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| 著者 | AMROZI AMROZI, KAMIMURA Shunichi, ANDO Takaaki, HAMANA Katsumi |
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Histoendocrinological Observation of Dominant Follicles and Subordinate Follicles in First Follicular Wave in Japanese Black Cows

AMROZI, Shunichi KAMIMURA[†], Takaaki ANDO and Katsumi HAMANA
(Laboratory of Theriogenology, Department of Veterinary Medicine)

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Introduction

On the basis of histological study of bovine ovaries, Rajakoski¹¹⁾ found that two waves of follicular activity occurred during the bovine estrous cycles. Twenty years later this finding that two follicular waves are present during the estrous cycle has been clarified using transrectal ultrasonic imaging¹⁰⁾. Since then, transrectal ultrasonic imaging has been used intensively to monitor follicular waves in cows^{3, 7, 8, 12, 13)}. Studies of ovarian follicular dynamics in cattle may lead to development new methods for improving bovine fertility, estrous synchronizing with more precision, and enhancing superovulatory responses.

The first wave dominant follicle and its cohorts increase in diameter and then decrease during the estrous cycle. Apoptosis is a process of selective cell deletion by which underlying ovarian follicular atresia^{2, 5)}. The histological appearance and quantity of apoptotic granulosa cells (GC) in healthy and atretic ovarian follicles in ewes have been identified. Results show that most degenerate GC in follicles undergoing atresia display morphological characteristics of apoptosis, suggesting that this is the most common pathway of cell deletion. Typical features of apoptosis are cells containing nuclei with marginated chromatin; cells with a single small densely staining nucleus (pyknotic appearance); cells with multiple smaller, densely staining nuclear fragments; and densely staining membrane-bound bodies (apoptotic bodies) either single or in clusters⁶⁾. Furthermore, involvement of apoptosis in small, medium and large bovine follicles has been studied, and results show that apoptosis in GC occurs in any size of follicles and even appears in some healthy follicles¹⁶⁾. Nevertheless, apoptosis of bovine GC from dominant and largest subordinate follicles of the first follicular wave previously identified *in vivo* has never been studied.

The aims of the present research are to determine the first follicular wave in Japanese black cows and to quantify the apoptotic characteristics of dominant and largest subordinate follicles previously identified *in vivo* on Day 7 and Day 10.

Materials and Methods

Animals, experimental design, ultrasonography and ovariectomy

The animals used in this study were 6 Japanese black cows (10.5 ± 1.5 years old) in the

[†] Correspondence to:

S. Kamimura (Laboratory of Theriogenology, Kagoshima University),
Tel/fax: (099) 285-8737; E-mail: kamimura@vet.agri.kagoshima-u.ac.jp

Kagoshima University farm. They were in good body conditions (with scores of 2.5-4.0 out of 5) and had calved 8.7 ± 1.8 times. The reproductive status that is characterized by a large ovulatory follicle (12.5 ± 0.7 mm in diameter) and regressing corpus luteum was determined by means of ultrasonography, and confirmed by measurement of plasma progesterone concentrations. The cows were divided into 2 groups by day of ovariectomy: a Day 7 group ($n = 3$) and a Day 10 group ($n = 3$). Day 7 is considered the stage of developing dominant follicle (DF) and early regressing largest subordinate follicle (SF), and Day 10 is considered static or early regressing DF and regressing SF. During the experiment, animals were kept in stanchion.

In order to induce ovulation, PGF_{2 α} was injected im twice a day at 07.00 (15 mg) and 19.00 (10 mg) during the luteal phase (Day 7-14 of the estrous cycle). The dynamics of the corpus luteum and the dominant and subordinate follicles in the ovaries were monitored twice daily at 07:00 and 19:00 by ultrasonography before and after PGF_{2 α} treatment until the day of ovariectomy. A real time B-mode ultrasound scanner (EUB-405, Hitachi-Medical Co., Tokyo) equipped with a 7.5 MHz transducer (EUP-033J, Hitachi-Medical Co., Tokyo) was used. Sequential identification of individual follicles more than 2 mm in diameter was carried out. Appropriate images of follicles were arrested on screen and maximum diameters of follicles were measured using the built in caliper system. The images of follicles were hard-printed. Ultrasonic images of the ovaries were recorded on a follicular map and on a video processing unit for retrospective analysis. Number of follicles were counted and follicles were classified based on their diameter as measurement by the ultrasonic image caliper system Class I (2-5 mm), Class II (6-9 mm), and Class III (≥ 10 mm). Ovariectomy was performed by the flank laparotomy under epidural anesthesia in the morning on Day 7 or Day 10 of the estrous cycle.

Processing of ovaries

Following ovariectomy, the ovaries were placed in physiological saline. Thereafter, ultrasonography was carried out to confirm the location of DF and SF, which had been identified by the ultrasonic examinations before ovariectomy. Following this confirmation, DF and SF were dissected free from extraneous tissue using a stereomicroscope. An individually dissected follicle was placed in a glass jar and was opened to collect follicular fluid, and follicular wall.

Detection of apoptosis by TUNEL (Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling): Light microscopy

Follicular wall samples of dominant and subordinate follicles were immediately put into 10% phosphate-buffered formalin. For negative controls, normal calf ovary sections were used. Follicular tissues were processed for histology, and embedded in paraffin wax. Follicular walls were sectioned at 5 μ m and were mounted on slides for hematoxylin and eosin staining. Series of other sections were mounted on aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, USA) coated slides dried at 40°C for 12 h, dewaxed, and hydrated. The slides were immersed in a plastic jar containing 0.1 M citrate buffer pH 6.0 and microwave irradiation (450 W) was performed for 3 minutes. This was followed by rapid cooling with immediately added distilled water and washing with PBS (Sigma Chemical, St Louis, USA) three times, for 5 minutes each time. The slides were immersed for 30 min in room temperature blocking solution containing 0.1 M Tris-HCl, 3% BSA, and 20% normal bovine serum, pH 7.5. Endogenous peroxidase was inactivated by dipping in 0.3% H₂O₂ in methanol for 30 min in RT, followed by PBS wash three times, for 5 minutes each time. Negative control slides were incubated in label solution without terminal transferase. The DNA 3'-end labeling was

performed after incubation in a humidified chamber containing 150 mM NaCl for 60 min at 37°C in enzyme solution, terminal deoxynucleotidyl (TdT) transferase (In Situ Cell Death Detection Kit, POD, Roche, Indianapolis, IN, USA), followed with PBS wash three times, for 5 minutes each time. The next step was to block nonspecific binding of the anti-fluorescein-antibody to the tissue with blocking solution containing 0.1 M Tris-HCl, 3% BSA, and 20% normal bovine serum, pH 7.5. After that, they were washed with PBS three times, for 5 minutes each time. For signal conversion and to enable analysis of stained cells under light microscope, the slides were incubated in a humidified chamber for 30 min at 37°C in converter POD, prediluted 1:2 in blocking solution. After washing in PBS three times for 5 minutes each time, the POD retained in immune complex was visualized as a color reaction by incubation with DAB substrate for 1 min in RT, followed by washing in tap water extensively. Counterstaining was done with Gill's hematoxylin for 5 s. The slides were mounted with mounting media tube for cover glass (Daigo Sangyo, Tokyo, Japan). Numbers of TUNEL-labeled cells in the GC and theca cell (TC) layers were counted at least in 10 fields per follicle under light microscope at x400. Cells were considered TUNEL-positive when brown-stained, often combined with characteristic apoptotic appearance (marginated chromatin, pyknosis or multiple fragmentations). Less staining cells were expelled from analysis. Apoptotic bodies that occurred in clusters adjacent to one another were counted as single event.

Blood plasma sampling and hormone determinations

Blood from the jugular vein was collected daily before PGF_{2α} treatment and twice daily following PGF_{2α} treatment at the same time as ultrasonography. Plasma was collected by centrifugation of the blood samples at 4°C, 3000 rpm for 20 minutes and then plasma samples were stored at -20°C. Double antibody RIA was used to determine the concentrations of progesterone using antisera to progesterone (GDN # 337)¹⁴⁾. The intra- and interassay coefficients for progesterone were 4.2 and 8.0%.

Data analysis

The number of animals observed until Day 7 was 6 and after that it was 3. Values were expressed as mean ± SD. Mean values based on the number of follicles that emerged at respective times and the diameters of each follicular class were analyzed statistically using Student's *t*-test. A value of $p < 0.05$ was considered significantly different.

Results

The results showed that injection of 25 mg PGF_{2α} during the luteal phase induced ovulation 90 ± 19 h later. The numbers of follicles in various diameter categories after PGF_{2α} treatment until day 10 post ovulation was depicted in Figure 1. The mean number of follicles per day for Class I and Class III differed significantly ($p < 0.05$). From wave emergence the total number of Class I and Class II follicles increased until one day later and then decreased. From Day 1 pm to Day 5 pm, mean number of follicles in Class II and Class III was significantly different ($p < 0.05$) for the appropriate categories. After Day 5 pm, no significant difference in the mean number of follicles was detected between Class II and Class III categories.

At the wave emergence, a mean number of 13.5 ± 9.5 follicles in Class I was found. On average, future DF and SF appeared as 4.9 ± 0.8 mm and 4.9 ± 0.9 mm follicles, respectively. Deviation between DF and SF occurred on Day 3 pm ($n = 6$) with mean diameters of the DF and SF being

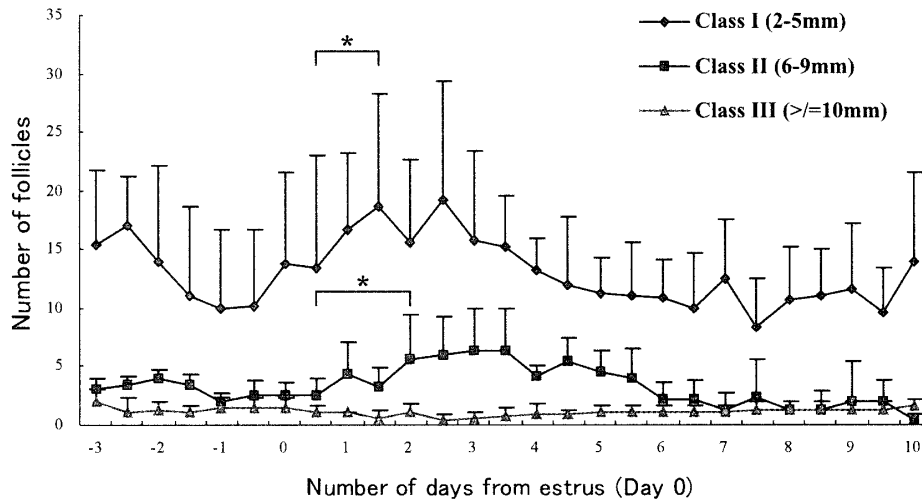


Figure 1. Mean number of follicles in the various diameter categories for each day. Mean numbers were significantly different per day for follicles in Class I and Class II ($p<0.05$). A mean number of follicles in Class I increased from Day 0 pm until Day 1 pm (*: $p<0.05$). A mean number of follicles Class II increased from Day 0 pm until Day 2 am (*: $p<0.05$). From Day 1 pm to Day 5 pm, mean number of follicles in Class II and Class III were significantly different ($p<0.05$) in the appropriate categories.

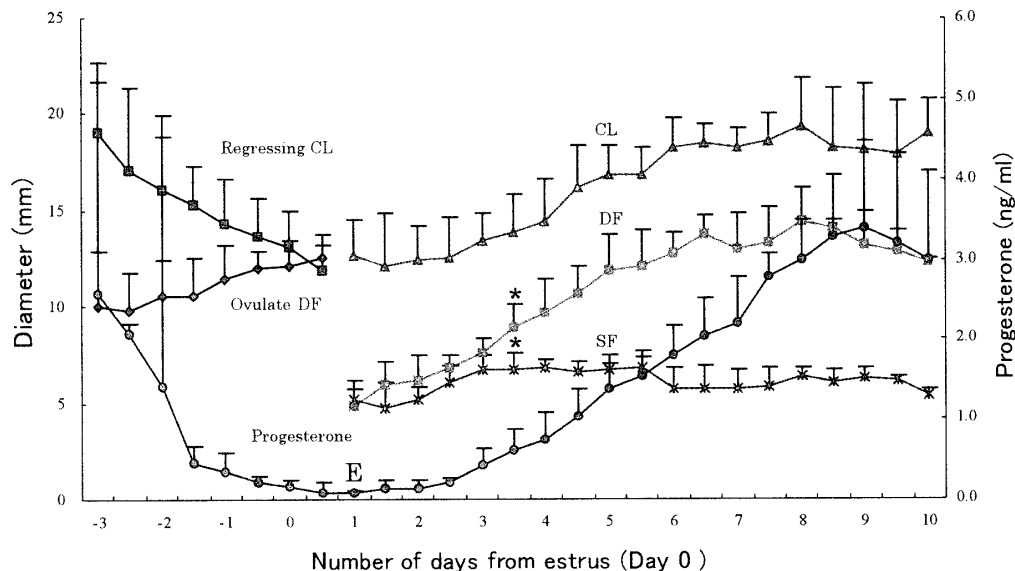


Figure 2. Mean diameters of the ovulatory DF, regressing CL, developing CL, DF, SF and progesterone levels. Ovulation of DF was followed by development of CL which concomitant with increasing progesterone level showed that the cows have normal estrous cycle. Deviation between DF and SF occurred on Day 3 pm when the growth rates of DF and SF were significantly different (*: $p<0.05$). E = dominant follicles emergence.

8.9 ± 1.3 mm and 6.8 ± 0.9 mm ($p<0.05$), respectively. The DF grew till Day 8 reaching a maximum diameter of 14.6 ± 2.1 mm ($n = 3$) and then regressed.

HE staining of follicular walls of DF showed the characteristics of healthy follicles on Day 7, slight atresia in DF on Day 10, and heavy atresia in SF on Days 7 and 10 (Figure 3). Detection of apoptosis by TUNEL labeling consistent with the presence of fragmented DNA was evident in cells containing marginated chromatin, and showing internalization by neighboring cells, apoptotic bodies, densely stained nucleus, and phagocytosing dying basal granulosa cells by macrophages

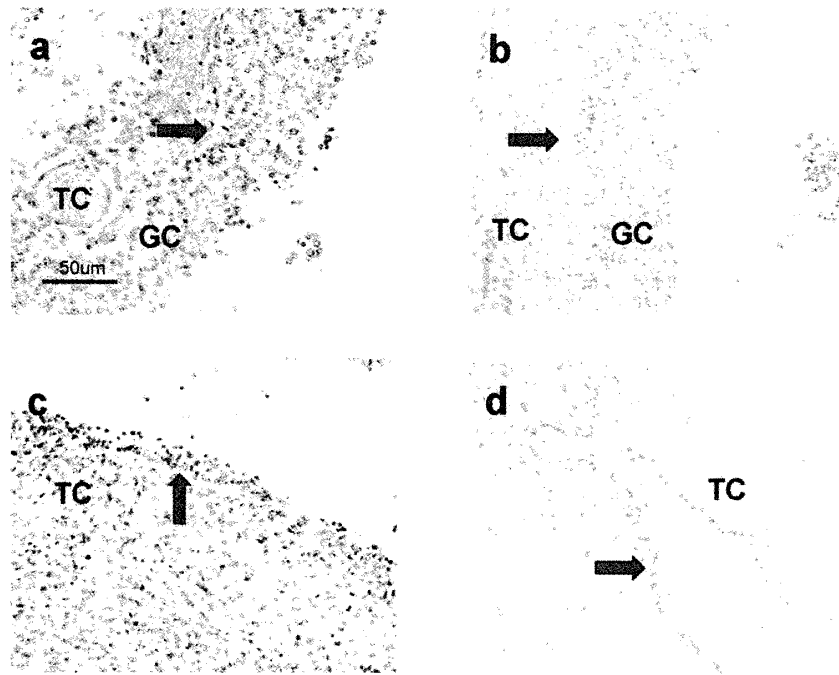


Figure 3. Representative photomicrographs (x200; HE) of healthy DF on Day 7 with intact and well-organized multi laminar GC layers (a) and slight atretic DF on Day 10 with disorganized GC layers partially detached from the base membrane (b). The atretic SF on Day 7 (c) showing thinned GC layers partially detached from the base membrane; and (d) atretic SF on Day 10 showing thinned GC layers wide spreads detached from the base membrane and connective tissue cells predominant in theca. Arrows denote base membrane.

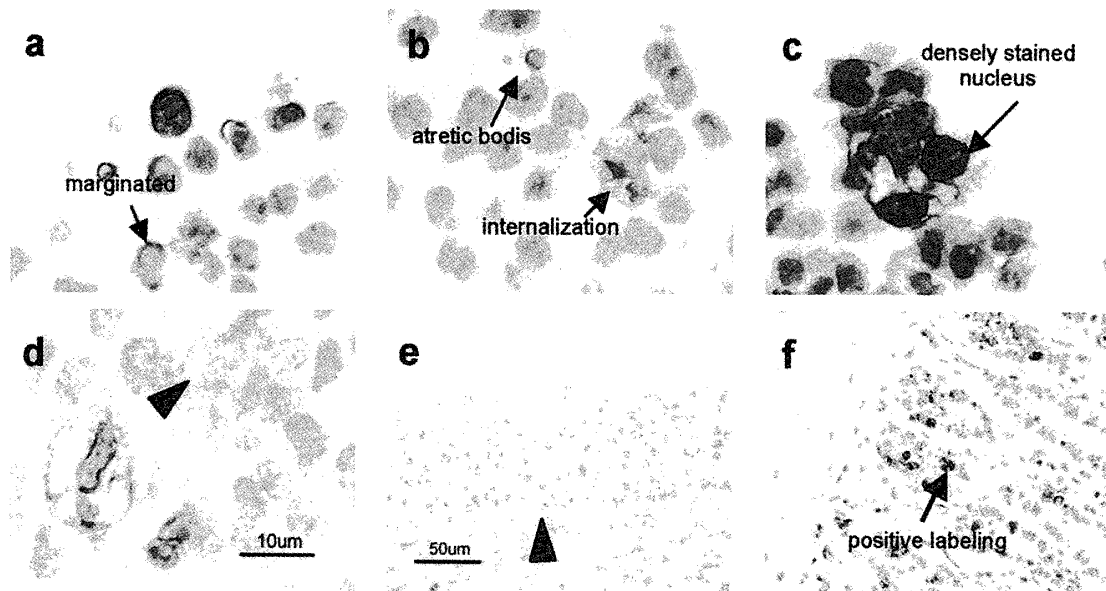


Figure 4. Characteristic apoptotic cells and/or apoptotic bodies in the membrane granulosa and theca interna of early atretic DF on Day 10 fixed in 10% buffered formalin and stained with *in situ* 3' end-labeling (brown staining), counterstained with Gill's hematoxylin (x1000). Labeling consistent with the presence of fragmented DNA was evident in cells containing marginated chromatin (a), internalization by neighboring cells and apoptotic bodies (b), densely stained nucleus (c), macrophages phagocytosing dying basal granulosa cells (d). Negative control (x400) from calf ovary (e) showed no labeling. Positive control (x400) from small intestine of rat (f) showed positive labeling. Arrowheads denote base membrane.

(Figure 4). The ratios of apoptotic cells in GC in DF on Day 7 and Day 10 were $3.1 \pm 2.7\%$ and $20.9 \pm 0.6\%$ respectively, and in theca cells the ratios were $2.5 \pm 2.8\%$ and $6.5 \pm 1.7\%$, respectively (Figure 5).

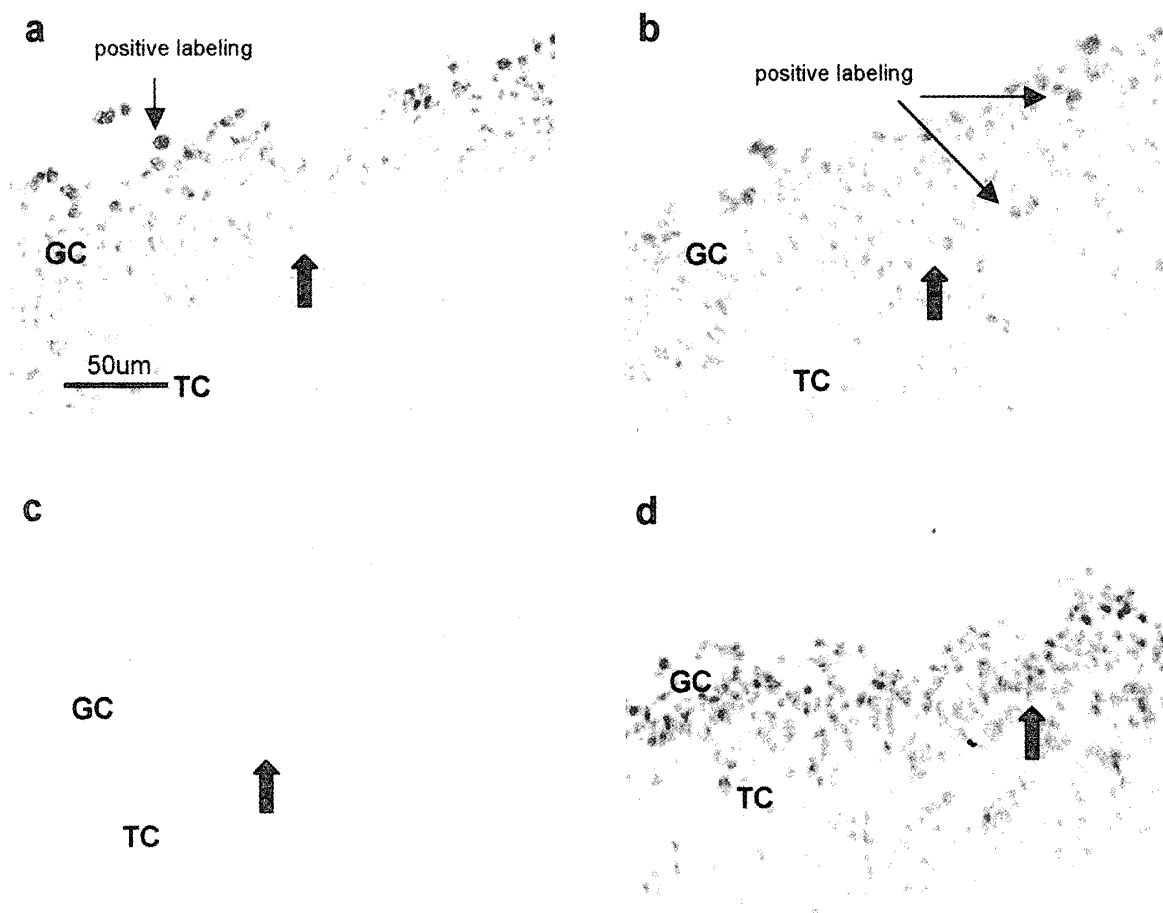


Figure 5. *In situ* 3' end-labeling of apoptotic cells in the GC and TC of 10% buffered formalin-fixed healthy DF on Day 7 (a) and early atretic DF on Day 10 (b). An adjacent section DF on Day 7 was stained with hematoxylin (c) and on Day 10 with hematoxylin eosin (d). x400. Arrows denote base membrane.

Discussion

Ultrasonography of the first follicular waves every 12 hr showed the emergence of follicles in Class I 12 hr after the ovulation of DF, and these increased in numbers from Day 0 pm until Day 1 pm. The first recruited small follicles emerged and were followed by the development of cohorts. At the same time, the mean numbers of follicles in Class II increased, concurrent with the increasing number of follicles in Class I in one day. The mean number of follicles in both classes decreased two days after follicle wave emergence. The decreasing number of follicles in Class I and II has a correlation with decreased FSH concentrations between days 1 and 4¹¹ and estradiol increase at wave emergence⁸¹. The results suggested (Figure 1) that recruitment of small follicles from the pool of primary follicles occurred continuously from wave emergence until one day later.

Dominant follicle ovulation induced by PGF_{2α} injection followed by the development of the corpus luteum, together with the progesterone concentrations observed suggested that the animals used in this research have normal estrous cycles (Figure 2). At the time of emergence, the future DF and the SF were 4.9 ± 0.8 mm and 4.9 ± 0.9 mm in diameter, respectively. At every 12 h ultrasonography, there was a gradual divergence in mean growth rates of the future DF and the SF for 60 h after wave emergence (Figure 2). There was no significant difference in growth rates between the two follicles until deviation. Deviation was characterized by the cessation of growth of SF with a slower growth rate for 2 days before the SF attained maximum diameter. A study on

heifers reported deviation of DF and SF occurred at 61 h after wave emergence when two largest follicles were 8.3 ± 0.2 mm and 7.8 ± 0.2 mm in diameter⁹⁾. These were similar to the present results where DF and SF of deviation were 8.9 ± 1.3 mm and 6.8 ± 0.9 mm, respectively.

The hematoxylin eosin staining was performed to determine histologic alterations in DF and SF. The results for DF showed healthy follicles with intact and well-organized multi-laminar GC layers on Day 7 (Figure 3). Dominant follicles on Day 10 showed characteristics of slight atretic with GC layers partially detached from the base membrane and a few pyknotic GC. Atretic images of SF showed thinned GC layers partially detached from the base membrane on Day 7 (Figure 3). The atretic images of SF on Day 10 showed thinned GC layers with widespread detachment from the base membrane, a few pyknotic GC or no pyknotic nuclei in the lumen, and connective tissue cells predominant in the theca interna (Figure 3). These are the characteristics of heavy atresia^{2, 5, 11)}. Clearly, DF and SF showed different histologic characteristics on Day 7 and Day 10. SFs between Day 7 and Day 10 showed different histological images.

Furthermore, the differences between DF and SF were observed with the TUNEL staining method. The characteristics of the apoptotic cells and/or apoptotic bodies in the membrana granulosa and theca interna were indentified (Figure 4). TUNEL labeling consistent with the presence of fragmented DNA was evident in cells containing marginated chromatin, and showing internalization by neighboring cells, apoptotic bodies, densely stained nucleus, phagocytosing dying basal granulosa cell by macrophages^{6, 15, 16)}. In DF on Day 7, apoptotic cells were present in the antral granulosa as a cluster of apoptotic cells, and a few ($2.5 \pm 2.8\%$) were found in the theca cells (Figure 5). The prevalence of apoptotic granulosa cells in DF on Day 10 ($20.9 \pm 0.6\%$) was higher than in DF on Day 7, where apoptotic cells were distributed in the antral and mural GC. Interestingly, on Day 10 some apoptotic GCs were clumping as shown in Figure 3c. A similar image was observed by Rajakoski¹¹⁾ where numerous pyknotic nuclei, often gathered into large clumps, floated in the liquor folliculi as a characteristic of first degree atresia. In addition, in the theca interna, labeling was found within macrophages as vacuoles and located near the base membrane. In an earlier study⁴⁾ of the basal lamina atretic follicles were often breached by macrophages, where phagocytosed dying basal GC.

In conclusion, the results showed that: 1) the first follicular wave in cows is characterized by one day of recruitment of small follicles and a gradual divergence in mean growth rates of the future dominant and largest subordinate follicles; 2) early regression of DF is preceded by severe apoptosis. Furthermore, results suggested that macrophages may be involved in follicle atresia by phagocytosing dying cells.

Summary

The aims were to determine the turnover of the first follicular wave in Japanese black cows and to quantify immunohistological characteristics of identified DF and SF identified *in vivo* and extracted by means of ovariectomy on Day 7 and Day 10 (Day 0 = day of estrus). Six cases of the first follicular wave in Japanese black cows were observed using ultrasonic detection. The number of follicles, diameter of DF and SF, and prevalence of apoptotic cells in the membrana granulosa and theca calls were studied.

Injection of 25 mg PGF_{2α} induced ovulation 90 ± 19 h later. At the follicular wave emergence, 13.5 ± 9.5 (mean \pm SD) follicles of 2 - 5 mm in diameter were found. Retrospectively, future dominant follicle and largest subordinate follicle were observed 4.9 mm in diameter for the first

appearance. Deviation of the DF and SF occurred on Day 3 pm ($n = 6$) with mean diameters of 8.9 ± 1.3 mm and 6.8 ± 0.9 mm, respectively. DF developed until day 8 reaching a maximum diameter 14.6 ± 2.1 mm ($n = 3$) and then regressed. The follicular wall of DF showed characteristics in keeping with healthy follicles on Day 7, becoming slightly atretic in DF on Day 10, while SF showed heavy atresia both on Day 7 and Day 10 under hematoxylin and eosin staining. As to the prevalence of apoptotic cells, DF showed $3.1 \pm 2.7\%$ and $20.9 \pm 0.6\%$ on Day 7 and on Day 10 respectively in membrane granulosa, and $2.5 \pm 2.8\%$ and $6.5 \pm 1.7\%$ on Day 7 and on Day 10 respectively in theca cells. In conclusion, these results showed that: 1) the first follicular wave in cows is characterized by one day recruitment of small follicles and a gradual divergence of growth rates in the future dominant and largest subordinate follicles; 2) early regression of the DF is preceded by severe apoptosis.

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