Concentrations of Progesterone, Follistatin, and Follicle-Stimulating Hormone in Peripheral Plasma Across the Estrous Cycle and Pregnancy in Merino Ewes That Are Homozygous or Noncarriers of the Booroola Gene

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ABSTRACT

The circulating concentrations of progesterone, FSH, and follistatin across the estrous cycle and gestation were compared in Australian merino sheep that were homozygous for the Booroola gene, FecB, or were noncarriers. The Booroola phenotype is due to a point mutation in the bone morphogenetic protein receptor IB. Progesterone concentrations began to rise earlier and were higher in the Booroola ewes than in the noncarriers on most days of the luteal phase but not during the follicular phase of the cycle. Follistatin concentrations remained unchanged across the estrous cycle in both groups of ewes, with no differences between genotypes. FSH concentrations were higher in Booroola ewes than in noncarrier ewes on most days of the estrous cycle, with a significantly higher and broader peak of FSH around the time of estrus. Progesterone concentrations were significantly higher in early and midgestation in Booroola ewes but were lower toward the end of gestation than those in noncarriers. FSH declined in both groups across gestation, with lower concentrations of FSH in Booroola ewes during midgestation. Follistatin remained unchanged across gestation in Booroola ewes and noncarrier ewes with a twin pregnancy but declined across gestation in noncarrier ewes with a singleton pregnancy. These results suggest that follistatin concentration is not regulated by the FecB gene during the estrous cycle and pregnancy but is influenced by the number of fetuses. However, the FecB gene appears to positively affect both progesterone and FSH during the estrous cycle and across pregnancy, which suggests that bone morphogenetic proteins play an important role in the regulation of both hormones.

INTRODUCTION

Booroola sheep possess a major gene, FecB, that influences their ovulation rate [1]. Homozygotes (BB), heterozygotes (B+), and noncarriers (++) of the FecB gene are segregated on the basis of ovulation rates of ≥5, 3 or 4, and 1 or 2, respectively [2]. The gene effects are due to a point mutation in the bone morphogenetic protein (BMP) receptor type IB [3–5], although the exact mechanism by which this mutation induces the increased ovulation rate remains to be determined.

In sexually mature ewes, differences in the morphology of the antral follicles have been consistently observed, with follicles maturing and ovulating at significantly smaller diameters in ewes carrying the Booroola gene than in normal merino ewes [6, 7]. The action of the Booroola gene has been generally associated with higher concentrations of FSH during the estrous cycle [8–10].

No differences were observed between Booroola and ordinary Merino ewes in peripheral concentrations of estradiol and androstenedione on the days samples were taken (Days 2, 9, and 16) during the estrous cycle [11]. Booroola ewes have been reported to have up to 25% higher progesterone concentrations than ordinary Merino ewes during the midluteal phase of the estrous cycle, but this difference was not significant [12] because of the small sample size. In the follicular phase of the estrous cycle induced by prostaglandin F2α, there were no gene-specific differences in concentrations of progesterone [13–15]. No differences were found in ovarian secretion rates of immunoreactive inhibins around luteolysis or in daily plasma concentrations throughout the estrous cycle, as measured by the Monash RIA [14]. More recently, plasma inhibin A concentration was determined using a specific two-site ELISA during the follicular and early luteal phases in ewes with an ovarian autotransplant, but no differences were noted between genotypes [13]. FSH and LH concentrations in plasma were not different between genotypes after treatment of ovariectomized ewes with estradiol, progesterone, or bovine follicular fluid, indicating the sensitivity of the hypothalamic-pituitary axis to ovarian hormones are similar for Booroola and ordinary Merino ewes [15].

Follistatin, originally identified and isolated from both bovine and porcine follicular fluids on the basis of its inhibition of pituitary FSH [16, 17] secretion, is a monomeric glycosylated polypeptide chain that binds activin with high affinity and neutralizes most but not all of its biological actions [18–20]. Follistatin also neutralizes the biological activity of several BMPs [21].

In a previous study, Klein et al. [22] showed that the peripheral concentrations of plasma follistatin in sheep increased during the luteal phase above follicular phase concentrations, peaking 10 days after the LH surge. However, the assay was subsequently shown to be affected by the presence of activin [23], leaving some doubt as to the validity of the results. Follistatin mRNA has been detected in ovine granulosa cells of preantral, antral, and early atretic follicles at all stages of the estrous cycle and in the corpora lutea (CL) at the early and midluteal stages of the cycle. However, only low levels of follistatin mRNA were observed in the presumptive preovulatory follicle [24, 25].
gestation and decrease after delivery in humans [26–28], suggesting that follistatin plays an important role during pregnancy and perhaps parturition.

The aim of this study was to examine in detail the changes in peripheral concentrations of progesterone and FSH and the role of follistatin. We used an assay that is not affected by activins to identify gene-specific differences during the estrous cycle and pregnancy between Booroola and ordinary Merino ewes.

MATERIALS AND METHODS

Animals and Sample Collection

The ewes used in this study were classified as BB and ++ based on ovulation rate. One group of BB ewes (n = 5) and one group of ++ ewes (n = 5) were grazed on open paddocks and run with castrated male Merinos (wethers) that had received injections of 50 mg of a mixture of testosterone esters (Durateston; Intervet, Lane Cove, NSW, Australia) over the previous 2 mo. The wethers were harnessed with marking crayons so that the day of estrus (Day 0) could be determined in the ewes. Blood samples were taken daily from Day 0 by jugular venipuncture until the ewes were marked again, indicating the next estrus. Another set of BB ewes (n = 5) and ++ ewes (n = 10) were run with intact rams harnessed with marking crayons to determine the conception date. Blood samples were obtained weekly by venipuncture during the period of gestation, and all the plasma samples were stored at −20°C until assayed. The ewes were further divided on the basis of the number of lambs born into singleton pregnancy (SG; n = 5) or twin pregnancy (BB and TG; n = 5) groups to determine whether there are any FecB-specific differences in progesterone, follistatin, and FSH during pregnancy or whether the differences were related to litter size. Some BB ewes had litters of >2, but these ewes were not included in the study because of small sample size. All experiments were approved by the University of New England Animal Ethics Committee and conform to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes.

Hormone Assays

Follistatin was determined in plasma using an assay as previously described [28]. The assay included a rabbit antiserum (204; donated by D.M. deKretser, Institute of Reproduction & Development, Monash University, Clayton, VIC, Australia) raised against purified 35-kDa bovine follistatin. A human recombinant follistatin (FS288) was used as both standard and tracer. The dissociation reagents were modified to 10% Triton X-100, 3% sodium deoxycholate, and 0.5% SDS. Cross-reactivity with inhibin and activin was <0.5%. Plasma pools gave dose-response curves parallel to the standard curve, and the recovery of follistatin from spiked samples was 98% ± 5%. The detection limit was 1.5 ng/ml, and the intra- and interassay coefficients of variation were 6% and 18%, respectively.

Samples were assayed for progesterone using a procedure similar to that previously described [29]. The antiserum was prepared using progesterone-11α-HS-BSA (230; Bioquest Limited, North Ryde, NSW, Australia). Each sample was extracted in duplicate with a 10X volume of diethyl ether. The sensitivity of the assay was 25 pg/ml. The interassay variation was 11%, and the intra-assay variation was 4%.

FSH was measured using an RIA kit from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The detection limit of the assay was 0.8 ng/ml (ofFSH NIDDK-RP-1), the interassay variation was 9%, and the intra-assay variation was 3%.

Data Analysis

The concentrations of progesterone, follistatin, and FSH across the estrous cycle and pregnancy between BB and ++ ewes were analyzed as repeated measures and fitted to a generalized linear mixed model, with systematic effects of group and time, random effects of animal, and an animal by time interaction. Exploratory data plots indicated that the error should be modeled by a gamma distribution, and the log-link function was used. Trends over time in both the systematic and random models were represented by spline curves whose smoothness is regulated by the correlations among the residuals. The models were fit to the data using the ASREML statistical software [30]. The results are presented as weighted smooth splines of group trends, and the observed group means were plotted at each sampling time.

RESULTS

Estrous Cycle

The mean concentrations of progesterone, follistatin, and FSH based on samples taken daily throughout a natural estrous cycle are shown in Figures 1–3. Progesterone in both BB and ++ ewes, as expected, increased rapidly in the first week, plateaued through the second week, and then fell rapidly in the follicular phase from Day 14 to Day 17. In the BB ewes, progesterone began increasing earlier than
in the ++ ewes, but in both groups progesterone began declining at the end of the luteal phase at the same time. The progesterone concentrations tended to be higher during the luteal phase and were significantly higher \( (P < 0.05) \) in BB ewes than in ++ ewes during the early luteal phase from Day 1 to Day 4, but they were not different during the follicular phase (Fig. 1). Follistatin fluctuated across the estrous cycle without significant change with time either in BB or in ++ ewes (Fig. 2). The overall concentrations were 11.22 ± 2.11 ng/ml for BB ewes and 11.86 ± 1.89 ng/ml for ++ ewes. FSH concentrations were higher in BB than in ++ ewes during the estrous cycle on most days (Fig. 3). In both groups, FSH was highest on the day of estrus, but in the BB ewes the peak was relatively higher and broader than that in the ++ ewes.

**Pregnancy**

In this study, the average (±SD) gestation periods were 149.1 ± 2.0 days for BB ewes, 149.8 ± 2.49 days for the singleton ++ group (SG), and 150.4 ± 1.14 days for the twin ++ group (TG) and were not significantly different among groups \( (P > 0.05) \).

The profiles of maternal plasma progesterone, follistatin, and FSH across gestation are shown in Figures 4–6. The concentrations of progesterone in BB ewes (Fig. 4) increased from 3.8 ± 0.4 ng/ml to a peak of 7.1 ± 0.5 ng/ml at 105 days of gestation. In ++ ewes (Fig. 4), progesterone concentrations were not different across gestation between the SG and TG groups. Progesterone in the ++ ewes increased from 1.9 ± 0.4 ng/ml in the SG and TG groups to a peak of 7.9 ± 0.8 ng/ml at Day 105. Progesterone concentrations in plasma tended to be higher in the BB ewes for the first half of pregnancy and then were not significantly different from those in the ++ ewes for the remaining period of gestation.

The trend in follistatin concentrations differed between groups across gestation (Fig. 5). The trend in follistatin concentrations in BB ewes was to remain unchanged, with large confidence intervals due to the variation in trends between animals in the group. Follistatin increased in two ewes over the 21 wk, remained unchanged in one ewe, and decreased in two ewes. In the SG ++ ewes (Fig. 5), follistatin declined significantly from 10.65 ± 1.03 ng/ml on Day 55 to 6.81 ± 1.17 ng/ml at term. However, in the TG
++ ewes, follistatin concentrations remained unchanged across gestation, with an overall mean of 10.16 ± 0.75 ng/ml. As term approached, follistatin in the SG ++ ewes was significantly lower than that in the BB or TG ++ ewes.

FSH concentrations declined during pregnancy, but with different profiles for each genotype (Fig. 6). In BB ewes, FSH tended to decline more rapidly earlier in pregnancy than in the ++ ewes, and lower concentrations were noted during mid gestation. FSH concentrations in both groups of ++ ewes (Fig. 6) were similar but declined more rapidly than those in BB ewes toward the end of pregnancy. In comparison with the ++ ewes, lower concentrations were observed in BB ewes from Day 69 to Day 97.

DISCUSSION

We present the first reports of plasma profiles of progesterone, FSH, and follistatin in the prolific Australian Booroola strain of Merino sheep compared with those of its nonprolific counterpart across the estrous cycle and pregnancy. Plasma progesterone concentrations did not differ significantly between BB and ++ ewes during the follicular phase. However, on most of days in luteal phase, the progesterone concentrations were higher in BB ewes than in ++ ewes. These data contrast with previous reports that there were no FecB-specific differences in jugular plasma concentrations or ovarian secretion rates of progesterone in middle or late luteal phase and the prostaglandin F2α-induced follicular phase [13], although there were differences in the mean numbers of CL during the midluteal phase between the genotypes [7]. This inconsistency between the present and previous findings is likely to be due to the small windows of study used by other research groups.

Booroola ewes are reported to ovulate about 7.5 h earlier than the ordinary Merino after the onset of estrus. No differences have been noted in the time from the onset of estrus to the preovulatory LH surge nor in the duration of the preovulatory LH surge itself [31]. These findings suggest that the early increase in progesterone during the luteal phase may be due at least in part to the earlier development of the CL. However, this explanation does not account fully for the rise occurring nearly 2 days early, which is likely due to the impairment of the BMP 1B receptor [5], reducing the dampening effect of BMPs on progesterone production by granulosa cells in the maturing follicles.

The development of immunoassays for follistatin has been complicated by the characteristics of this molecule, such as presence of multiple molecular forms in biological fluids in a variety of species, varying degrees of glycosylation, and the existence of complexes of follistatin with activins and perhaps other growth factors. There is no widely accepted follistatin assay, and a number of different immunoassays have been reported using different standards and antibodies. In these assays, some researchers have used human recombinant FS288 or FS315 as standards, and others have used purified individual porcine or bovine isoforms or mixtures of these isoforms [27, 28]. Therefore, it is impossible to fully reconcile all the data emerging from studies in which these various assays have been used.

Gilfillan and Robertson [32] reported that during the human menstrual cycle there were significantly lower total serum follistatin concentrations during the luteal phase than during the follicular phase. In contrast, others have found no significant variation across the human menstrual cycle [35, 27]. In the Klein et al. study of the estrous cycle in sheep [22], a significant increase in plasma follistatin concentrations was observed between Day 4 and Days 10–11 after the LH surge, and this increase was followed by a gradual decrease between Day 11 and Day 3 of the follicular phase of the next cycle. However, the assay used in that study was later found to be significantly affected by the presence of activin [23], which in the light of our study suggests that the rise in follistatin observed by Klein et al. [22] was likely a reflection of changes in activin concentrations.

In the present study, follistatin concentrations remained unchanged throughout the estrous cycle in BB and ++ ewes. Based on the results of the present and most previous studies, it is clear that the gonads are not a major source of circulating follistatin, and peripheral plasma follistatin concentrations are not an indicator of cyclic ovarian activity. Therefore, we concluded that follistatin during the estrous cycle is not affected by the presence of the FecB gene.

During pregnancy, major differences were observed between BB and ++ ewes that could not be accounted for by the increased number of fetuses. The concentrations of progesterone increased across gestation in both genotypes, with the pregnancy increase in progesterone occurring much earlier in BB ewes that in either of the ++ groups. This finding and the estrous data strongly suggest that progesterone concentrations are influenced by the FecB gene, which implicates BMPs as having an important role in pregnancy. Maternal plasma concentrations of follistatin remained unchanged in the BB and TG ++ ewes during gestation, in contrast to the decline observed in the SG ++ ewes. However, the high level of variability between animals in the BB group warrants further study. Although these ewes gave birth only to twins, their high ovulation rate suggests that other fetuses died at various stages of development, consequently complicating the follistatin profiles. Nevertheless, the different profiles in maternal follistatin observed between the BB ewes and ++ ewes may be due to the rapid rise occurring nearly 2 days early, which is likely due to the impairment of the BMP 1B receptor [5], reducing the dampening effect of BMPs on progesterone production by granulosa cells in the maturing follicles.
due to the number of fetuses and may be independent of the FecB gene. FSH concentrations, as expected, declined across gestation in all three groups. However, FSH declined more rapidly during midgestation in the BB group than in the + + groups. The higher concentrations of FSH observed during the estrous cycle and the differences in FSH secretion during pregnancy in BB ewes compared with + + ewes lead us to conclude that FSH secretion is influenced by the FecB gene.

The gene-specific profiles in maternal plasma progesterone could reflect differences in the number and maturity of CL during early pregnancy. At the beginning of pregnancy, the number of CL is likely to be higher and the CL develop more quickly in BB than in + + ewes. Progesterone concentrations in BB ewes on Day 6 were already similar to those during the normal luteal phase, but they did not reach equivalent levels until Day 20 in + + ewes.

In SG + + ewes, follistatin fell significantly between Day 55 and Day 76 while progesterone started increasing. In the study of Knight et al. [34], inhibin A fell about 7-fold between Day 60 and Day 90 of gestation in crossbred ewes. Thus, many endocrine events occur in the transition from the corpus luteum-dependent phase in early gestation to the placenta-dependent phase in late gestation.

Our findings concerning the follistatin profiles in Booroola or ordinary Merino ewes contrast with those associated with human pregnancy, during which circulating follistatin is greatly increased in the third trimester. Inhibin A profiles during gestation were found to be different between sheep and humans, with inhibin A concentrations declining in ewes [34] but increasing in humans across gestation [35]. These data suggest that activins/inhibins are differently regulated in sheep and humans.

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