Evaluation of numbers of microscopic and macroscopic follicles in cattle selected for twinning[1]

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ABSTRACT: We hypothesized that the number of microscopic follicles present in the ovaries of cattle selected for twin births (Twinner) would be greater than in the ovaries of contemporary Controls. Ovaries were collected from seven Control and seven Twinner cows at slaughter. The number of Small (1 to 3.9 mm), Medium (4 to 7.9), and Large (> 8 mm) surface follicles was counted and one ovary was fixed for histological evaluation. Fifty to sixty consecutive 6-μm slices were taken from a piece of cortical tissue, approximately 1 cm × 1 cm in area, located between the surface follicles. Microscopic follicles were classified as primordial (oocyte surrounded by a single layer of squamous pregranulosa cells), primary (oocyte surrounded by a single layer of one or more cuboidal granulosa cells), secondary (oocyte surrounded by two or more layers of granulosa cells), or tertiary (oocyte surrounded by multiple layers of granulosa cells with initiation of antrum formation to ≤1 mm in diameter). The total number of follicles was counted in 200 fields (2 mm × 2 mm) per ovary. A field containing no follicles was classified as empty. There were significantly more secondary follicles in Twinner compared with Control ovaries (12.9 vs 6.3; $P < .05$). Twinners also tended to have more small surface follicles (35.4 vs 49.0; $P < 0.1$). We conclude that ovaries of Control and Twinner cows do not differ in the number of primordial follicles or in the number of follicles activated into the growing pool; however, Twinner cows are able to maintain more growing follicles at the secondary and subsequent stages of development.

Key Words: Bovine, Follicle, Folliculogenesis, Twinning

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Introduction

Relatively few studies have examined relationships between numbers of preantral follicles and antral follicles in the bovine ovary (Cushman et al., 1999; Erickson, 1966a; Erickson, 1966b; Erickson et al., 1976). Erickson (1966b) classified heifers by whether they had more or fewer than 100,000 primordial follicles in both ovaries and concluded that heifers with more than 100,000 primordial follicles also had a greater number of growing and vesicular follicles.

We reported that the number of microscopic preantral follicles present in one bovine ovary removed 7 to 8 d after estrus was related positively to the number of small (1 to 3 mm) follicles (**Small**) present in that ovary and to ovulatory response of the contralateral ovary when superovulated with FSH beginning 2 d later (Cushman et al., 1999). From those results, we concluded that the numbers of growing preantral follicles in the bovine ovary influenced the number of small surface follicles.

Echternkamp et al. (1990) have selected a line of cattle (Twinner) that have an increased frequency of multiple ovulations. These Twinner cattle also have a greater number of follicles ≥ 4 mm in diameter 48 to 50 h after $PGF_{2\alpha}$ than unselected cattle (**Control**). In a more recent study examining selection of follicles in Twinner cattle, Echternkamp and Gregory (1998) demonstrated that Twinner cattle have more **Small** (1 to 3.9 mm), **Medium** (4 to 7.9 mm), and **Large** (>8 mm) antral follicles at 24 to 72 h after $PGF_{2\alpha}$. This would indicate that the Twinner cattle have a larger pool of small follicles and are able to maintain more follicles throughout the selection process. The objective of the present study was to examine the relationship between the numbers of microscopic follicles and the number of surface follicles in ovaries from Twinner and Control populations. Specifically, we hypothesized that cattle

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selected for twin births would have a greater number of preantral follicles present in their ovaries.

Materials and Methods

Experimental Animals and Tissue Collection. Seven cows selected for multiple ovulations (Twinner) and seven crossbred cows (Control) from composites containing breeds in the Twinner line at the Roman L. Hruska U.S. Meat Animal Research Center at Clay Center, NE were used in this experiment. The cows ranged from 2 to 5 yr of age. They were fed 22.7 kg/d of a ration containing 84.8% corn silage, 11.3% corn, and 3.8% mineral-vitamin supplement. At random stages of the estrous cycle, the cows were injected with GnRH (100 mg, Cystorelin, Sanofi Animal Health, Inc., Overland Park, KS) followed 7 d later by 30 mg of $PGF_{2\alpha}$ (Lutalyse, Pharmacia and Upjohn, Inc., Kalamazoo, MI) to synchronize estrus and the first follicular wave. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Tissue Handling and Processing. Ovaries were collected at slaughter on d 6 to 8 after estrus to be consistent with the stage of the cycle examined by Cushman et al. (1999). The ovaries were collected within 5 to 10 min of slaughter, and the surface follicles were counted and classified as described previously (Echternkamp et al., 1990). Briefly, follicles were classified as small (1 to 3.9 mm in diameter), medium (4 to 7.9 mm), or large (> 8 mm). The number of CL present on the ovaries was counted. One ovary was chosen randomly and fixed in 3% glutaraldehyde for histological evaluation. After fixation, a random piece of ovarian cortex approximately 1 cm × 1 cm × 350 μm was selected from among the surface follicles, excised, and dehydrated using a graded series of ethanol, and then cleared with Clearite (Richard-Allan Medical, Richland, MI) and embedded in paraffin (Paraplast Plus; Baxter). The tissue was oriented in the paraffin block such that the outer curvature of the ovarian cortex faced the cutting surface of the block. Fifty to 60 consecutive sections (6 μm) 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 as previously described (Cushman et al., 1999). Briefly, a 1 cm × 1 cm grid divided into 25 2- × 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 to make tracking primordial and primary follicles easier. For each section, every completely filled 2- × 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 squamous pregranulosa cells; primary, an oocyte surrounded by one or more cuboidal 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, retrospective examination of previous slides for the presence of the nucleus of the oocyte was performed. If it was not present, then the data for that follicle were recorded. A follicle was counted in the first field in which the oocyte of the nucleus appeared. If the oocyte was on the line between two sections, the follicle was counted in the field that contained the majority of the oocyte. If the majority of the oocyte fell within a field that was not to be counted, then the follicle was not counted. If a 2- × 2-mm field was filled with cortical tissue but contained no follicles, it was classified as Empty. Because of the outer curvature of the ovary, initial sections were smaller than 1 × 1 cm and in sections where portions of antral follicles > 1 mm in diameter appeared, the fields containing these follicles were ignored. A total of 200 fields (38 to 60/sections per ovary) were counted for each ovary, regardless of whether they were empty or contained follicles. This represented the total depth of the cortex because cortical depth varies greatly between cows (van Wezel et al., 1999). No attempt was made to assess atresia, because the rate of atresia in preantral follicles is very low.

We have chosen to use this method to compare numbers of microscopic follicles between populations because it provides an accurate method of subsampling the ovary without having to section the entire ovary. Unfortunately, because of the antral follicles present in the ovary it is difficult to estimate the total cortical volume, and, therefore, no calculations for total numbers of microscopic follicles in the ovary can be made. This method simply provides a method for comparing the numbers of follicles within an equal area of cortical tissue.

Statistical Analysis. The number of microscopic and macroscopic follicles and the number of CL were analyzed by least-squares ANOVA with genetic line as the independent variable. Partial correlation analyses, adjusted for genetic line, were performed among all follicular data using the PROC CORR of SAS (SAS, 1988).

Results

Genetic Lines. Differences were detected between genetic lines in numbers of microscopic and macroscopic follicles. Twinner cows had more secondary follicles than Control cows ($P < .05$, Table 1); however, there were no differences between genetic lines in the number of primordial, primary, or tertiary follicles or in the number of empty fields. Overall, there tended to be fewer total surface follicles in the Control line than in the Twinner line ($P < .1$, Table 2). This decrease was primarily due to a tendency for a decreased number of small surface follicles in the Control line ($P \leq .1$). The

Table 1. Mean number of microscopic follicles per 200 fields per cow within genetic lines

		Microscopic follicle class			
Line	n	Primordial	Primary	Secondary	Tertiary
Control	7	104.7	58.7	6.3[a]	2.0
Twinner	7	153.7	84.3	12.9[b]	2.0
SEM	—	27.4	17.3	2.0	.5

[a,b]Within a column, means lacking a common superscript letter differ ($P < .05$).

number of medium or large follicles did not differ ($P > 0.1$) between genetic lines.

Overall Correlations. Number of primordial follicles was correlated positively with number of primary and secondary follicles ($P < .05$, Table 3), and the number of primary follicles was correlated positively with the number of secondary follicles ($P < .05$). No histological classifications were correlated significantly with number of tertiary follicles ($P > .1$). Correlation analyses showed that among populations of microscopic follicles, number of empty fields was correlated negatively with number of primordial, primary, and secondary follicles ($P < .05$).

Correlations among the populations of small, medium, and large surface follicles on the ovary were not significant ($P > .1$, data not shown). Similarly, populations of surface follicles were not correlated significantly with number of CL ($P > .1$, data not shown).

Comparisons between microscopic and macroscopic follicles revealed that number of small surface follicles was correlated positively with the number of primordial, primary, and secondary follicles ($P < .05$, Table 3), whereas correlations between the number of medium or large follicles, CL, and the histological populations were not significant ($P > .1$, data not shown).

Discussion

Cattle selected for twin births had twice the number of secondary follicles of contemporary Controls. There was also a tendency ($P < 0.1$) for the number of small surface follicles and CL to be greater in cattle selected to double-ovulate. We did not observe a difference between genetic lines in the number of medium or large surface follicles; however, in previous studies, a greater number of follicles ≥ 4 mm in diameter was observed in Twinner cattle when ovaries were collected following treatment with $PGF_{2\alpha}$ (Echternkamp and Gregory, 1998; Echternkamp et al., 1990). These follicular differences may be due to differences in time of the follicular waves at the time of ovariectomy in the two experiments. In the previous studies (Echternkamp and Gregory, 1998; Echternkamp et al., 1990), the ovaries were collected shortly after $PGF_{2\alpha}$-induced luteolysis, when the selection process was occurring, whereas, in the present study, ovaries were collected approximately 10 d after $PGF_{2\alpha}$-induced luteolysis, late in the first wave of the following estrous cycle.

Results of the present study combined with those of previous studies (Echternkamp and Gregory, 1998; Echternkamp et al., 1990) suggest that, although there is no difference in the numbers of primordial and primary follicles, Twinner cattle are capable of maintaining more follicles in the secondary and subsequent stages. Therefore, although there may be no difference in the size of the primordial pool or the ability to activate follicles, it appears that the Twinner cows are able to keep more follicles growing beyond the primary stage, leading to increased numbers of secondary follicles, small surface follicles, and surface follicles ≥ 4 mm in diameter. Echternkamp et al. (1990) have demonstrated that the Twinner cows have increased serum and follicular fluid IGF-I concentrations. In the IGF-I knockout mouse, follicles do not grow beyond the early antral stage and have decreased ovarian FSH receptor concentrations (Baker et al., 1996; Zhou et al., 1997). It is possible that in the Twinner cows the increased serum and(or) follicular fluid IGF-I concentrations increase ovarian FSH receptor concentrations and result in maintenance of a greater number of secondary follicles to the antral follicle stage.

In our previous study (Cushman et al., 1999), a positive correlation was found between the number of primordial follicles and the number of primary and tertiary follicles, but the correlation between primordial follicles and secondary follicles was not significant. In the present study, primordial follicles were correlated positively with primary and secondary follicles but not with tertiary follicles. A possible explanation for this discrepancy is that follicular waves, well documented in antral follicles (Adams et al., 1992; Ginther et al., 1989), may

Table 2. Mean number of surface follicles and CL per ovarian pair per cow within genetic lines

			Surface follicle class			
Line	n	CL	Total	Small (1 to 3.9 mm)	Medium (4 to 7.9 mm)	Large (> 8 mm)
Control	7	1.1[a]	40.7[a]	35.4[a]	2.9	2.4
Twinner	7	1.6[b]	54.9[b]	49.0[b]	3.3	2.6
SEM	—	.2	5.8	5.6	.9	.4

[a,b]Within a column, means lacking a common superscript letter tend to differ ($P \leq .1$).

Table 3. Correlations among total numbers of preantral follicles, Empty fields, and Small (1 to 3.9 mm) surface follicles

	Classification of fields or follicles within fields				
Correlated trait	Empty	Primordial	Primary	Secondary	Tertiary
Primordial	–.87[a]	—	—	—	—
Primary	–.78[a]	.95[a]	—	—	—
Secondary	–.78[a]	.82[a]	.74[a]	—	—
Tertiary	.01	.12	.16	.11	—
Small	–.36	.61[a]	.50[a]	.52[a]	.37

[a]$P < .05$.

also occur in secondary and tertiary follicles. If a group of primordial follicles is activated into the growing pool at the same time, they would pass into the secondary stage as a group and then into the tertiary stage at approximately the same time. In the first study (Cushman et al., 1999), we may have collected ovaries at a time when the majority of the ovaries had a wave passing through the tertiary stage, whereas, in this study, we may have collected ovaries when a majority of the ovaries had a wave passing through the secondary stage. Further evidence for the concept of preantral follicles growing in waves comes from a recent study by Mizunuma et al. (1999). Their results demonstrated that activin from secondary follicles inhibited the growth of primary follicles and suggested that as the number of secondary follicles declined due to atresia or movement into the antral pool, a new group of primary follicles would grow to the secondary stage.

Some differences in the correlations between microscopic follicle numbers and small surface follicle numbers were observed in the present study as compared with the previous study (Cushman et al., 1999). However, it should be noted that in the previous study (Cushman et al., 1999) small follicles were classified as 1 to 3 mm, and in the present study small follicles were classified as 1 to 3.9 mm. Differences in classification could explain the differences in correlations between the two studies.

Implications

From these results, it can be concluded that ovaries of Twinner females contain the same numbers of primordial follicles, and Twinners do not activate more follicles into the growing pool than Control cows. However, Twinner cattle are able to keep more follicles growing in the secondary and antral stage during the recruitment and selection process.

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