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MITOTIC ACTIVITY OF BOVINE PREOVULATORY FOLLICLES ON DAY 2 OR DAY 3 OF THE FIRST FOLLICULAR WAVE

Chainarong Navanukraw* and Somchit Guntaprom

Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand.

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ABSTRACT

The objectives of this study were to characterize and investigate the mitotic activity of bovine preovulatory follicles on day 2 or day 3 of the first follicular wave. Eight mature crossbred beef heifers (18 months of age; 494 ± 18 kg body weight) received injections of a prostaglandin-F2 alpha analogue (Estrumate®; 2 injections, 11 days apart, 0.5 mg each) to synchronize estrus. Ovaries were collected at slaughter on day 2 or 3 of the subsequent estrous cycle. Follicles were classified as estrogen-active or estrogen-inactive based on follicular fluid estradiol and progesterone concentrations. Proliferating cell nuclear antigen immunostaining was performed using a monoclonal antibody to determine the labeling index (an index of the rate of cell proliferation) of the estrogen-active and estrogen-inactive follicles. The granulosa and thecal cell-labeling index were determined morph metrically with the aid of the image analysis. The rate of cell proliferation in the granulosa and thecal cell layers of the estrogen-inactive follicles was markedly less than that of estrogen-active follicles (6.93, 17.63 vs. 33.96, 37.89 %, respectively; $P < 0.05$). This result indicates that a significant decrease in granulosa and thecal cell proliferation occurs in the estrogen-inactive follicles before or during selection of the dominant follicle.

Keywords: Mitotic activity, preovulatory follicles, labeling index, bovine

INTRODUCTION

The studies on physiology of the ovary dealing with the terminal stages of follicular development are one of interests with extensive knowledge on the hormonal and cellular mechanisms regulating astral follicle growth (Richards, 2001). However, knowledge of the factors controlling the initiation of the follicular wave starting from recruited follicles to dominant follicle is extremely limited, particularly in monoovular species such as cattle. Follicular growth during the estrous cycle in cattle is usually characterized by two or three follicular waves (Fortune et al., 2001). Each wave of follicular growth is characterized by recruitment of a group of follicles, which continue to grow to approximately 6 to 8 mm in diameter. In monoovulatory species, only one follicle continues

* Corresponding author. E-mail address: chanav@kku.ac.th.

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to grow, in a linear manner, larger than the remaining follicles and become the dominant follicle, while subordinate follicles of the wave cease to grow and undergo atresia. The dominant follicle continues to grow and has a greater capacity to produce estradiol than the largest subordinate follicle (Evans and Fortune, 1997). Follicular vasculature and its mitogenic activity have been found to play a key role in maintaining follicular health (Redmer et al., 1991). One example is a reduction in DNA synthesis of follicular endothelial cells, which was associated with reduced follicular vascularity (Greenwald, 1989). A greater density of capillaries than smaller follicles (Zeleznik et al., 1981) surrounds maturing follicles. The gonadotropins provided to the maturing follicles by this vasculature suggest that the degree of vascularity may play a role in the maturation of the preovulatory follicle (Zeleznik et al., 1981). It has been shown that granulosa cells of estrogen-active follicle preovulatory follicles in cows secrete mitogenic factors that may stimulate follicular cell proliferation and angiogenesis (Redmer et al., 1991). Thus, vascular development of individual follicles may also play an important role in the mechanisms of follicular health, growth, and perhaps in follicular dominance (Redmer and Reynolds, 1996). However, to date, little is known about the mitotic activity of bovine preovulatory follicles, which one of characteristics is to recruit a pool of growing follicles and select the competent preovulatory follicles. Due to the fact that cell numbers within theca and granulosa layers of follicle are dictated by relative rates of division and death. Proliferate and anti-apoptotic effects of estrogen-active follicles are critical determinants of the dominant follicle selection (Lund et al., 1999). Our hypothesis was that there were some differences in cell proliferation or mitotic activity, as determined by using labeling index, of these preovulatory follicles. Therefore, we designed the present study to characterize the mitotic activity of the bovine preovulatory follicles expecting that this study will make a substantial contribution to understanding of not only growth of preovulatory follicles but also phenomenon of cell proliferation during the selection of dominant follicle.

MATERIALS AND METHODS

reproductively mature crossbred beef heifers (18 months of age; 494 ± 18 kg body weight) were us ed for this study. Estrous cycles were synchronized using two injections of prostaglandin-F2 alpha analogue (Estrumate®, Animal Health Corp., Union, NJ) given 11 days apart. The estrus in all animals was observed within 1-4 days after the second prostaglandin-F2 alpha injection. The day of first estrus was designated as day zero of the subsequent first follicular wave. Ovaries were harvested at 48 to 72 h after onset of estrus that corresponded to a time before the first day of dominance (Austin et al., 2001). Surface diameter of all visible follicles (≥ 3 mm) was determined in two axes and expresses as a mean ± standard error. The location of all follicles wit hin an ovary was diagrammed, and follicular fluid was gently aspirated from all follicles by using a 1 cc syringe with a 25 gauge, 5/8" hypodermic needle. Follicular fluid volumes were recorded, centrifuged immediately and stored until concentrations of estradiol and progesterone could be determined to classify the follicles as estrogen-active (estradiol: progesterone ≥ 1) or estrogen-active (estradiol: proges terone ≤ 1) (Redmer et al., 1991; Sunderland et al., 1994).

The concentrations of estradiol were measured by radioimmunoassay in 100 µl of unextr acted follicular fluid using a previously validated assay (Redmer et al., 1991). Follicular fluid was diluted 1:25, 1:100, or 1:225 with gel-phosphate buffer saline (gel-PBS; 0.01 M phosphate, 0.14 M saline and 0.1% gelatin; pH 7.4). Sensitivity of this assay was one pg/ml. All samples were analyzed in a single assay, and the intraassay coefficient of variation was 2.7%. Radioimmunoassay measured concentrations of progesterone in 100 µl of unrestri cted follicular fluid using a previously validated assay (Redmer et al., 1991). Follicular fluid was diluted 1:20 with gel-PBS. Sensitivity of this assay was 0.25 ng/ml. All follicular fluid samples were analyzed in a single assay, and the intraassay coefficient of variation was 3.8%.

Immunohistochemical detection of proliferate cell nuclear antigen was performed to determine the labeling index (an index of the rate of cell proliferation)

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of the estrogen-active and estrogen-inactive follicles. Paraffin-embedded sections (6 μm) from each ovary were used for detection of proliferate cell nuclear antigen using a specific monoclonal antibody (MAB24R, Chemi-Con International, Temecula, CA) as described previously (Navanukraw et al., 2001). Sections were incubated overnight at 4 °C with the primary antibody diluted 1:50 in blocking buffer. The avidin biotinylated enzyme complex (Vector Laboratories, Burlingame, CA) detected primary antibody. For the control specimens, the primary antibody was replaced with purified normal mouse IgG (normal mouse IgG is produced from serum of mice that have not been immunized and can be used as a control reagent for immunoassays using mouse monoclonal antibodies). The granulosa and thecal cell-labeling index were determined morphometrically with the aid of the image analysis system (VIDAS version 2.5; Roche Image analysis system, Los Altos, CA) as previously reported (Zheng et al., 1994; Navanukraw et al., 2001). Tissue sections were positioned under the 40-x objective lens so that either the granulosa or thecal layer was brought into view. The labeling index for thecal cells included all labeled cells, and no attempt was made to distinguish between thecal cell types. The labeling index was calculated as the number of proliferative cell nuclear antigen labeled nuclei expressed as percentage of total nuclei.

Statistical analyses

Follicles were classified into two groups, i.e., estrogen-active and estrogen-inactive, and concentrations of steroids in follicular fluid and follicular diameter, and thecal and granulosa labeling index were analyzed using a one-way analysis of variance with the general linear model procedure of SAS. When the F-test was significant ($P < 0.05$), differences among means were evaluated by a Bonferroni *t* test (SAS Institute, Inc., 2001).

RESULTS AND DISCUSSION

The follicles from the growing follicles of each of the eight animals were chosen for further evaluation as follows. It was shown that a single follicle in one of eight animals was clearly larger than the remaining follicles in the growing follicles. Its size was approximately 10 mm compared to 4-6 mm diameter, and was estrogen-active with estradiol: progesterone ≥ 1; actual estradiol: progesterone ratio i n follicular fluid was 6.39. This follicle was, therefore, the only one evaluated. In another of the animals, all 17 follicles in the growing follicles were estrogen-inactive with estradiol: progesterone < 1. Thus, the largest follicle with approximately 15 mm in diameter was the only one evaluated for this animal. In the remaining six animals, 2-4 follicles were chosen based on the first criteria that they were the largest or second largest follicles. Three of these follicles were estrogen-inactive with 8, 10.5, and 13.5 mm diameter, respectively. It was found that 14 follicles with sizes ranging from 6-9 mm in diameter were estrogen-active Thus, 14 estrogen-active and 4 estrogen-inactive follicles were evaluated as shown in Table 1.

Follicular diameters, follicular fluid estradiol, follicular fluid progesterone, and the ratio of estradiol: progesterone concentrations were different ($P < 0.05$) between the estrogen-active and estrogen-inactive follicles as shown in Table 1. All follicles exhibited granulosa and thecal cell proliferation by proliferative cell nuclear antigen staining (Figures 1A and 1B). The rate of cell proliferation in the granulosa and thecal cell layers of the estrogen-inactive follicles was markedly less than that of estrogen-active follicles (6.93, 17.63 vs. 33.96, 37.89 %, respectively; $P < 0.05$).

In this study, the size of the estrogen-active follicles was smaller than estrogen-inactive follicles, which was different from other reports (Ginther et al., 1997; Mihm et al., 2000) that reported the future dominant follicle was slightly larger than others in the cohort. However, the difference in size was not always predictive of future dominance, which suggests that intrafollicular mechanisms within the dominant follicle allow it to amplify the effects of follicle stimulating hormone and, thus, respond more robustly than the subordinate follicles to the rise in circulating follicle stimulating hormone (Fortune et al., 2001).

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Table 1. Follicular diameter, follicular fluid estradiol, progesterone, granulosa and thecal labeling indexes of bovine preovulatory follicles.

Follicle types	Diameter r(mm)*	Estradiol (ng/ml)	Progesterone (ng/ml)	Estradiol: Progesterone ratio	Granulosa cell labeling index++ (%)	Thecal cell labeling index++ (%)
Estrogen-active (n=14)	8.18 ± 0.4[a]	349.93±117[c]	47.51±5.1[a]	6.06±1.3[c]	33.96±1.37[c]	37.89±1.96[c]
Estrogen-inactive (n=4)	11.25±1.8[b]	2.20±0.9[d]	379.8±117.7[b]	0.03±0.02[d]	6.93±6.90[d]	17.63±7.90[d]

*Diameter based on average diameter of each follicle.
[a, b]Within a column, means with different superscripts differ ($P < 0.01$).
[c, d]Within a column, means with different superscripts differ ($P < 0.05$).
++Labeling index = number of proliferating cells expressed as a proportion of the total number of cells.

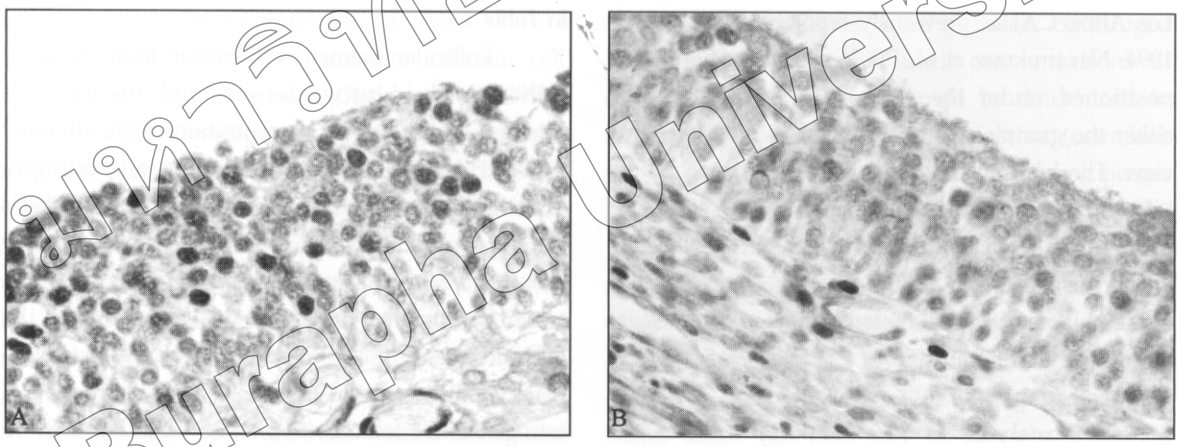

Figure 1. Immunolocalization of proliferative cell nuclear antigen: (A) in a section of the estrogen-active follicle, and (B) in a section of the estrogen-inactive follicle.

Proliferative cell nuclear antigen is an auxiliary protein of DNA polymerase delta, an enzyme vital for DNA replication (Foley et al., 1991). Proliferative cell nuclear antigen has been used to establish cell-labeling techniques for investigating cell proliferation. Although we did not observe differences among regions of the granulosa and theca cell layer, we did observe a difference between the estrogen-active and estrogen-inactive follicles. Rate of cell proliferation in the granulosa and thecal cell layers of the estrogen-inactive follicles was markedly less than that of estrogen-active follicles. Earlier morphometric studies suggested that estadiol hormone may stimulate activation of follicles in cattle (Peluso and Hirschel, 1988), but an important question that remained was whether estadiol hormone did this through induction of hypertrophy or hyperplasia of granulosa cells (Hulshof et al. 1995). When we stained sections for proliferative cell nuclear antigen, the results showed clearly that estrogen induced hyperplasia, causing an increase in granular cell nuclei, and protected cells from

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oxidative stress-induced apoptosis (Lund et al., 1999). The synergism between granulosa and theca cells are based on a 2-cell, 2-gonadotropin hypothesis for biosynthesis of estrogen.

The rate of cell proliferation in the granulosa and thecal cell layers of the estrogen-active follicles are up regulated by estrogen (Uilenbrock et al., 1997) and will be involved in the mechanism of follicular health, growth, and follicular dominance (Redmer and Reynolds, 1996). In addition, these two cell types are the progenitors of luteal cells, the principal source of circulatory progesterone. Attenuation of estrogen production by preovulatory follicle necessitates formation of a fully competent corpus luteum and can enhance fertility (Niswender et al., 1994). This result is supported by a similar pattern of cell proliferation in cystic, atretic, and healthy antral follicles in other studies (Jablonka-Shariff et al., 1996; Isobe and Yoshimura, 2000), and indicates that a significant decrease in granulosa and thecal cell proliferation occurs in the estrogen-inactive follicles before or during selection of the dominant follicle. The results of this study also indicate that the proliferative cell nuclear antigen can be used as an immunohistochemistry technique to study the mitotic activity of follicular development throughout the estrous cycle in all farm animal species.

SUMMARY

Our findings indicate that a significant decrease in granulosa and thecal cell proliferation occurs in the estrogen-inactive follicles before or during selection of the dominant follicle. These data provide additional understanding of follicular growth and development, including the mitogenic activity of follicles in cows.

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