Effects of immunization against two inhibin antigens on daily sperm production and hormone concentrations in ram lambs

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ABSTRACT: The gonadal hormone inhibin regulates daily sperm production (DSP) indirectly through negative feedback control of FSH secretion and may also affect DSP via direct actions within the testis. Studies attempting to increase DSP through the immunization against inhibin have yielded equivocal results. The current study compared 2 inhibin antigens for effects on DSP and hormone secretion. Hampshire ram lambs (BW = 42 ± 2 kg; age = 113 ± 3 d) were assigned randomly to 3 groups: 1) control (n = 4); 2) α-peptide conjugate (PTC, n = 6); and 3) α-subunit (SUB, n = 6). Antigen PTC consisted of an α-inhibin, N-terminal, 25-amino acid peptide conjugated to ovalbumin. Antigen SUB was the complete inhibin α-subunit. Lambs were immunized on d 0 (June 19, 2006), 18, 38, and 63. Body weight was recorded on immunization days and scrotal circumference on d 63. Blood samples were collected on d 0, 7, 14, 18, 28, 35, 38, 49, 56, 63, and 70. Rams were slaughtered on d 71. Testes were weighed, and parenchyma was obtained for DSP determination. Plasma α-inhibin antibody titer and LH, FSH, and testosterone concentrations were measured. α-Inhibin antibody titer was first detectable on d 14 in both PTC- and SUB-immunized ram lambs and generally increased thereafter. Mean DSP per gram of testis (DSP/g) was increased (P < 0.01) 26% in PTC- and SUB-immunized ram lambs over that in control ram lambs. Total DSP per ram lamb and testes weight did not differ among the 3 treatment groups. Variation in DSP per ram lamb and testes weight were greater (P = 0.05) in PTC- and SUB-immunized ram lambs than in control ram lambs. Plasma FSH concentrations were similar in PTC- and SUB-immunized ram lambs. Immunization against either α-inhibin antigen did not alter LH, testosterone, BW, or scrotal circumference. Findings indicate that 1) the 2 α-inhibin antigens increase DSP/g to similar extents; 2) α-inhibin antibody may act at least in part through an intratesticular mechanism because DSP/g was increased in some animals without concomitant increases in FSH; and 3) immunization against α-inhibin may affect testes weight by actions independent of those that regulate DSP/g.

Key words: immunization, inhibin, ram lamb, sperm production, testes weight

INTRODUCTION

Drummond et al. (2004) summarized 38 studies in which sheep and cattle had been immunized against α-inhibin antigens. Rams and bulls had been immunized in 6 of the studies. Findings were inconsistent. In some studies immunization against inhibin antigens increased sperm production, whereas in others it had little effect. The authors concluded that the eventual commercial success of inhibin-based fertility vaccines will depend on the design of new more potent immunogens that consistently enhance reproductive performance. Phillips (2005) concluded from his review of the literature that it is not clear whether inhibin immunization will ever be routinely used to increase fertility of valuable stud males.

Rams were immunized against α-inhibin antigens in 2 previous studies. McKeown et al. (1997) immunized 16 rams against a peptide that corresponded in sequence to the N-terminal 26 amino acids of the mature segment of the bovine inhibin α-subunit. The peptide had been conjugated to human serum albumin. Immunization did not consistently increase sperm concentration or semen volume. Voglmayr et al. (1990) immunized 4 rams against the entire human inhibin α-subunit. Immunization tended (P = 0.08) to increase epididymal sperm reserves by 26%.
Use of winter-born ram lambs during the following fall-breeding season is advantageous in that it allows for more rapid genetic progress and reduces the number of rams to be maintained for breeding. Most ram lambs have not obtained full testicular development at the onset of the fall breeding season (Ley et al., 1990). This limits breeding capacity and may be especially evident when ram lambs are used in single-sire breeding groups and with extended ram-to-ewe ratios (Dinsmore and Lewis, 1994).

The current study was conducted to compare effects of immunization against an inhibin α-subunit peptide or the entire inhibin α-subunit on daily sperm production (DSP) in winter-born ram lambs before the start of the fall breeding season.

**MATERIALS AND METHODS**

**Animals**

Animal usage procedures were in compliance with the University of Minnesota Institutional Animal Care and Use Committee.

Hampshire ram lambs (n = 17) were housed in a 6.1 × 12.2-m pen within a barn and exposed to ambient photoperiod and temperature through adjacent open sliding doors. Lambs were fed daily 0.91 kg/ram of a pellet concentrate-corn mix (22% Sheep Balancer B136, Land O'Lakes, Purina Feed LLC, Shoreview, MN, and 78% corn). The amount of mix fed was increased by 0.09 kg at 10-d intervals until 1.36 kg/ram was fed and maintained at this amount. Ram lambs had access to hay and fresh water ad libitum. On June 19, 2006 (d 0), ram lambs were 42 ± 2 kg BW and averaged 113 ± 3 d of age. Average daily gain was 0.29 ± 0.018 kg during the treatment period (d 0 to 71).

**Experimental Procedure**

Animals were assigned randomly to 3 groups: 1) control (n = 5); 2) α-inhibin peptide conjugate (PTC, n = 6); and 3) α-inhibin subunit (SUB, n = 6). One ram lamb assigned to the control group died on d 28 from an unknown cause. Ram lambs were immunized on d 0, 18, 38, and 63. Body weights also were recorded on these days. Scrotal circumference (SC) was measured on d 63. Blood samples (4 mL) were collected into chilled heparinized (20 IU) tubes on d 0, 7, 14, 18, 28, 35, 38, 49, 56, 63, and 70. Blood samples were centrifuged at 1,500 × g for 20 min. Plasma was stored at −20°C until assayed for anti-α-inhibin titer, FSH, LH, and testosterone concentrations.

**Inhibin Antigens**

Antigen PTC was synthesized and conjugated to ovalbumin using carbodiimide (EDC, BioSynthesis Inc., Lewisville, TX). The peptide, α-(1-25), matched in sequence the N-terminal 25 AA of the mature α-subunit of ovine inhibin. The C-terminal had a glycine-tyrosine extension to facilitate radioiodination. The peptide:ovalbumin ratio was 1:1:1.0 (wt/wt). Antigen SUB was a recombinant fusion protein comprised of the mature α-subunit (134 AA) and a 21-AA N-terminal extension of bovine inhibin, which contained an affinity tag for purification. Cloning, expression, purification to 90%, and sequence verification were conducted by M. Williams (Protein Expression Facility, Biotechnology Institute, University of Minnesota, St. Paul, MN). Antigens were emulsified in a mixture of saline and Freund’s adjuvant (1:2, vol/vol) at a concentration of 0.25 mg/4 mL. Ram lambs were administered 0.25 mg of PTC or SUB at each immunization delivered in four 1-mL s.c. injections spread over the back. Freund’s complete adjuvant was used for primary immunizations, and Freund’s incomplete adjuvant was used for boosters. Control ram lambs were immunized against Freund’s complete adjuvant (primary) and Freund’s incomplete adjuvant (boosters) without inhibin antigen.

**Assays**

**Anti-α-inhibin Titer.** Antibody (Ab) titer was measured in duplicate 20-μL plasma aliquots. Aliquots were incubated 24 h at 4°C with 180-μL of 0.1% BSA-0.1 M PBS, pH 7.0 (BSA-PBS) and 125I-α-(1-25) (45,000 cpm in 100-μL of BSA-PBS, ~191 μCi/nmol), after which 1 mL of cold absolute ethanol was added. Tubes were vortexed and centrifuged (20 min at 1,500 × g). Supernatants were aspirated and pellets were air-dried before quantification of radioactivity using a gamma counter. Results are expressed as percent of total cpm bound. A single assay was conducted. Sensitivity was 2.0% binding, and the intraassay CV was 6.0%. Log-logit binding-inhibition slopes developed from the assay of 5- to 40-μL of plasma from ram lambs that had been immunized against PTC or SUB were linear and not different.

**Hormones.** A single RIA was conducted for each hormone. Gonadotropin concentrations were measured in duplicate 100-μL plasma aliquots following the NIDDK oFSH and oLH RIA double-Ab protocols. Immunoreagents were NIDDK oFSH-RP-2 and oLH-I-4 for reference, oFSH-19-SIAFP and oLH-I-4 for iodination, and anti-oFSH-1 and anti-oLH-1 immune sera. Assay sensitivities and CV were 1.25 ng/mL and 7.0% for FSH, and 1.0 ng/mL and 8.9% for LH, respectively. Testosterone was measured in duplicate 50-μL plasma samples using an RIA kit (Coat-A-Count, Diagnostics Product Co., Los Angeles, CA) as described previously (Wheaton and Godfrey, 2003). The assay sensitivity was 0.04 ng/mL, and the CV was 8.7%.

**Daily Sperm Production.** Procedures followed those described by Jones and Berndtson (1986). On d 71, ram lambs were transported to the abattoir and slaughtered in random order. Testes were removed, trimmed, blotted, weighed, and sliced into dorsal and
ventral halves. The tunica albuginea was separated from the parenchyma, cleared of adhering tissue, and weighed. Parenchymal weight was calculated by subtracting the weight of the tunica albuginea from the testis weight. A 10- to 15-g sample of central parenchyma was removed, weighed, snap-frozen, and stored at -20°C.

In random order, frozen samples were thawed partially, minced using scissors, and washed into a Waring blender with 150-mL of cold physiological saline. The mixture was blended for 1 min, allowed to settle for 30 s, blended again for 1 min, and poured into an Erlenmeyer flask. The blender container was rinsed with an additional 50-mL of saline, which was added to the homogenate. Homogenates were stored at 4°C for 4 to 24 h before counting homogenization-resistant spermatids. For counting, homogenates were stirred while an aliquot was withdrawn and applied to a hemacytometer. Two individuals, working independently and without knowledge of the sample treatment group, each removed and counted 4 aliquots from each homogenate using a Nikon (Melville, NY) E200 microscope under 400x magnification. Each aliquot was counted in 10 of 25 squares (0.1 mm²/25 squares). The intrapanel CV (4 aliquots per homogenate) was 2.7%. The interindividual CV was 8.1%. Counts per 10 squares was multiplied by \(5 \times 10^6\) and divided by the sample weight and 4.99 to convert to DSP per gram of testis (DSP/g). The 4.99 divisor corresponded to the number of days of the ram seminiferous cycle in which spermatids are generated (Amann et al., 1974). Daily sperm production per gram testis was multiplied by testes weight (TW) to calculate total DSP per ram lamb (DSP/ram).

Statistics

Statistical procedures were conducted using SAS (SAS Inst. Inc., Cary, NC). Data sets having a single observation per ram lamb (DSP/g, DSP/ram, TW, SC, ADG, and BW on d 63) were tested for mean differences using PROC ANOVA. Means that differed significantly \((P \leq 0.05)\) were compared with each other using Tukey’s Studentized range test. Variation in DSP/g, DSP/ram, and TW was tested for treatment effect using Levene’s test for homogeneity of variances (hovtest). When group variances differed (DSP/ram, TW), means were tested for differences using PROC NPAR1WAY, Kruskal Wallis test. Serial observations (Ab titer, LH, FSH, testosterone) were analyzed using PROC MIXED with a repeated statement and the AR(1) autoregressive covariance structure. Variation in TW in the α-inhibin-immunized ram lambs was examined for associations with hormone and Ab concentrations using PROC CORR. Correlation coefficients were calculated within the PTC- and SUB-immunized groups and pooled using Fisher’s z transformation. Values used for hormone and Ab concentrations were area under the curve calculated from d 18 to 70. Anti-body titers were detectable during this time period. Correlation coefficients were not calculated for the control group because of the small number of animals. Mean differences due to treatment were considered nonsignificant at \(P > 0.05\).

RESULTS

Daily sperm production per gram testis differed \((P = 0.0003)\) among ram lambs in the 3 treatment groups. Mean DSP/g was increased \((P < 0.01) 26%\) in PTC- and SUB-immunized ram lambs compared with the mean DSP/g in control ram lambs (Table 1, Figure 1). Variation in DSP/g was similar in control, PTC-, and SUB-immunized ram lambs. Mean DSP/ram did not differ among animals in the 3 treatment groups. Variation in DSP/ram was greater \((P = 0.05)\) in PTC- and SUB-immunized rams than in control ram lambs. In PTC- and SUB-immunized ram lambs, DSP/ram ranged from 72 to 261% of the mean DSP/ram in control ram lambs.

Average daily gain, BW on d 63, and TW were similar among ram lambs in the 3 treatment groups. Variation in TW was greater \((P = 0.05)\) in PTC- and SUB-immunized rams than in control rams. In PTC- and SUB-immunized rams, TW ranged from 53 to 198% of the mean TW in control ram lambs. Variation in TW was not correlated with variation in DSP/g, BW, or days of age. It was correlated with SC on d 63 \((r = 0.69, P = 0.003)\).

Table 1. Age, BW, scrotal circumference (SC), testes weight (TW), and daily sperm production (DSP) in ram lambs immunized against an α-inhibin peptide-ovalbumin conjugate (PTC), the entire inhibin α-subunit (SUB), or Freund’s adjuvant (control)1

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>PTC</th>
<th>SUB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, d</td>
<td>184 ± 4</td>
<td>181 ± 6</td>
<td>188 ± 7</td>
</tr>
<tr>
<td>BW, kg</td>
<td>63.8 ± 1.9</td>
<td>59.2 ± 3.1</td>
<td>59.5 ± 3.6</td>
</tr>
<tr>
<td>SC, cm</td>
<td>31.5 ± 0.6</td>
<td>32.0 ± 1.2</td>
<td>32.0 ± 0.7</td>
</tr>
<tr>
<td>TW, g</td>
<td>180 ± 4</td>
<td>246 ± 38</td>
<td>191 ± 27</td>
</tr>
<tr>
<td>DSP/g, millions</td>
<td>11.5 ± 0.3</td>
<td>14.5 ± 0.4</td>
<td>14.5 ± 0.4</td>
</tr>
<tr>
<td>DSP/ram, billions</td>
<td>2.1 ± 0.0</td>
<td>3.6 ± 0.6</td>
<td>2.8 ± 0.4</td>
</tr>
</tbody>
</table>

1Within a row, means without a common superscript letter differ \((P < 0.01)\).
2Age = age on d 71.
3BW = BW on d 63.
Figure 1. Variation in daily sperm production per gram testis (DSP/g), DSP per ram lamb (DSP/ram), and testes weights. Each bar represents a value for an individual ram lamb. Control ram lambs were immunized against Freund’s adjuvant, and PTC and SUB lambs were immunized against an α-inhibin peptide-ovalbumin conjugate or the entire inhibin α-subunit, respectively. Mean DSP/g was greater (\( P < 0.01 \)) in PTC- and SUB-immunized lambs than in control lambs. Variation in testes weights and DSP/ram was greater (\( P = 0.05 \)) in PTC- and SUB-immunized lambs than in control lambs.

DISCUSSION

Active immunization of ram lambs against PTC or SUB resulted in similar effects on sperm production, Ab titers, and hormone concentrations. Both α-inhibin antigens increased DSP/g by 26%. Increases ranged from 14 to 41% in individual ram lambs. Neither antigen increased total DSP/ram. Daily sperm production per ram lamb, the product of DSP/g × TW, reflected the greater variation in TW (CV = 38%) than in DSP/g (CV = 7%). Variation in TW was similar among ram lambs within the 2 groups of α-inhibin-immunized ram lambs and was greater than in control ram lambs. Antibody titers developed against PTC and SUB were similar in profile and magnitude when measured using \( ^{125}I \)-α-(1-25) as ligand. Additional α-inhibin epitopes present in the SUB antigen may have elicited formation of Ab not detected in the titer assay. This possibility may have led to the greater FSH concentrations in SUB than in control ram lambs. Neither antigen altered plasma LH or testosterone concentrations. The similar effects of PTC and SUB α-inhibin antigens indicate that responses are not markedly different following active immunization against conjugated N-terminal α-peptides or the full-length α-inhibin subunit. The former has advantages over the latter in cost of synthesis and purification.

A stimulatory effect of immunization against α-inhibin on DSP/g has not been reported previously for sheep. An increase in DSP/g is consistent with the 26% increase in epididymal sperm reserves per gram of testis reported by Voglmayr et al. (1990). In that study, rams were immunized against the entire inhibin α-subunit for 108 d, and epididymal sperm reserves were measured during the breeding season. McKeown et al. (1997) immunized rams against a conjugated α-inhibin peptide and measured sperm output from De-
December to August, a period of time which excluded most of the breeding season. In August the sperm concentration in ejaculates was increased 14% in the α-inhibin-immunized rams compared with contemporary control rams. Sperm concentration was not increased when evaluated over the entire collection period. In α-inhibin-immunized goats the sperm concentration in ejaculates was increased during the early portion of the breeding season, but thereafter the increase was not sustained (Medan et al., 2006). Increases in DSP/g have been reported previously for α-inhibin-immunized bulls. Mean DSP/g was increased 2-fold in bulls immunized for 150 d against an α-inhibin peptide conjugate (Martin et al., 1991). A 32% increase ($P < 0.1$) in DSP/g was reported by Schanbacher (1991) for bulls that had been immunized against an α-inhibin peptide conjugate for over 1 yr. Present results and the aforementioned cited literature point to a stimulatory effect of immunization against α-inhibin antigens on DSP/g in rams and bulls. The effect is detectable within 71 to 150 d following the primary immunization. In rams the effect seems to be manifest only during the breeding season.

The mechanism of action may be paracrine as the increase in DSP/g in ram lambs was not dependent upon concomitant changes in systemic gonadotropin or testosterone concentrations. A local mechanism of action is consistent with results from rodent studies. The intratesticular administration of inhibin decreased numbers of intermediate and B1 spermatogonia (van Dissel-Emiliani et al., 1989), and the addition of inhibin to cultures of seminiferous tubules inhibited DNA synthesis in intermediate spermatogonia (Hakovirta et al., 1993). Sertoli cells are the main source of inhibin in rams based on the expression of inhibin $\alpha$, $\beta_A$, and $\beta_B$-subunit proteins (McNeilly et al., 2002). Rat Sertoli cells secrete inhibin bidirectionally, basally into testicular interstitial fluid and apically into seminiferous tubule fluid (Maddocks and Sharpe, 1990). Inhibin in tubular fluid is resorbed into the bloodstream from the rete testis. Bioactive and immunologically active inhibin has been semipurified from ram rete testes fluid (Vaughan et al., 1989). Maddocks and Sharpe (1990) speculated that inhibin secreted basally has a predominantly paracrine role, targeting Leydig cells and spermatogonia. Presence of α-inhibin Ab in the testicular interstitium may have decreased the effective local concentration of inhibin. A possible consequence in keeping with the function of inhibin as an activin antagonist may have been to enable activin to increase numbers of spermatogonia (Mather et al., 1990; Hakovirta et al., 1993; Cook et al., 2004).

Active immunization against α-inhibin increased DSP/g but not total DSP/ram. The variation in DSP/ram was greater in α-inhibin immunized than in control rams and reflected the greater variation in TW in α-inhibin immunized than in control rams. The CV for TW in the control-, PTC-, and SUB-treated ram lambs were 4.3, 38.3, and 34.1%, respectively. Although the difference in variation was likely attributable to treatment, the possibility remains that the difference in variation came from the random assignment of 4 ram lambs to the control group that subsequently developed testes of similar weights. Testes weights in 2 groups of 4 untreated Il-de-France and Romanov rams had CV of 26.1 and 16.7%, respectively (Hochereau-de Reviers et al., 1985). Voglmayr et al. (1990) presented epididymal sperm reserves on a per gram rather than per testis basis because of wide variation in TW. No mention was made or data provided to ascertain whether the variation in TW differed between the α-inhibin immunized and control rams. Variation in TW in α-inhibin immunized bulls was numerically greater.
than that in control bulls, but the difference was not as marked as that in the current study (Schanbacher, 1991). Variation in TW in the α-inhibin-immunized ram lambs was correlated negatively with FSH and positively with testosterone. Testosterone and FSH were inversely related. Schanbacher (1991) also reported a negative correlation between TW and FSH. One possibility to account for the TW-hormonal correlations is that testosterone secretion was proportional to TW and fed back to decrease FSH secretion (Tilbrook et al., 1993). A study is underway to confirm present findings that immunization against α-inhibin alters TW. Evidence supports some independent regulation of sperm production and TW. In bulls DSP/g was increased 2-fold without a change in SC (Martin et al., 1991). Conversely, in rams SC was increased without an increase in sperm concentration (McKeown et al., 1997).

Anti-α-inhibin titer in PTC- and SUB-immunized ram lambs was detectable by d 14 and then generally increased during the duration of the study. At peak titer, approximately 0.002 nmol of α-inhibin peptide was bound by 1-mL plasma. The plasma binding activity was calculated using the volume of plasma assayed, specific activity of the tracer, and the radioactivity bound at or near peak titer. Peak Ab titer in the current study was much less than that in a previous study in which St. Croix ram lambs were immunized against α-inhibin beginning 2-wk after birth (Wheaton and Godfrey, 2003). Estimates of anti-α-inhibin titers reported by other investigators were made using available information and likewise showed extensive variation among studies. Differences in estimated titers among studies were not obviously ranked with antigen dose, route of administration, or the number, interval, or duration of booster immunizations. Based on present results, DSP/g can be increased with a relatively low α-inhibin titer but not gonadotropin concentrations. In studies with estimated midlevel titers, FSH was increased (Voglmayr et al., 1990; Bame et al., 1999) and positively associated with Ab titer (Schanbacher, 1991; McKeown et al., 1997). Effects on LH were inconsistent, being increased (Voglmayr et al., 1990), increased in peak amplitude (Schanbacher, 1991; McKeown et al., 1997), or decreased (Bame et al., 1999). In studies with higher titers, LH was decreased (Martin et al., 1991; Wheaton and Godfrey, 2003). Evidently, the threshold for the stimulatory effect on DSP/g is less than that needed to increase FSH secretion. Greater titers may decrease LH concentrations. α-Inhibin titer-dependent effects may underlie some of the variation in responses reported in the literature.

Immunization against PTC or SUB increased DSP/g but not DSP/ram. To date no increase in DSP/testis or sperm output has been reported for rams or bulls. Present results indicate that this may be due to treatment-induced variability in TW. Potential exists to increase total sperm production as DSP/ram was more than doubled in ram lambs with heavier testes. A better understanding of mechanisms that regulate DSP/g and TW is needed in order to develop methods to reliably increase total sperm production.

LITERATURE CITED


