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Superovulatory Response of One Ovary Is Related to the Micro- and Macroscopic Population of Follicles in the Contralateral Ovary of the Cow

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ABSTRACT

We hypothesized that the ovulatory response of one ovary to FSH would be related positively to the size of the primordial and growing pools of follicles in the other ovary. Nonlactating cows (n = 26) were unilaterally ovariectomized and 2 days later were superovulated. The superovulatory response was classified as Low (< 5 corpora lutea [CL]), Medium (5–14 CL), or High (> 14 CL). Surface follicles on the ovary removed before superovulation were classified as small (1–3 mm), medium (3–7 mm), or large (> 7 mm), and the ovary was then fixed and serially sectioned. Follicles ≤ 1 mm in diameter in 388 ± 38 fields (2 × 2 mm) per cow were classified as primordial, primary, secondary, or tertiary. By classification, Suboptimal ovaries contained < 100 follicles ≤ 1 mm and Optimal ovaries contained > 250 follicles ≤ 1 mm. Number of CL was correlated positively with total number of primordial, tertiary, and medium surface follicles. Number of Empty fields (2 × 2-mm fields containing no follicles) was correlated negatively with superovulatory response and number of primordial follicles. Number of CL was related to number of tertiary follicles in a positive linear manner and the number of medium follicles in a positive quadratic manner (r^2 = 0.66). Numbers of primordial, tertiary, small surface follicles, medium surface follicles, and total surface follicles were lower ($p \leq 0.06$) in the Low superovulatory response group than in the Medium or High group. Suboptimal ovaries had fewer small surface follicles and fewer CL than Optimal ovaries ($p < 0.05$). We conclude that superovulatory response in cattle is related positively to the pools of primordial and growing follicles in the bovine ovary.

INTRODUCTION

The bovine ovary contains a large pool of primordial follicles at birth, but less than 0.1% of the follicles present in the ovary ovulate during the reproductive lifetime of a cow [1–3]. Most follicles that enter the growing pool are destined to undergo atresia, but short-term treatment with gonadotropins (superovulation) stimulates advanced antral follicles to grow to ovulatory size and ovulate. There is a large amount of variation in response to superovulatory treatments, because cows may ovulate from 0 to 40 follicles following a 4- to 5-day treatment with FSH [4]. Variation in the number of FSH-responsive follicles present in the ovary at the initiation of superovulatory treatment has been estimated to account for 70% of the variation in superovulatory response [5].

There is a similarity between the two ovaries in the number of follicles present in the cow [1], the sow [6], the ewe [7], and the monkey [8]. Erickson [1] observed that in most specimens he examined there was only about a 10% difference in the number of germ cells between the two ovaries in cows. From this, he predicted that the population of follicles in one ovary might be a good indicator of the number of follicles present in the other ovary. Moreover, when Erickson [2] classified heifers by whether they had more or less than 100 000 primordial follicles in both ovaries, he observed that heifers with more than 100 000 primordial follicles also had a greater number of growing and vesicular follicles. He [1, 2] concluded that there was a large variability among cows in the number of germ cells present in both ovaries, and suggested that variation in reproductive capacity may be partially due to variation in germ cell numbers among animals.

One method for studying follicular dynamics would be to remove one ovary for examination and then to superovulate the remaining ovary. However, removal of one ovary results in an acute compensatory increase in the number of large follicles present in the other ovary in multiple ovulators (rat [9]; pig [10]). In cattle that have been hemicastrated, this compensatory ovarian hypertrophy seems to be manifested as an increase in the number of follicles that progress from the small to medium antral stage. Saiduddin et al. [11] observed an increase in the number of follicles greater than 5 mm in diameter when cattle were unilaterally ovariectomized. In another study in which prepubertal heifers were unilaterally ovariectomized, there was no difference in the total number of visible follicles 7 days later; however, there was an increase in the number of follicles 5–6 mm and ≥ 9 mm in diameter [12]. Lussier et al. [13] also observed an increase in the number of medium follicles (3.67–8.6 mm) in heifers that had been hemicastrated for 6 days. Selection of the dominant follicle and ovulation may also be affected by unilateral ovariectomy [14]. Mohan and Rajamahendran [14] used ultrasonography to demonstrate that unilateral ovariectomy resulted in sustained growth of the subordinate follicle and ovulation of 2 follicles approximately 75% of the time during the three subsequent ovulatory periods.

We are unaware of any research that has examined how the number of follicles that ovulate after superovulation is related to the sizes of various-sized pools of follicles in the bovine ovary. Therefore, the objective of the present study was to examine the relationship between the micromorphology of one bovine ovary and the superovulatory response of the contralateral ovary. Specifically, we hypothesized that the ovulatory response to FSH of one ovary would be related positively to the size of the primordial and growing pools of follicles in the other ovary.

MATERIALS AND METHODS

Experimental Animals and Tissue Collection

Nonlactating Holstein and Jersey cows (n = 26) were maintained on a mixed pasture of fescue, white clover, and Bermuda grass. These cattle were used in an experiment to determine the effects of long-term treatment with estradiol

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TABLE 1. Correlations between histological populations and CL number.

Correlated trait	Classification of fields or follicles within fields				
	Empty	Primordial	Primary	Secondary	Tertiary
Primordial	−0.80[a]				
Primary	−0.29	0.51[a]			
Secondary	0.02	0.35	0.32		
Tertiary	−0.34	0.52[a]	0.23	0.66[a]	
CL	−0.50[a]	0.44[a]	0.16	0.31	0.68[a]

[a] $p < 0.05$.

and recombinant bovine somatotropin on superovulation [15]; however, these treatments did not alter follicle numbers, and therefore treatments were pooled for the current study to examine the relationship between follicle numbers and superovulatory response. The ovary contralateral to the corpus luteum (CL) was removed by colpotomy 48 h before initiation of superovulatory treatment [16]. Beginning on Day 9.7 ± 0.17 of an estrous cycle, cows were treated with 26 mg of FSH-P (Schering-Plough Animal Health Corp., Kenilworth, NJ) in a decreasing dosage over 4 days. On the third day of FSH treatment, cows were given (i.m.) 25 mg of prostaglandin $F_{2\alpha}$ (Lutalyse; Pharmacia and Upjohn Inc., Kalamazoo, MI) to induce luteolysis. Seven days after estrus, the animals were slaughtered and the reproductive tracts were recovered to determine the number of CL. All experimental procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Tissue Handling and Processing

Immediately after unilateral ovariectomy, surface follicles of the excised ovary were counted and classified as small (1–3 mm), medium (3–7 mm), or large (> 7 mm). The ovary was divided into quarters, and two quarters were fixed in Bouin's solution. The tissue was dehydrated using a graded series of ethanol, cleared with Clearite (Richard-Allan Medical, Richland, MI), and embedded in paraffin (Paraplast Plus; Baxter, West Chester, PA). Thirty to fifty consecutive sections (6 μm) from one quarter were mounted onto glass slides and stained with a periodic acid-Schiff reaction and hematoxylin counterstain.

Morphometrics

Histological sections were examined using a superimposed counting grid. Briefly, a 1-cm × 1-cm grid divided into twenty-five 2-mm × 2-mm counting fields was printed onto acetate film (3M Corporation, Austin, TX). Grids were glued (Krazy Glue; Borden, Columbus, OH) onto the underside of each slide below each section. The edges of each section and antral follicles were used as landmarks to ensure that the grid was positioned the same on consecutive sections. For each section, every completely filled 2-mm × 2-mm field that contained cortical tissue was counted. Follicles were classified into one of the following stages: primordial, an oocyte surrounded by a single layer of pregranulosa cells; primary, an oocyte surrounded by a single layer of activated granulosa cells; secondary, an oocyte surrounded by two or more layers of granulosa cells; and tertiary, an oocyte surrounded by two or more layers of granulosa cells but no larger than 1 mm in diameter with a distinct antrum. To avoid counting follicles more than once, follicles were counted by tracking back to see whether the nucleus of the oocyte had appeared in the previous section; if not, it was counted. If a 2-mm × 2-mm field was filled with cortical tissue but contained no follicles it was classified as Empty.

TABLE 2. Correlations between surface populations and CL number.

Correlated trait	Surface follicle class			
	Small (1–3 mm)	Medium (3–7 mm)	Large (>7 mm)	Total follicles
Medium (3–7 mm)	0.01			
Large (>7 mm)	0.07	0.61[a]		
Total	0.60[a]	0.79[a]	0.64[a]	
CL	0.29	0.50[a]	0.34	0.59[a]

[a] $p < 0.05$.

TABLE 3. Correlations between histological populations and surface populations.

Correlated trait	Surface follicle class		
	Small (1–3 mm)	Medium (3–7 mm)	Large (>7 mm)
Empty	−0.38[a]	−0.31	−0.32
Primordial	0.50[a]	0.24	0.17
Primary	0.56[a]	−0.02	−0.25
Secondary	0.44[a]	0.03	−0.15
Tertiary	0.44[a]	0.28	0.27

[a] $p < 0.05$.

Statistical Analysis

Partial correlation analyses, adjusted for the effects of estradiol, bovine somatotropin, and their interaction, were performed among all micromorphological, macromorphological, and superovulatory response data using the PROC CORR of the Statistical Analysis System (SAS) [17]. Number of CL was regressed on micromorphological and macromorphological counts of follicles using the General Linear Models (GLM) procedure of SAS. Variables that were not significant were eliminated from the model, and the remaining variables were fitted to linear and quadratic equations.

Based on the frequency of CL from all cows, superovulatory response was classified as Low (< 5 CL, n = 9), Medium (5–14 CL, n = 9), or High (> 14 CL, n = 8). Least-squares ANOVA (PROC GLM, SAS) was used to determine whether there were differences among these classes in number of microscopic and macroscopic follicles. To further determine whether populations of microscopic follicles could be used to predict superovulatory response, the total number of microscopic follicles was classified as Suboptimal (< 100 microscopic follicles, n = 5) or Optimal (> 250 microscopic follicles, n = 21) based on a bimodal distribution of the counts. There were no ovaries with a total number of microscopic follicles between 100 and 250. Least squares ANOVA was used to determine whether there were differences among these classes in number of macroscopic follicles and number of CL.

RESULTS

Correlations

Correlation analyses showed that among populations of microscopic follicles, number of tertiary follicles (≤ 1 mm) had the strongest positive correlation with number of CL (Table 1). Number of CL also was correlated positively

TABLE 4. Mean number of microscopic follicles within superovulatory response (SR) groups.

SR	Microscopic follicle class			
	Primordial	Primary	Secondary	Tertiary
Low	77.4[a]	64.8[x]	4.1[x]	1.6[a]
Medium	178.2[b]	137.3[y]	9.1[y]	3.8[b]
High	173.7[b]	95.7[y]	9.1[y]	3.8[b]
SEM	22.4	22.1	1.7	0.6

[a,b] $p < 0.05$; within a column.
[x,y] $p < 0.1$; within a column.

with number of primordial follicles and negatively with number of Empty fields.

The number of primordial follicles was correlated positively with the number of primary and tertiary follicles and negatively with the number of Empty fields. There was no significant statistical correlation between the number of primary and the number of secondary or tertiary follicles, but the number of secondary follicles was positively correlated with the number of tertiary follicles.

There were significant correlations between the populations of surface follicles on the ovary excised before superovulation and the superovulatory response (number of CL) after superovulation of the contralateral ovary (Table 2). Total number of surface follicles was correlated positively with superovulatory response ($r = 0.59$, $p < 0.05$). Among the surface classifications, medium follicles (3–7 mm) were correlated positively with superovulatory response, and there was a tendency ($p < 0.1$) toward a positive correlation between large follicles and superovulatory response. Number of small surface follicles (1–3 mm) was not correlated with superovulatory response, number of medium (3–7 mm) follicles, or number of large (> 7 mm) follicles.

Comparisons between microscopic and macroscopic follicles revealed that number of small surface follicles was correlated positively with the number of primordial, primary, secondary, and tertiary follicles and negatively with the number of Empty fields (Table 3). There were no significant correlations between the number of medium or large follicles and the histological populations.

Relationship between Follicles and CL Number

Figure 1 shows a surface plot resulting from multiple regression of number of CL on number of tertiary and medium follicles. There was a positive linear relationship (partial $r = 0.11$) between the number of tertiary follicles counted and the number of CL present on the ovary after superovulation. There was a quadratic relationship (partial $r = 0.55$) between the number of medium follicles present on one ovary before superovulation and the number of CL present on the other ovary following superovulation.

TABLE 5. Mean number of surface follicles within superovulatory response (SR) groups.

SR	Surface follicle class			
	Total	Small (1–3 mm)	Medium (3–7 mm)	Large (>7 mm)
Low	6.1[a]	2.4[a]	2.5[a]	1.3
Medium	22.4[b]	11.6[b]	9.3[b]	1.6
High	21.7[b]	8.3[b]	11.1[b]	2.3
SEM	3.1	1.9	2.4	0.7

[a,b] $p \leq 0.06$; within a column.

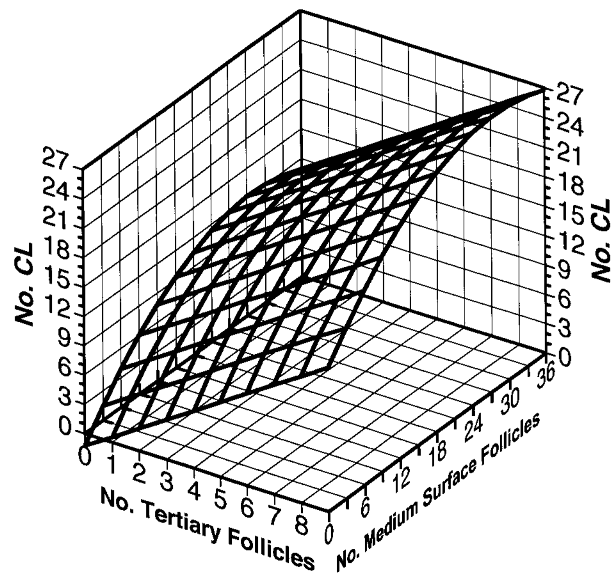

FIG. 1. Effect of the number of tertiary follicles and medium follicles on the number of CL after superovulation. Number of CL = $-1.240 + (1.760 \times \text{tertiary}) + (0.9965 \times \text{medium}) - (0.0174 \times \text{medium}^2)$.

Response Groups

There was no difference in the number of fields that were counted among the levels of superovulatory response (388 ± 38 fields per cow, $p = 0.19$); however, there was a greater percentage of Empty fields in the Low responders than in either the Medium or High responders ($p = 0.003$, Fig. 2).

Number of microscopic and macroscopic follicles differed among the three superovulatory response groups. There were fewer primordial follicles in the Low responding group than in either the Medium or High responders ($p < 0.05$, Table 4), and there was a tendency ($p < 0.1$) toward fewer primary and secondary follicles in the Low responders. There were fewer tertiary follicles (antral to 1 mm) in the Low responders than in the Medium or High responders ($p < 0.05$).

Overall, there were fewer total surface follicles in the Low responders than in the Medium or High responders ($p < 0.05$, Table 5). There were fewer small (1–3 mm) surface follicles in the Low responders than in the Medium or High responders ($p < 0.05$). There was a tendency ($p = 0.06$)

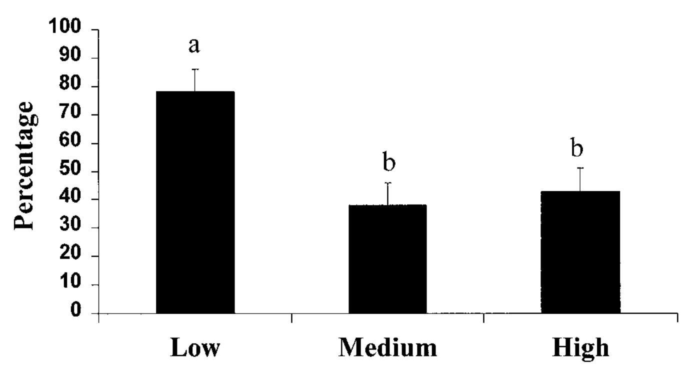

FIG. 2. Average percentage of Empty fields per cow within the three superovulatory response groups. There was a significantly greater percentage ($p < 0.05$) of Empty fields in the Low superovulatory response group than in the Medium or High group. The data represent means ± SEM of 8–9 cows per response group.

TABLE 6. Mean number of surface structures in cattle with Sub-optimal or Optimal numbers of microscopic follicles.

	Surface structure			
	Small (1–3 mm)	Medium (3–7 mm)	Large (>7 mm)	CL
Sub-optimal	1.8 ± 2.5[a]	4.7 ± 3.2	1.3 ± 0.9	4.3 ± 3.1[a]
Optimal	9.4 ± 1.3[b]	8.8 ± 1.8	1.9 ± 0.5	12.5 ± 1.7[b]

[a,b] $p < 0.05$; within a column.

for the Low responders to have fewer medium (3–7 mm) follicles, but there were no differences in the number of large (> 7 mm) follicles among the three response groups.

Classification by Optimal or Suboptimal Number of Microscopic Follicles

When ovaries were classified by total number of microscopic follicles, there were fewer small (1–3 mm) follicles and CL in the Suboptimal group ($p < 0.05$, Table 6).

DISCUSSION

We believe that this is the first study to examine relationships between histological and surface populations of follicles in one ovary and superovulatory response of the other ovary in cattle. Clearly, we have demonstrated that cows that had a low response to superovulation with FSH-P had fewer primordial and tertiary follicles. These cows also had fewer total surface follicles, mainly attributable to fewer small (1–3 mm) and medium (3–7 mm) follicles. Moreover, cows that had fewer microscopic follicles had fewer small (1–3 mm) follicles and CL.

The finding that the number of tertiary follicles (≤ 1 mm) was strongly positively correlated with superovulatory response ($r = 0.68$, $p < 0.05$) shows that the pool of follicles that are not yet at a stage that would respond to exogenous FSH may subsequently contribute in a linear manner to the responsive pool. These follicles do not represent the population of antral follicles that would be responding to FSH 48 h after unilateral ovariectomy, because Lussier et al. [18] demonstrated that it takes about 42 days for the earliest antral follicles to grow to ovulatory size in cattle. This would seem to indicate that the pool of microscopic antral follicles is a good predictor of how an animal will respond to superovulation in the future. Although one cannot establish cause-and-effect relationship through correlation analysis, support for a causal link comes from the observations that the regression of the number of CL on the number of tertiary follicles showed a positive linear relationship and that Low responders had fewer tertiary follicles than Medium or High responders.

The number of primordial follicles was correlated with the number of primary ($r = 0.52$, $p < 0.05$) and tertiary follicles ($r = 0.51$, $p < 0.05$). This would indicate that cows that had a larger pool of dormant follicles would subsequently have more follicles moving into the growing pool, would have a larger pool of tertiary follicles, and would be better superovulators. From these data, it appears that it might be possible to take a needle biopsy of ovarian tissue and, on the basis of the number of primordial and tertiary follicles present, predict the type of superovulatory response that could be obtained from a heifer before she ever reached breeding age. This might also be a technique for evaluating the ovarian status of infertile cows or cows that respond poorly to superovulation.

Based on the total number of microscopic follicles for each cow, cows were classified as having Optimal or Suboptimal populations of follicles. Classification was based on a clear demarcation in the frequency of cows with different numbers of follicles. Thus, cows in the Suboptimal category had a maximum of 100 microscopic follicles whereas those in the Optimal category had a minimum of 250 microscopic follicles. The cause of this difference is unknown, but cows in the Suboptimal category had fewer FSH-responsive follicles as indicated by the lower superovulatory response. These differences in number of follicles probably originated during early folliculogenesis or before puberty, because Erickson [2] found differences among heifers as early as 2 mo of age.

There were no significant correlations between the number of primary follicles and the number of secondary or tertiary follicles. The reason could be that movement from the primary stage to the secondary stage is a key control point in folliculogenesis. A large rate of atresia during the secondary stage could explain the lack of significant correlations between the number of primary follicles and the subsequent microscopic stages. Proof that atresia may occur in bovine preantral follicles can be found in the work of Erickson [1]. In a period of 40 days, the number of follicles in the fetal ovary decreased from 2 500 000 to 108 000. There would have to be some atresia of preantral follicles because not all these follicles could grow to the antral stage in this short time. While the current study did not examine atresia rates, Hirshfield [19] demonstrated that in the rat there is very little atresia during the first several generations of granulosa cell division, with the rate of atresia increasing in the late secondary stage. It is possible that the lack of correlation between primary and secondary follicles in the current study may be due to the experimental sampling method.

The total number of surface follicles was correlated with superovulatory response ($r = 0.59$, $p < 0.05$). Similar correlations between the total number of surface follicles on the ovary at the initiation of superovulation and superovulatory response have been reported by Monniaux et al. [5]. We also observed a positive correlation ($r = 0.50$, $p < 0.05$) between the number of medium follicles (3–7 mm) and the superovulatory response. Gong et al. [20] noted a similar correlation between the number of small surface follicles and superovulatory response; however, in their study, follicles were measured by ultrasound and the size classifications were slightly different from ours. In our study, dimensions of individual small and medium surface follicles were not recorded; thus, we were unable to reclassify the data to compare directly our results with those of Gong et al. [20]. The fact that the number of medium follicles was correlated with superovulatory response is in good agreement with other data of Gong et al. [21], demonstrating that the pool of antral follicles that grow in response to FSH is selected from follicles that are about 3–4 mm in diameter. This is further supported by the finding that the number of small follicles present on the surface of the ovary 48 h before treatment with FSH was not correlated with superovulatory response. The number of small surface follicles was positively correlated with all microscopic classes, but with neither the medium nor the large follicles. Therefore, we speculate that FSH is selecting follicles from the 3- to 4-mm size range. There was only a tendency for the number of large follicles to be correlated with superovulatory response. The larger of these follicles probably regressed when the FSH-P was administered [22].

The quadratic relationship between the number of medium surface follicles and the number of CL suggests that an increase beyond approximately 21 medium follicles does not add to the number of follicles that are ovulated in response to FSH-P. At this point, another factor, such as the amount of FSH administered or spacial restrictions on the surface of the ovary, may become rate limiting to the number of follicles that can grow to ovulatory size. The linear relationship between number of CL and number of tertiary follicles was based on counting a sample of tissue, not on an estimate of tertiary follicles in the whole ovary. Nevertheless, the positive linear regression indicates that the population of tertiary follicles contributes to the number of CL by providing a directly related pool of FSH-responsive follicles. Collectively, these data suggest that the superovulatory response is largely dependent upon the population of follicles in the ovary up to some threshold, beyond which the response plateaus or declines.

When cows were classified in response groups, there were fewer primordial and tertiary follicles in the Low responders than in the Medium or High responders. There were also fewer surface follicles, primarily small (1–3 mm) and medium (3–7 mm) follicles, in the Low superovulatory response group. On the basis of the concept that folliculogenesis is a long process in the cow [18], we are currently pursuing the idea of long-term modification of the pool of growing follicles to improve superovulatory response [15, 23]. Typically, superovulation treatments have been used to increase the number of follicles growing to ovulatory size within a 3- to 5-day window of time during diestrus. Although the size of the primordial pool is set at birth [1], it might be possible to increase the rate of activation of primordial follicles into the growing pool and subsequently increase the number of follicles that survive to the tertiary stage at which they can respond to superovulatory treatments. In this manner, a cow that would normally be a Low responder could be converted to a Medium or High responder.

The concept that folliculogenesis in cattle is a long process, coupled with the variation that we observed in the size of different follicle pools, also might help explain the poor repeatability of superovulation [24]. Numbers of medium follicles, which represent the size class responsive to FSH stimulation, were correlated well with superovulatory response, as was number of tertiary follicles, which should reach ovulatory size within about 42 days [18]. Number of primordial follicles was not correlated as well with superovulatory response. The time required for early preantral follicles to reach ovulatory size has been estimated, by extrapolation, to be 80–100 days in the cow [25], 6 mo in the ewe [26], and 84 days in the sow [27]. Therefore, in a situation when a donor cow might be superovulated every 2–3 mo, environmental factors, such as nutritional stress or heat stress, could have an effect on the growth of these preantral follicles and affect the subsequent superovulatory response when the follicles reach the antral stage. In contrast, there is a higher repeatability among number of follicles available for ovum pickup twice weekly [28]. This shows that in the short term, the size of cohorts within a cow is highly repeatable. Even with ovum pickup, however, there are long-term trends toward changes in the size of cohorts [28], suggesting environmental influences on the preantral follicle population.

From this study, we conclude that the ovulatory response to FSH reflects the size of the primordial pool and the pool of tertiary follicles. Cows that had a lower superovulatory response had fewer primordial follicles and tertiary follicles. Further studies will involve long-term modification of folliculogenesis to attempt to increase the size of the tertiary pool and improve subsequent superovulatory response.

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