\alpha$ -(1–25) peptide. The  $\alpha$ -(1–25)-Ab titer is expressed as the percentage of precipitated  $^{125}\text{I}$ - $\alpha$ -(1–25) peptide/20,000 cpm. Components of the FSH RIA were NIDDK-anti-oFSH-1 serum, NIAMDD-oFSH-13 for reference, and USDA-oFSH-19-SIAFP-I-2 for radioiodination. Sensitivity was 2.5 ng/ml and the intra-assay CV was 5.8%. Components of the LH RIA were NIADDK-anti-oLH-1 serum, NIH-LH-S19 for reference, and NIADDK-oLH-I-3 for radioiodination. Sensitivity was 0.5 ng/ml, and the intra-assay CV was 7.9%. Testosterone concentrations were assayed in plasma samples collected at 1 h intervals during the 8-h blood sampling period conducted at 20 weeks of age. A Coat-A-Count kit (Diagnostic Products Co., Los Angeles, CA) was used as described previously [8]. Sensitivity was 2.0 pg/ml and the intra-assay CV was 8.5%.

Serial hormonal data were analyzed for treatment and age effects using the General Linear Models (GLMs) procedure for repeated measures [9]. Pearson's correlation coefficients were determined using the PROC CORR procedure. The number of LH pulses during the 8-h blood sampling period was determined using the Pulsar computer program [10]. Cut-off values ( $G_n$ ) for 1–5 point peaks were 3.8, 2.3, 1.6, 1.1, and 0.8, respectively. Treatment means were compared using the PROC TTEST procedure.

### 3. Results

Plasma samples collected from all ram lambs at 3 and 4 weeks of age and from control lambs throughout the study contained little if any  $\alpha$ -(1–25)-Ab as binding of  $^{125}\text{I}$ - $\alpha$ -(1–25) was nondetectable with few exceptions. In inhibin-immunized lambs,  $\alpha$ -(1–25)-Ab titer was detected consistently in the plasma sample collected 1 week following the second immunization, which was administered at 7 weeks of age. Thereafter,  $\alpha$ -(1–25)-Ab titer increased to 25 weeks and then plateaued (Fig. 1). Each boost was followed within 1 week by an increase in  $\alpha$ -(1–25)-Ab titer. Magnitude of the immune response varied ( $P < 0.001$ )

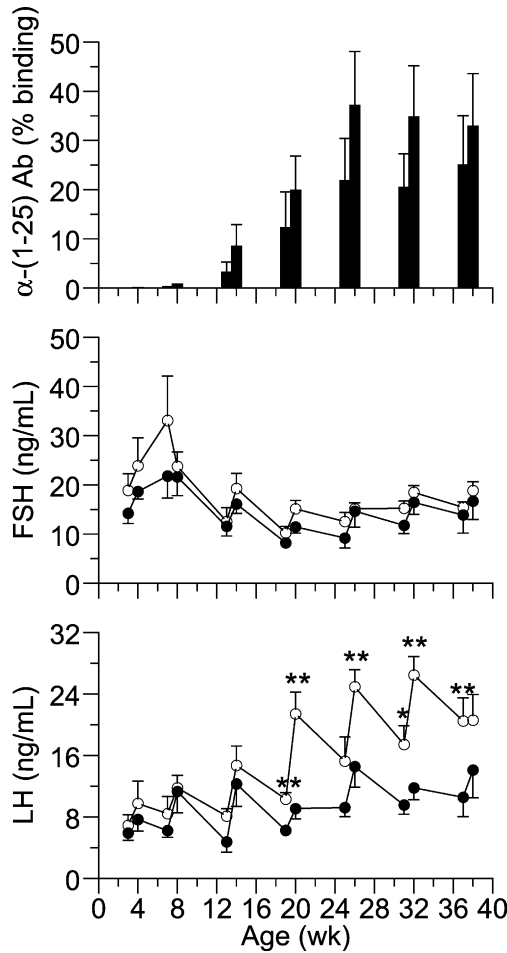


Fig. 1. The top panel depicts plasma binding of  $^{125}\text{I}$ - $\alpha$ -(1–25) peptide in inhibin-immunized ram lambs at the time of each immunization (first bar) and 1 week later (second bar). The middle and bottom panels present plasma FSH and LH concentrations in inhibin-immunized (●) and control (○) ram lambs at the time of immunization and 1 week later. Asterisks indicate statistically significant differences between corresponding means (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

among inhibin-immunized rams, ranging from 3.5 to 77.3% (mean of within ram values from weeks 25 to 38).

Body weights of control and inhibin-immunized lambs were similar from 3 to 37 weeks of age (Fig. 2), as were rates of gain (control =  $0.83 \pm 0.05$  kg/week, inhibin-immunized =  $0.80 \pm 0.04$  kg/week). Body weights of control and inhibin-immunized lambs also were similar at the time spermatozoa were first detected in ejaculates, and at the onset of puberty (Table 1). At pubertal onset, inhibin-immunized lambs were older than control lambs ( $P < 0.05$ ). SC and semen characteristics were similar in control and inhibin-immunized lambs at pubertal onset and 1 week later (Table 1).

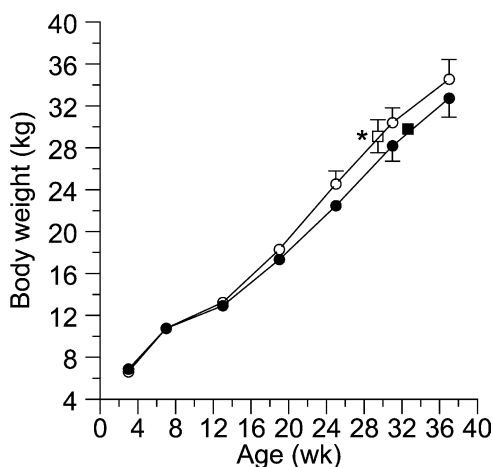


Fig. 2. Body weights of inhibin-immunized (●) and control (○) ram lambs. The square symbols mark the age and body weight of inhibin-immunized and control ram lambs at the onset of puberty. The asterisk denotes a statistically significant difference between means for age at the onset of puberty ( $P < 0.05$ ).

Plasma FSH concentrations were similar in control and inhibin-immunized lambs (Fig. 1). Concentrations differed ( $P < 0.001$ ) by age without a significant treatment by age interaction. Plasma LH concentrations were greater ( $P < 0.01$ ) in control than inhibin-immunized lambs (Fig. 1). Concentrations increased with age to a greater extent in control than in inhibin-immunized lambs (treatment by age interaction,  $P < 0.01$ ). In both control

Table 1

Reproductive responses (mean  $\pm$  S.E.M.) in ram lambs actively immunized against an inhibin  $\alpha$ -subunit peptide (inhibin-immunized,  $n = 7$ ) or carrier protein (control,  $n = 8$ ) at 3, 7, 13, 19, 25, 31, and 37 weeks of age

Reproductive parameters	Control	Inhibin-immunized
Age at 1st sperm <sup>1</sup> (week)	26.2 $\pm$ 0.6	27.1 $\pm$ 0.9
Body weight at 1st sperm (kg)	25.5 $\pm$ 1.1	26.1 $\pm$ 0.9
Age at puberty <sup>2</sup> (week)	29.5 $\pm$ 0.7 <sup>a</sup>	31.9 $\pm$ 0.5 <sup>b</sup>
Body weight at puberty (kg)	29.1 $\pm$ 1.6	30.2 $\pm$ 1.1
Reproductive parameters at puberty		
Scrotal circumference (cm)	23.5 $\pm$ 0.7	24.6 $\pm$ 0.5
Ejaculate volume (ml)	1.3 $\pm$ 0.3	1.0 $\pm$ 0.2
Sperm concentration ( $\times 10^6$ )	239 $\pm$ 81	101 $\pm$ 10
Motility (%)	28.1 $\pm$ 6.0	29.2 $\pm$ 6.9
Reproductive parameters post-puberty (1 week)		
Scrotal circumference (cm)	23.8 $\pm$ 0.8	24.9 $\pm$ 0.7
Ejaculate volume (ml)	1.1 $\pm$ 0.2	1.2 $\pm$ 0.2
Sperm concentration ( $\times 10^6$ )	343 $\pm$ 106	388 $\pm$ 73
Motility (%)	46 $\pm$ 9	59 $\pm$ 10

Superscripts (a, b): means differ ( $P < 0.05$ ).

<sup>1</sup> Spermatozoa first observed in an ejaculate.

<sup>2</sup>  $\geq 50 \times 10^6$  sperm cells in an ejaculate.

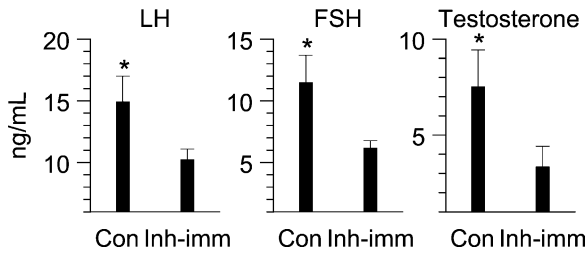


Fig. 3. Mean LH, FSH, and testosterone concentrations in inhibin-immunized and control ram lambs. Plasma samples were collected at 20 min intervals for 8 h at 20 weeks of age. Asterisks indicate a statistically significant difference between corresponding means ( $P < 0.05$ ).

and inhibin-immunized ram lambs, FSH and LH concentrations increased ( $P < 0.01$ ) within 1 week following immunization against h $\alpha$ -G (control) or  $\alpha$ -(1–25)-h $\alpha$ -G (inhibin-immunized). In inhibin-immunized ram lambs, correlation coefficients were insignificant between the mean  $\alpha$ -(1–25)-Ab titer from weeks 25 to 38 and the mean LH, FSH, age at first sperm, age at puberty, body weight at puberty, SC, and sperm concentration.

During the 8-h blood collection period at 20 weeks of age, plasma LH, FSH, and testosterone concentrations were lower ( $P < 0.05$ ) in inhibin-immunized than control ram lambs (Fig. 3). The number of LH pulses in control and inhibin-immunized ram lambs was similar. The range was 0–3 with most animals having 1 or 2 pulses per 8 h.

#### 4. Discussion

Active immunization against an inhibin  $\alpha$ -subunit peptide conjugate did not promote early or enhanced sperm output in St. Croix ram lambs. Ram lambs were immunized against  $\alpha$ -(1–25)-h $\alpha$ -G beginning at 3 weeks of age. Plasma  $\alpha$ -(1–25)-Ab titer was detectable within 1 week following the first booster immunization administered at 7 weeks of age. Thereafter,  $\alpha$ -(1–25)-Ab titer increased and subsequently plateaued at a level that varied among rams. Characteristics of the immune response were similar to those reported previously in 6-month-old Suffolk ewe lambs that had been immunized against  $\alpha$ -(1–25)-h $\alpha$ -G [7].

Development of  $\alpha$ -(1–25)-Ab in St. Croix ram lambs did not result in increased FSH concentrations. Evidence indicates that in ram lambs [1] and adults [11,12] increased FSH secretion mediates effects of inhibin immunoneutralization on SC and sperm production. Rams apparently differ from ewes in this regard as in ewes immunoneutralization of inhibin can stimulate gonadal activity without an obligatory increase in FSH secretion [2,7,13]. Lack of increase in FSH secretion in inhibin-immunized ram lambs may be attributable to the type of immunogen utilized. Al-Obaidi et al. [1] actively immunized ram lambs against PP inhibin at 3 and 6 weeks of age. Increased FSH concentrations were detected beginning at 7 weeks of age. In the same study, other ram lambs received their primary immunization at 9 instead of 3 weeks of age. In these animals subsequent FSH concentrations were not elevated. Al-Obaidi and co-investigators suggested that Sertoli cells may begin to secrete inhibin before 9 weeks of age, and that the concomitant presence

of inhibin Ab may chronically diminish inhibin feedback. In the present study, St. Croix ram lambs were immunized initially at 3 weeks of age in keeping with the findings of Al-Obaidi et al. [1]. At 7 and 8 weeks of age titer was relatively low and possibly insufficient to effectively neutralize inhibin bioactivity. Furthermore,  $\alpha$ -(1–25)-Ab may not have cross-reacted with native inhibin to the same extent as Ab generated against the preparation of PP inhibin used by Al-Obaidi et al. [1]. The latter likely contained multiple forms of inhibin [14]. In ewes, Ab developed against inhibin  $\alpha$ -subunit peptides do not appear to be as effective as those generated against PP inhibin in increasing FSH concentrations [2,15].

Development of  $\alpha$ -(1–25)-Ab was coincident with a divergence in LH levels in inhibin-immunized and control ram lambs. In the former, plasma LH levels did not increase with age as they did in the latter. Results indicate that the depressed LH secretion in inhibin-immunized ram lambs was not due to increased negative feedback by testosterone. Testosterone concentrations were lower in inhibin-immunized than control ram lambs at 20 weeks of age. The lower LH levels and lower mean FSH concentrations (which were significantly lower at 20 weeks of age) in inhibin-immunized compared to control ram lambs were similar to the gonadotropin response in 6-month-old ewe lambs following active immunization against  $\alpha$ -(1–25)-h $\alpha$ -G [7]. These ewe lambs continued to receive boosters, and when examined a year later,  $\alpha$ -(1–25)-Ab titer had increased ninefold and LH levels now were similar and FSH concentrations were greater in inhibin-immunized than control ewes [15]. Other investigators have reported decreased LH concentrations following immunization against inhibin. Al-Obaidi et al. [1] detected lower LH levels in Merino ram lambs that had been immunized against PP bovine inhibin beginning at 9 weeks of age. Anderson et al. [2] reported decreased LH concentrations in ewe lambs that had been immunized against an inhibin  $\alpha$ -subunit peptide, but not in ewe lambs that had been immunized against PP porcine inhibin. The cause of the contrasting gonadotropin responses (i.e. decreased LH without a marked change in FSH, and increased FSH without a change in LH) is unclear. It may be related to the titer and affinity of Ab developed against the inhibin immunogen. A relatively low titer may affect LH more than FSH by blocking a positive action of inhibin on the up-regulation of pituitary GnRH receptors [16], yet be unable to neutralize the inhibitory action of inhibin on the synthesis of the FSH $\beta$  subunit [17]. Another possibility is that immunoneutralization of a particular inhibin form or related peptide may decrease LH secretion. The ovine fetal and adult testis express inhibin  $\alpha$ -,  $\beta_A$ -, and  $\beta_B$ -mRNA and protein, and thus have the potential to secrete free inhibin  $\alpha$ -subunit, inhibin A, and inhibin B [18,19]. Inhibin Ab raised against inhibin  $\alpha$ -subunit peptides would be expected to bind with relatively high affinity endogenous peptides that possess an unhindered amino acid sequence analogous to that in the N-terminal region of  $\alpha C$  inhibin. It is noteworthy that such peptides have been identified in ovine rete testis fluid, of which one has been termed “LHRH Statin” for its dampening effect on the hypothalamic GnRH pulse rate [20]. Immunoneutralization of LHRH Statin would be expected to increase LH pulse frequency and LH concentrations, neither of which occurred in inhibin-immunized St. Croix ram lambs. Present results point to the possible existence of an immunologically related inhibin  $\alpha$ -subunit peptide that increases, rather than decreases, LH secretion. Further speculation is that the peptide may be transiently produced during pubertal development. Concentrations of LH in adult rams and ewes were not decreased following active immunization against inhibin  $\alpha$ -subunit peptides [12,15].

Plasma FSH and LH concentrations increased in both inhibin-immunized and control ram lambs 1 week following immunization. The increase may be attributable to a nonspecific response to Freund's adjuvant, which constituted the bulk of both inhibin-immunized and control immunogens. Injection of the emulsion may have activated a central stress-responsive corticotropin-releasing hormone (CRH) pathway. It has been shown that CRH stimulates GnRH pulse frequency and increases LH secretion in testosterone-treated castrate rams [21].

Present findings show that active immunization against an inhibin  $\alpha$ -subunit peptide neither advanced puberty nor increased sperm production in ram lambs. On the contrary, it decreased LH and testosterone secretion and delayed the onset of puberty. A better understanding of underlying inhibin physiology may lead to the development of an inhibin immunogen and immunization protocol that consistently increases FSH secretion in ram lambs. If so, the approach would need to be reassessed for effects on the onset and rate of sperm production.

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