Antral Follicle Count Reliably Predicts Number of Morphologically Healthy Oocytes and Follicles in Ovaries of Young Adult Cattle


Molecular Reproductive Endocrinology Laboratory, Laboratory of Mammalian Reproductive Biology and Genomics, Department of Animal Science, and Physiology Department, Michigan State University, East Lansing, Michigan 48824 Department of Internal Medicine, Erasmus MC, Rotterdam 3000 DR, The Netherlands School of Agriculture, Food Science, and Veterinary Medicine, and Conway Institute, College of Life Sciences, University College Dublin, Dublin 4, Ireland

ABSTRACT

Methods to predict numbers of healthy oocytes in the ovaries of young adults could have important diagnostic relevance in family planning and animal agriculture. We have observed that peak antral follicle count (AFC) determined by serial ovarian ultrasonography during follicular waves is very highly reproducible within individual young adult cattle, despite 7-fold variation among animals. Herein, we tested the hypothesis that AFC is positively associated with the number of morphologically healthy oocytes and follicles in ovaries and with serum concentrations of anti-Müllerian hormone (AMH), an indirect marker for number of healthy follicles and oocytes in ovaries. In the present study, age-matched young adult cattle (12–18 mo old) were subjected to serial ultrasonography to identify animals with a consistently high (≥25 follicles that were ≥3 mm in diameter) or low (≤15 follicles) AFC during follicular waves. Differences in serum AMH concentrations, ovary weight, and number of morphologically healthy and atretic follicles and oocytes were determined. The phenotypic classifications of cattle based on AFC during follicular waves or AMH concentrations both predict reliably the relative number of morphologically healthy follicles and oocytes in ovaries of age-matched young adult cattle.

anti-Müllerian hormone, antral follicle count, bovine, follicle waves, ovarian reserve, ovary size, reproductive longevity, total number of healthy oocytes, variation in oocyte numbers

INTRODUCTION

The number of morphologically healthy oocytes in the ovaries of mammals is remarkably variable at birth, ranging, for example, from 350 000 to 1 100 000 in humans [1–3] and approximately 14 000 to 250 000 in cattle [4, 5]. Moreover, the number of primordial follicles, which comprise the vast bulk of the different follicle types in ovaries, decreases rapidly during aging [4, 6], and is probably never replenished. Noninvasive methods to quantify the number of morphologically healthy follicles and oocytes in the ovaries of young adults could have significant diagnostic relevance in reproductive medicine. For example, such methods could potentially be used to improve family planning; gauge the impact of nutrition, disease, and therapies on the number of healthy oocytes and reproductive lifespan; and develop new breeding schemes to select for high-fertility farm animals.

Antral follicle numbers are positively associated with a variety of indirect measures of fertility in single-ovulating species, like humans [7–16] and cattle [4, 17–23]. Although these observations imply a potentially important diagnostic value for counting follicles, they should be interpreted with caution for several reasons; 1) antral follicles grow in a wavelike fashion during the reproductive cycles of women [24, 25] and cows [26]; 2) only single-point-in-time quantification of antral follicle numbers at unknown stages of follicular waves has usually been used to assess follicle numbers; and 3) it is unknown whether the variation in antral follicle numbers during follicular waves is reflective of the inherently high variation in the number of morphologically healthy oocytes in the ovaries of young adult women or cows.

Cattle were chosen as the appropriate model to examine the physiological significance of the variation in antral follicle count (AFC) during follicular waves for two reasons. First, cattle are a single-ovulating species with two or three waves of antral follicle growth and atresia during relatively long reproductive cycles [26], similar to women [2, 3, 24, 25, 27]. Second, despite varying 7-fold among cattle, AFC during follicular waves is highly reproducible (0.85–0.95) within healthy individuals regardless of breed, age, season, or management conditions [28, 29]; thus, cattle can be phenotyped reliably based on AFC during a follicular wave. For example, we routinely observed some healthy age-matched young adults in our studies with as few as eight follicles growing during consecutive follicular waves of estrous cycles, whereas others had as many as 56 growing follicles [28]. Anti-Müllerian hormone (AMH), a member of the transforming growth factor β (TGFβ) superfamily of growth factors, is produced primarily by granulosal cells of healthy growing follicles [30], and circulating AMH concentrations are positively associated with follicle numbers in mice [31] and women [30, 32, 33]. However, it is unknown whether AMH concentrations are associated with AFC during follicular waves in cattle. The pronounced variation in AFC during follicular...
wolves in cattle is hypothesized to be positively associated with the inherently high variation in the number of morphologically healthy follicles and oocytes in ovaries and with circulating AMH concentrations. To test this hypothesis, the present study took advantage of the bovine model [28, 29] to determine whether the total number of morphologically healthy follicles and oocytes in ovaries and circulating concentrations of AMH differed between age-matched young adults with consistently relatively low or high AFCs during follicular waves.

MATERIALS AND METHODS

Animals

Cattle used in experiments were located at either the Michigan State University Beef or Dairy Cattle Teaching and Research Centers, or the Lyons Research Farm, University College Dublin, Ireland. All experiments were performed in compliance with protocols approved by the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876), and the European Union Directive 86/609/EC, or the Institutional Animal Care & Use Committee at Michigan State University.

Identification of Cattle with a Low Versus a High AFC During Follicular Waves

Ovaries from each animal were monitored with an Aloka SSD-900 linear array transrectal probe (7.5-MHz transducer), and follicles were counted as described previously [28, 29]. In brief, each ovary was scanned from end to end to identify positions of the corpus luteum and antral follicles. Images for different ovarian sections were captured on the ultrasound monitor, and the diameter and total number of follicles ≥3 mm in diameter were drawn on an ovarian map. Two separate perpendicular measurements of diameter were averaged for each follicle, and the diameter and the total number of follicles ≥3 mm in diameter per pair of ovaries—hereafter referred to as the AFC—were recorded for each animal. Cattle that consistently had a relatively low versus high peak AFC during follicular waves were identified as previously explained [28, 29]. Briefly, adult animals of similar ages and weights were injected twice with prostaglandin F2α (PGF2α) spaced 11 days apart to initiate luteolysis and synchronize occurrence of ovulation. Ovaries were then analyzed once or twice daily to ultrasound determination to AFC, beginning on Days 1–2 after the last PGF2α injection and continuing until completion of the study. Peak AFCs were determined for three to five follicular waves, and the average peak value for AFC per wave was used to classify cattle arbitrarily into the low (<15 follicles), intermediate (16–24 follicles), and high (≥25 follicles) groups [28, 29].

Association of AFC with Variation in Number of Follicles and Oocytes in Ovaries

This study was conducted to determine whether AFC during follicular waves was positively associated with the number of morphologically healthy follicles and oocytes in ovaries. Whether ovary size and weight; total number of healthy primordial, transitional, primary, secondary, and antral follicles; and total number of atretic and poliovular follicles differed between animals with a low versus a high AFC during follicular waves was determined hereford × Angus × Charolais crossbred beef heifers (18.9 ± 0.6 mo of age, n = 90 animals) located at University College Dublin’s Lyons Research Farm were used to complete this study. After ultrasound analysis, 14 (15.6%) of 90 animals were classified in the low group, 65 (72.2%) in the intermediate group, and 11 (12.2%) in the high group. A subset of animals in each group (low, n = 4 animals; intermediate, n = 8; high, n = 4) were randomly selected, synchronized with PGF2α, and subjected to daily ultrasonography to monitor follicle numbers and day of ovulation. Jugular blood samples obtained during a previous study [29] were taken at 1100 h via venipuncture at 24-h intervals beginning on Day 6 of the estrous cycle and continuing until 1 day after ovulation (Day 2 of estrous cycle). Blood samples 6–9 days preceding ovulation, which corresponded with ovulatory follicular waves, and on the day of ovulation were analyzed for serum AMH concentrations.

AMH Assay

The commercially available human MIS/AMH ELISA kit (DSL-10-14400; Beckman- Coulter Inc.) was used to measure serum AMH concentrations in cattle, as kit’s instructions. The bovine AMH assay does not cross-react with other members of the TGFβ superfamily, including TGFβ3, BMP4, or activin [31]. Because AMH concentrations are relatively low in bovine compared with humans [47], the only modification in the assay was to measure
duplicate 40-μl rather than 20-μl volumes of serum during assays. Interassay and intraassay coefficient of variation for an overall average AMH concentration of 1.62 ± 0.07 ng/ml was ≤15.2% (n = 10 assays).

To validate the bovine serum AMH assay, parallelism of different doses of the following sources of bovine sera or follicular fluid with the AMH standard curve was evaluated: ovariectomized Holstein cows (n = 4 animals), beef steers (castrated bulls, n = 2), old Holstein cows (6.8 and 10 yr old, n = 2), and beef heifers with a low (n = 1) or a high (n = 1) AFC during follicular waves; fetal calf serum; and a pool of bovine follicular fluid obtained from small antral follicles (≤5 mm in diameter). As depicted in Figure 2, AMH concentrations in the different volumes of bovine sera from young adults, fetuses, or follicular fluid are parallel with the AMH standard curve, and AMH was undetectable in sera from castrated male or female, or relatively old, cows (6.8 and 10 yr of age).

Statistical Analysis

Analysis of variance was used to test whether AMH concentrations, number of follicles in ovaries, body and ovary weight, and ovarian length and height differed between animals with different AFCs during follicular waves. The Bonferroni t-test was used to determine whether statistical differences existed between individual means [48].

RESULTS

Ovary Size and Number of Follicles and Oocytes in Ovaries of Animals with a Low Versus a High AFC During Follicular Waves

Body weights (406 ± 14 kg versus 417 ± 15 kg) of animals were similar (P > 0.20) between groups. However, wet weight of ovaries, ovarian height, and ovarian length were much smaller (P < 0.001 to P < 0.05) in animals with a consistently low AFC compared with a high AFC during follicular waves (Table 1). Furthermore, although the propor-
Alterations in Circulating AMH Concentrations During Ovulatory Follicular Waves of Animals with Low, Intermediate, and High AFCs

Circulating AMH concentrations were approximately 6- and 2-fold greater \( (P < 0.01) \) in animals with high or intermediate AFCs compared with a low AFC during follicular waves (Fig. 4, left), but did not change within groups \( (P > 0.20) \) during the 6- to 9-day bleeding period prior to ovulation. Overall average AMH concentration during ovulatory follicular waves per animal was highly correlated with both average peak AFC during the two or three waves of an estrous cycle \( (r = 0.88, P < 0.01; \text{Fig. 4, right}) \) and with the overall average for daily AFC during estrous cycles for each animal \( (r = 0.92, P < 0.01; \text{data not shown}) \).

**DISCUSSION**

To our knowledge, this is the first report to directly demonstrate a positive link between the variation in AFC during the follicular waves of reproductive cycles with total number of morphologically healthy and atretic follicles and oocytes in the ovaries of young adults in a single-ovulating species. This novel observation in the bovine model may, therefore, have human relevance. The most significant findings from the present study demonstrate that despite similarity in ages and body weights of young adult cattle, the marked variation in AFC during follicular waves was highly positively associated \( (0.80-0.90) \) with circulating AMH concentrations, ovary size, and total number of morphologically healthy oocytes in ovaries. Consequently, use of serial ovarian ultrasonography to determine peak AFC during follicular waves and measurement of AMH concentrations represents a simple, noninvasive method to predict reliably the relative number of morphologically healthy oocytes and follicles in ovaries of age-matched young adult cattle.

The results of the present study demonstrated that the number of morphologically healthy oocytes and follicles in ovaries and circulating AMH concentrations during ovulatory follicular waves are also markedly reduced in young adult cattle with a low versus a high AFC during follicle waves. Surprisingly, many of these same phenotypic differences between young adult cattle with low versus high AFCs are also reported for older versus younger women \( [2, 3, 7, 8, 10, 12-16, 27, 32, 49] \) or cattle \( [4, 18, 20-23, 50-52] \), young women with relatively high versus normal circulating FSH concentrations \( [53] \), and young women born small versus normal size for gestational age \( [54, 55] \). However, despite the positive association between AFC and a plethora of indirect markers of infertility, future studies will be necessary to determine whether more direct measures of fertility, such as the number of inseminations required for conception or pregnancy rate, are also lower for the healthy young adult cattle with a consistently low AFC during follicle waves. Answering this critical question will firmly establish that a positive link exists between the variation in AFC during follicle waves, the number of morphologically healthy follicles and oocytes in ovaries, and fertility in young adults. These potential findings would make the bovine model especially useful to not only identify new diagnostic markers for fertility, but also to ultimately unravel the genetic or

**TABLE 1.** Average peak AFC, ovary size, and total number of follicles in ovaries of cattle with consistently low \( (\leq 15 \text{ follicles} \geq 3 \text{ mm in diameter}) \) versus high \( (\geq 25 \text{ follicles}) \) peak AFC during follicular waves.

<table>
<thead>
<tr>
<th>Follicle parameters</th>
<th>Low AFC</th>
<th>High AFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak AFC per wave</td>
<td>( 11.95 \pm 1.2 )</td>
<td>( 39.61 \pm 2.3 )***</td>
</tr>
<tr>
<td>Ovary wet weight (g)</td>
<td>( 3.05 \pm 0.33 )</td>
<td>( 7.11 \pm 0.41 )***</td>
</tr>
<tr>
<td>Ovary height (mm)</td>
<td>( 12.3 \pm 1.1 )</td>
<td>( 15.5 \pm 0.8 )*</td>
</tr>
<tr>
<td>Ovary length (mm)</td>
<td>( 23.3 \pm 1.4 )</td>
<td>( 28.3 \pm 1.3 )*</td>
</tr>
</tbody>
</table>

* Statistical significance within row: \( *P < 0.05, **P < 0.01, ***P < 0.001. \)

**FIG. 3.** Number of morphologically healthy primordial, transitory, primary, secondary, and antral follicles in ovaries of cattle with a low versus a high AFC during ovarian follicular waves. Animals with low \( (n = 5) \) versus high \( (n = 5) \) AFCs during follicular waves were identified and were subjected to PGF\(_{2\alpha}\) to synchronize estrus and determine day of ovulation. The ovary contralateral to the recent ovulation was removed surgically 1–2 days after ovulation and cut into eight roughly equivalent longitudinal strips, and strips of ovarian tissue were serially sectioned at 8-μm intervals. Sections were stained with hematoxylin and picric methyl blue, and the number of morphologically healthy primordial, transitory, primary, secondary, and antral follicles was counted in every 40th section for two randomly chosen ovarian strips per animal. Criteria for the health status of follicles, follicle classifications, and how the total number of follicles in ovaries was determined are described in Materials and Methods and the legend for Figure 1. Each bar represents the mean (±SEM) for five animals per group. Asterisks \( *(P < 0.05, **P < 0.01) \) indicate difference between means for the low versus high group.
TABLE 2. Pearson correlation coefficients.\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>AFC during waves</th>
<th>Ovary weight (g)</th>
<th>Total no. of healthy follicles</th>
<th>Total no. of atretic follicles</th>
<th>Total no. of healthy &amp; atretic follicles combined</th>
<th>Total no. of polyovular follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFC</td>
<td>1</td>
<td>0.90***</td>
<td>0.89***</td>
<td>0.90***</td>
<td>0.90***</td>
<td>0.80**</td>
</tr>
<tr>
<td>Ovary weight (g)</td>
<td></td>
<td>1</td>
<td>0.94***</td>
<td>0.81***</td>
<td>0.82**</td>
<td>0.85**</td>
</tr>
<tr>
<td>Total no. of healthy follicles</td>
<td>1</td>
<td></td>
<td>0.89***</td>
<td>0.90***</td>
<td>0.91***</td>
<td>0.86**</td>
</tr>
<tr>
<td>Total no. of atretic follicles</td>
<td>1</td>
<td></td>
<td>1</td>
<td>0.99***</td>
<td>0.91***</td>
<td>1</td>
</tr>
<tr>
<td>Total no. of healthy and atretic follicles</td>
<td>1</td>
<td></td>
<td>1</td>
<td>0.99***</td>
<td>0.91***</td>
<td>1</td>
</tr>
<tr>
<td>Total no. of polyovular follicles</td>
<td>1</td>
<td></td>
<td>1</td>
<td>0.99***</td>
<td>0.91***</td>
<td>1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Because of high variation in numbers for each parameter measured among animals (n = 10), all data were log	extsubscript{10} transformed before statistical analysis (note: 3 of the 5 animals in the Low group had 0 polyovular follicles, which were converted to 1 before log transformation).

** P < 0.01.
*** P < 0.001.

epigenetic causes of the high variation in numbers of healthy oocytes in ovaries, and the mechanisms whereby the inherently high variation in the number of healthy follicles and oocytes may have an impact on fertility.

Reproducibility of AFC during follicular waves of menstrual cycles has not been reported \cite{24, 25}. Nevertheless, AFC is highly reproducible (0.73–0.83) within individual women \cite{33, 56}, even though in these studies only a single ultrasound measurement of AFC was made during the early follicular phase for each menstrual cycle. This finding implies that like cattle, peak AFC may be highly reproducible during follicular waves in women, and that young adult women with a consistently relatively low AFC during follicular waves may also have smaller numbers of morphologically healthy oocytes compared with their age-matched counterparts with higher AFCs.

The young adult cattle with consistently high versus low peak AFC in the present study had much greater rates of antral follicle growth and atresia, as implied by the 3.3-fold higher peak AFC during follicular waves. This high rate of follicular growth and atresia probably explains why the cattle with high AFCs also had 10-fold more atretic follicles in their ovaries. Nevertheless, despite the high rates of growth and atresia and the excessive numbers of atretic follicles, the proportion of morphologically healthy oocytes and follicles was similar between groups. Moreover, although absolute numbers are much greater, previous studies show that the proportion of high-quality oocytes aspirated from antral follicles and the proportion of blastocysts and transferable embryos produced following in vitro fertilization are also similar between cattle with low versus high AFCs \cite{29}. These combined observations imply that unlike older compared with younger individuals, the inherently high variation in number of morphologically healthy oocytes and follicles in ovaries per se of young adults may be very unlikely to negatively impact oocyte quality. The caveat in this interpretation, however, is that none of the aforementioned studies that morphologically evaluated oocyte quality also directly examined pregnancy outcome.

To our knowledge, only one other study has reported a total number of different follicle types in bovine ovaries \cite{4}. In this study, all follicles with a single layer of granulosal cells surrounding an oocyte were classified as primordial, and different criteria were used for atresia compared with the present study. Nevertheless, the total numbers of morphologically healthy primordial, transitory, and primary follicles combined and secondary follicles reported in the present study for the young adult beef cattle with low or high follicle numbers were within the range of values reported for total number of healthy primordial (approximately 1000–80 000) or secondary (approximately 40–456) follicles for 12-mo-old beef cattle in the Erickson study \cite{4}. In contrast, the range for total number of healthy antral follicles in ovaries (approximately 1–59) was much lower in the Erickson study \cite{4} compared with the animals with low and high AFCs during follicular waves in the present study, probably because Erickson did not use a microscope to detect this follicle type. However, the total number of antral follicles for cattle with low or high AFC during follicular waves in the present study was within the range (127–490) reported in a previous study also using histological analysis to determine the number of antral follicles in young adult cattle \cite{38}.

The discovery in the present study that young adult cattle with a high AFC during follicular waves also have approximately a 5-fold greater number of morphologically healthy
correlation between peak AFC during follicular waves with the same animals in the present study, the high degree of concentrations are almost static during menstrual cycles [46, 45]. These concentrations exist between pregnant mothers or their unknown in the bovine whether differences in circulating FSH concentrations, ovary size, and number of morphologically healthy follicles and oocytes in ovaries. Based on these findings, the variation in peak AFC during follicular waves and, correspondingly, circulating AMH concentrations are both concluded to be reliable phenotypic markers to predict the relative number of morphologically healthy follicles and oocytes in ovaries and, perhaps potential reproductive longevity in young adult cattle.

In summary, the results of this study demonstrate that variation in peak AFC during follicular waves is highly positively associated with alterations in circulating AMH concentrations, ovary size, and number of morphologically healthy follicles and oocytes in ovaries. Even though the number of follicles lost via atresia during each follicular wave was 7-fold greater for the animal with the highest versus the lowest AFC, the animal with the highest AFC had a 21-fold greater number of healthy oocytes in her ovaries. Thus, despite the inherently higher rate of follicle growth and atresia, the animal with the highest AFC during follicular waves theoretically also has a sufficient number of healthy oocytes to maintain a much longer reproductive lifespan compared with its age-matched cohort with a lower AFC and a greatly diminished number of oocytes in ovaries. Note, however, that the potentially enhanced reproductive longevity for young adults with high versus low AFCs and, correspondingly, much larger numbers of healthy oocytes in ovaries assumes that the ovaries of both groups of animals are impacted similarly during aging by factors such as nutrition, stress, disease, and environmental toxicants.

In the present study, ovarian ultrasonography combined with histological analysis of the same ovaries unexpectedly revealed that the high variation in AFC (range: 7–50 follicles ≥3 mm in diameter per wave) among animals was also highly positively correlated with the number of polyovular follicles. Polyovular follicles have not been previously reported in cattle, but they are observed, albeit infrequently, in women [57, 58], rodents [57], and other species [57, 59]. The origin of polyovular follicles is unknown but is likely caused by encasement of multiple oocytes into primordial follicles during folliculogenesis [57, 59, 60]. Incidence of polyovular follicles in mice is decreased by gonadotropin treatment [61] but enhanced by overexpression of inhibin-α [62], knockout of the Dmrt4 gene [60], or treatment with estrogen [61]. Based on these observations, it is tempting to speculate that the chronically lower circulating FSH concentrations during pregnancy (and, in turn, presumably lower ovarian estradiol and inhibin-α production, although this was not tested in the present study), which are expected in animals with consistently relatively high versus low AFCs [28, 29], coupled with the much (45%) higher density of oocytes per gram of ovary (present study), may have indirectly caused or contributed to the higher number of polyovular follicles observed in animals with a consistently high AFC in the present study. However, although FSH has a positive role in the development of primordial follicles during embryogenesis in hamsters [63], it is unknown in the bovine whether differences in circulating FSH concentrations exist between pregnant mothers or their embryos with low or high follicle numbers in ovaries.

Although the day-to-day variation in serum AMH concentrations was relatively minor in individual cattle in the present study, circulating AMH concentrations were nearly 6- and 2-fold higher for young adult cattle with a relatively high or intermediate versus a low AFC (Fig. 4). In addition, the average peak AFC for these animals was highly positively correlated with the average AMH concentrations. These findings support previous results demonstrating that AMH concentrations are almost static during menstrual cycles [46, 64] and are highly correlated with AFC in women [32, 33]. Although circulating AMH concentrations and the number of healthy follicles and oocytes in ovaries were not measured in the same animals in the present study, the high degree of correlation between peak AFC during follicular waves with the number of healthy follicles and oocytes in ovaries, coupled with a previous report showing that AMH concentrations decrease coincidently with the decline in the number of primary and growing follicles as rodents age [31], strongly imply that circulating AMH concentration is also a reliable phenotypic marker to predict the relative number of morphologically healthy follicles and oocytes in ovaries of age-matched young adult cattle.

REFERENCES
