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—Technical Note—

Classification of Bovine Follicles Based on the Concentrations of Steroids, Glucose and Lactate in Follicular Fluid and the Status of Accompanying Follicles

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Abstract. A simple and clear means to identify the physiological status of follicles is essential for study of follicular biology. In the present study, we verified a novel classification procedure based on analysis of the follicular population and glucose concentration in follicular fluid (FF) as an alternative method to classify bovine follicles. Paired ovaries were collected from heifers, and the number of follicles and stage of the CL were recorded. Follicles were initially divided into the following 3 groups according to diameter and the ratio of E2 and P4 (E/P): E2 active (E-A: E/P≥1), E2 inactive (E-I: E/P<1, ≥8.5 mm) and small follicles (E/P<1, <8.5 mm). E-A follicles were easily identified as E2-rich dominant follicles and were further classified according to diameter and stage of the CL as early dominant (EDF: <8.5 mm), dominant (DF: ≥8.5 mm, stages I-III) or preovulatory follicles (POF: ≥8.5 mm, stage IV). E-I follicles were classified as follows based on the status of the accompanying follicles: early atretic (EAF: without an E-A follicle), mid-atretic (MAF: with an EDF or DF) and late atretic follicles (LAF: with an EAF or POF). The follicular P4 concentrations of the MAF and LAF were significantly higher compared with that of the EAF, while follicular glucose concentration of the LAF was lower compared with those of EAF and MAF, indicating that this classification can be used to distinguish early atretic follicles from more advanced atretic follicles. Small follicles were classified as growing (GF: without E-A follicles) and suppressed small follicles (SSF: with E-A follicles). The SSF was easily identifiable by this procedure, but some GF populations likely contained SSF. To identify true GF, the ratio of E2 in the GF and accompanying EAF may be used. In conclusion, analysis of the follicular population in conjunction with biochemical indices such as steroid and glucose concentrations in FF provides a simple and accurate means of classifying bovine follicles.
Key words: Bovine, Follicular fluid, Follicle classification, Glucose, Lactate, Steroid

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Abattoir-derived ovaries have been used extensively as sources of oocytes for embryo production *in vitro*. Ovaries also provide useful materials for investigating the physiological functions of follicles. Although recent development of ultrasonography enables researchers to monitor development of follicles in real time, follicular samples derived from abattoir-harvested ovaries continue to be cheap and valuable materials for investigating the biochemical natures of follicular fluid (FF) and ovarian functions such as gene/protein expression. For this reason, a simple and accurate method to determine the physiological status of follicles is necessary. To date, some follicular classification methods have been advocated. These methods are based on morphological, histological and biochemical criteria, but none of them are without faults.

Histological examination employs the incidence of pycnosis in granulosa cell nuclei and the degree of granulosa/thecal integrity as the criteria [1]. Although this method is sensitive enough to detect a slight sign of cellular degeneration, this may not be an appropriate sign of follicular atresia, since even healthy ovulatory follicles sometimes show pycnosis in granulosa cells [2, 3]. Morphological analysis, which is based on macroscopic observation of follicles, is a reliable method of distinguishing healthy follicles from advanced atretic follicles [4, 5], but it cannot distinguish early atretic follicles from healthy follicles [5]. Moreover, histological and morphological methods require tedious and time-consuming preparations. The biochemical method utilizes the ratio of estradiol (E2) and progesterone (P4) or/and androgens in FF as criterion to determine follicular health [6–9]. It provides an objective and clear-cut means of distinguishing an "estrogen-active" healthy follicle from an "estrogen-inactive" atretic follicle; however, it cannot be applied to small follicles <6 mm in diameter [7]. These three techniques classify follicles individually without taking into account the physiological status of accompanying follicles.

In cattle, two or three wave-like follicular developments occur during the estrous cycle [10–12]. Each wave is initiated by simultaneous development of multiple follicles 4–5 mm in diameter followed by selection and continuous growth of a dominant follicle [13]. The dominant follicle suppresses development of subordinate follicles as well as emergence of a new follicular wave [11, 13, 14]. During the follicular phase, the dominant follicle ovulates, while during the luteal phase, it loses its dominant status and undergoes a

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Table 1. Criteria of follicular classification

Follicle class	Abbrev.	Size (mm)	E2/P4 (w/w)	Accompanying follicles	CL stage	Remarks
Suppressed small follicle	SSF	<8.5	<1	EDF/DF/POF	I–IV	
Growing follicle	GF	<8.5	<1	No EDF/DF/POF	I–III	May contain SSFs
Early dominant follicle	EDF	<8.5	≥1	No DF/POF	I–III	
Dominant follicle	DF	≥8.5	≥1		I–III	
Preovulatory follicle	POF	≥8.5	≥1		IV	
Early atretic follicle	EAF	≥8.5	<1	No EDF/DF/POF	II–III	Initially classified as EAF-EI/FAF+EI
Mid-atretic follicle	MAF	≥8.5	<1	EDF/DF	II–III	
Late atretic follicle	LAF	≥8.5	<1	EAF/POF	III–IV	Initially classified as LAF+EAF/LAF+POF

prolonged atretic process [10, 12]. Disappearance of the dominant follicle triggers initiation of the next follicular wave [11, 13, 14].

These events imply that the physiological status of the follicle can at least be partially estimated from the status of accompanying follicles. This approach, in combination with the biochemical method, may provide a better classification procedure for follicular status. In the present study, we examined the validity of this approach. We also examined the concentrations of glucose, the crucial energy substrate for ovarian activity [15, 16], and its metabolite lactate in FF to evaluate their usefulness as criteria for the physiological status of follicles.

Materials and Methods

Sample collection and storage

Paired ovaries were harvested from Holstein × Japanese Black F1 heifers (21–26 months old) at a abattoir. Only healthy ovarian pairs were used in the present study. The ovaries were kept on ice and brought to the laboratory within 30 min of slaughter. In the laboratory, the ovaries were examined macroscopically, and the number and status of follicles and corpora lutea (CL) were recorded. Follicular fluid was aspirated from all follicles larger than 8.5 mm in diameter (0.25 ml FF) and, when possible, follicles larger than 5.0 mm in diameter (0.04 ml FF) using a syringe fitted with a 20-G needle. The aspirates were centrifuged briefly to remove cellular debris and then kept at –30C. To avoid post-mortem glucose metabolism [17, 18], the ovaries were kept cold during the procedures.

Steroid hormone assay

The concentrations of E2 and P4 in the FF samples were determined by an enzyme immunoassay (EIA) after extraction as published previously [19, 20]. The ranges of the standard curves and the ED50 of the assays were 2–2000 pg/ml and 110 pg/ml for E2 and 0.05–50 ng/ml and 2.4 ng/ml for P4. The intra- and interassay coefficients of variation for these assays were 6.2 and 8.5% for E2 and 4.5 and 7.4% for P4, respectively.

Glucose and lactate assays

The concentrations of glucose and lactate in the FF were determined by a colorimetric method using commercial kits (glucose assay, Glucose CII Test-Wako; Wako, lactate assay, Determiner LA; Kyowa Medex, Tokyo, Japan) following the instructions supplied by the manufacturers. The intra- and interassay coefficients of variation were 2.8 and 9.6% for glucose and 4.1 and 9.6% for lactate, respectively.

Classification of corpora lutea and follicles

Corpora lutea were macroscopically assessed for color, vascularity and consistency using published criteria [21] and were classified into four stages (stages I–III, luteal phase; stage IV, follicular phase). Follicular diameters were estimated from the weight of the FF using the equation $y=12.96x^{0.31}$, where y=the diameter of the follicles (mm) and x= the weight of the FF (g) [22].

Follicles were classified initially into ten groups based on diameter (≥8.5 mm or <8.5 mm), relative concentrations of E2 and P4 in FF (E/P≥1 or <1) and the stages of the CL and accompanying follicles. They were later reclassified into eight groups (Table 1).

Large estrogen-active follicles (E-A: ≥8.5 mm, E/P≥1) were examined, and their dominance (i.e., a single E-A in a pair of ovaries) or codominance (i.e., two E-As in a pair of ovaries) was confirmed. They were further divided into dominant (DF: with stage I-III CL) and preovulatory follicles (POF: with stage IV CL). When a follicle was estrogen-active but smaller than 8.5 mm and not accompanied by a DF or POF, it was designated as an early dominant follicle (EDF).

Large estrogen-inactive follicles (E-I: ≥8.5 mm, E/P<1) were initially classified into five groups according to the status of the accompanying follicles. When an E-I follicle was accompanied by either an EDF or DF, it was designated as a mid-atretic follicle (MAF). Whereas an E-I follicle that was not accompanied by any E-A or other E-I follicles was temporarily designated as an early atretic follicle without an E-I follicle (EAF-EI). In many cases, multiple E-I follicles in apparently different stages of follicular atresia were present in pairs of ovaries. In all cases examined, one of the follicles biochemically resembled an EAF-EI and another resembled an MAF. We temporarily designated the former as an early atretic follicle with an E-I follicle (EAF+EI) and the latter as a late atretic follicle with an EAF (LAF+EAF). When an E-I was accompanied by a POF, it was designated as an LAF with a POF (LAF+POF).

Follicles smaller than 8.5 mm were divided into two groups. When they were accompanied by E-As, they were classified as suppressed small follicles (SSF), whereas follicles with EAFs were

Table 2. Size and biochemical characteristics of the classified follicles

Follicular category (n)	Size (mm)	E2 (ng/ml)	P4 (ng/ml)	E2:P4 ratio (ng/ng)	Glucose (mg/dl)	Lactate (mg/dl)	Lac:Glc ratio (mol:mol)
<8.5 mm in diameter							
SSF (40)	7.0 ±0.9 [a]	1.3 ± 1.8 [a]	12.1 ± 8.5	0.18 ± 0.23 [a]	49.7 ± 14.3	90.1 ± 13.4	4.2 ± 2.5 [a]
GF (35)	6.5 ±0.8 [b]	1.0 ± 1.5 [a]	15.0 ± 11.1	0.13 ± 0.18 [a]	42.7 ± 17.1	97.7 ± 14.9 [a]	5.7 ± 3.5 [b]
EDF (6)	8.1 ±0.3 [c]	19.9 ± 10.0 [b]	10.7 ± 4.3	2.04 ± 1.34 [b]	58.0 ± 11.6	80.6 ± 9.1 [b]	2.9 ± 1.0 [a]
≥8.5 mm in diameter							
DF (32)	12.1 ±2.2	34.2 ± 15.6 [a]	19.3 ± 9.4 [a]	2.04 ± 1.08 [a]	69.7 ± 14.3 [a]	59.3 ± 17.5 [a]	1.8 ± 0.9 [a]
POF (11)	13.7 ±3.4	68.0 ± 37.6 [b]	22.6 ± 7.7 [a,b]	3.15 ± 1.67 [a]	72.7 ± 5.9 [a]	60.6 ± 21.2	1.7 ± 0.7 [a]
EAF (29)	13.3 ±1.8	9.9 ± 9.1 [c]	41.6 ± 32.5 [b]	0.37 ± 0.31 [b]	75.2 ± 13.4 [a]	50.3 ± 13.5 [a]	1.4 ± 0.8 [a]
MAF (20)	13.0 ±1.9	1.3 ± 1.9 [d]	168.3 ± 159.3 [c]	0.03 ± 0.06 [c]	63.3 ± 18.4 [a]	56.6 ± 16.6	2.1 ± 1.3 [a]
LAF (18)	12.4 ±1.7	1.0 ± 2.0 [d]	288.0 ± 162.4 [c,d]	0.00 ± 0.01 [c,d]	45.7 ± 15.5 [b]	78.0 ± 21.7 [b]	5.8 ± 10.0 [b]

Values are expressed as means ± SD. Values with different superscripts within each column and the same size categories are significantly different (P<0.05).

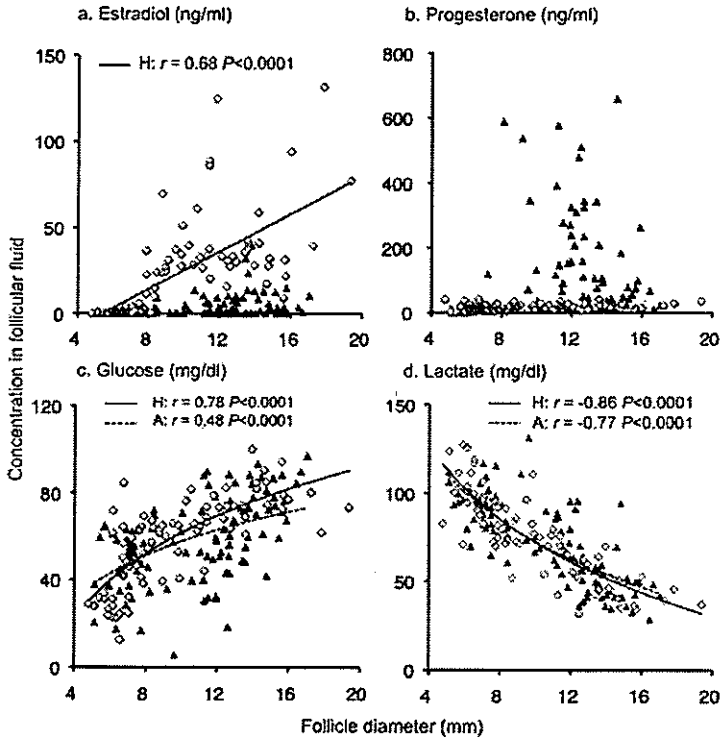

Fig. 1. Relationships between follicular diameter and the concentrations of estradiol (a), progesterone (b), glucose (c) or lactate (d) in the follicular fluid of bovine non-atretic (open diamond) and atretic (filled triangle) follicles. Significant correlations were found for estradiol in healthy follicles and for both glucose and lactate in healthy and atretic follicles.

classified as growing follicles (GF).

Statistical analysis

The data were analyzed using a one-way ANOVA followed by the Steel-Dwass multiple comparison test or Wilcoxon rank sum test. The relationships between the levels of steroids, glucose and lactate and sizes of follicles were analyzed by regression analysis. All data were presented as means ± SD with statistical significance set at P<0.05.

Results

One hundred ninety-one follicles from 73 heifers were analyzed in the present study. As mentioned in the materials and methods, large E-I follicles were initially classified into 5 groups. However, because there was no difference between EAF-EI and EAF+EI or between LAF+EAF and LAF+POF with regard to diameter or any biochemical indices in FF (data not shown), the E-I follicles were reclassified into three groups (EAF, MAF and LAF). The sizes and biochemical characteristics of the classified follicles are summarized in Table 2. In the healthy follicles (i.e., GF, EDF, DF, POF), the concentrations of E2 in FF increased abruptly when the follicles reached around 8 mm in diameter, and continued to increase as the follicles enlarged (Fig. 1a). There was a highly significant positive correlation between the concentration of E2 and follicular size in the healthy follicles (P<0.0001; Fig. 1a). The concentrations of E2 in the large

Table 3. Size and biochemical characteristics of small follicles and accompanying early atretic follicles

Follicular category (n)	Size (mm)	E2 (ng/ml)	P4 (ng/ml)	E2:P4 ratio (ng/ng)	Glucose (mg/dl)	Lactate (mg/dl)	Lac:Glc ratio (mol:mol)
GF							
G/E>1 (5)	7.4 ±0.5	3.9 ± 1.3 [a]	15.8 ± 8.9	0.34 ± 0.26 [a]	56.5 ± 18.0	89.9 ± 13.6	3.1 ± 2.0
G/E<1 (11)	6.4 ±1.0	0.4 ± 0.5 [c]	20.4 ± 15.6	0.08 ± 0.13 [b]	42.3 ± 14.1	93.6 ± 13.4	4.9 ± 1.8
EAF							
G/E>1 (5)	14.4 ±1.6	1.5 ± 1.0 [a]	34.4 ± 15.6	0.04 ± 0.02 [a]	75.4 ± 9.9	42.9 ± 3.2	1.2 ± 0.1
G/E<1 (11)	13.0 ±2.2	10.5 ± 8.3 [c]	57.8 ± 37.5	0.28 ± 0.26 [b]	75.9 ± 14.4	53.9 ± 14.0	1.5 ± 0.7

Values are expressed as means ± SD. G/E: E2 in the GF/E2 in the EAF of the same ovarian pair. Values with different superscripts within each column and the same follicle categories are significantly different (a,b; $P<0.05$: a,c; $P<0.01$). Only the largest GF and accompanying EAF in each ovarian pair were used in this analysis.

E-I follicles (i.e., EAF, MAF, LAF) were significantly lower than those in the E-A follicles (i.e., EDF, DF, POF, $P<0.05$; Table 2). Among the large E-I follicles, the concentrations of E2 in the advanced atretic follicles (i.e., MAF, LAF) were significantly lower than that in EAFs ($P<0.05$; Table 2). The concentrations of P4 in the healthy follicles never exceeded more than 50 ng/ml, while they were drastically increased in MAFs and LAFs ($P<0.05$; Table 2).

The concentrations of glucose and lactate were less variable among the follicular categories (Table 2); however, regardless of the physiological status of the follicles, we found highly significant positive and negative correlations between follicular size and the concentrations of glucose (healthy, $r=0.78$; atretic, $r=0.48$) and lactate (healthy, $r=-0.86$; atretic, $r=-0.77$), respectively ($P<0.0001$; Fig. 1c and d). The concentrations of glucose and lactate showed an inverse relationship in both the healthy and atretic follicles (healthy, $r=-0.75$; atretic, $r=-0.70$; $P<0.0001$).

There were no significant differences in the concentrations of steroids, glucose and lactate between SSFs and GFs (Table 2). Unlike SSFs, which can be easily identified by the presence of dominant follicles, small growing follicles are more difficult to identify. In the present study, the follicular population categorized as GF likely contained both regressing SSFs from the previous wave and growing follicles from the new wave. Thus, we attempted to distinguish these two populations by using the ratio of E2 in the largest GF and accompanying EAF (G/E ratio). Using 16 pairs of EAFs and GFs, we observed an inverse relationship in the E2 concentrations of the GFs and EAFs ($r=-0.74$; $P<0.005$), indicating that E2 production increases in growing follicles and decreases in EAFs (i.e., more aged EAFs). Consequently, we classified GFs into two groups of follicles based on G/E ratios >1 (i.e., presumptive growing follicle) and <1 (i.e., SSF; Table 3). The concentration of E2 was significantly higher in GFs with G/E >1 than in those with G/E <1 ($P<0.01$). The concentrations of P4, glucose and lactate did not differ between these follicles.

Discussion

The aim of the present study was to establish a simple and clear-cut method of classifying bovine follicles harvested from abattoir-derived ovaries. For classification, we used two established criteria, namely, the size of the follicle at follicular deviation [12, 23, 24] and the ratio of E2 and P4 in the FF (E/P ratio) [6–8], as arbitrary thresholds.

The emergence, dominance and regression of the dominant follicle are the key events that determine the fate of other follicles. Thus, we identified the presence or absence of dominant follicles (i.e., EDF, DF, POF) in each pair of ovaries and used this information to identify other types of follicles.

The DF and POF can easily be identified as a single large E-A follicle or sometimes two large E-A follicles in a pair of ovaries. They appear to be in the growth phase of a follicular wave that lasts for 3–5 days after follicular selection [10] (Fig. 2D and P).

A follicle can attain dominant status before it reaches 8.5 mm in diameter. In the present study, we classified follicles around 8 mm in diameter with E/P ratios ≥1 as early dominant follicles (EDFs). These EDFs were accompanied by subordinate follicles that were 0.4–1.3 mm smaller. The size difference between the largest (dominant) and second largest (subordinate) follicles reaches 2 mm within a day after follicular deviation [25]. Therefore, the follicles classified as EDFs were likely young dominant follicles that just attained the dominant status and were rapidly increasing in E2 production [25, 26] (Fig. 2E).

Under our present classification scheme, large E-A follicles in the early growth phase of the ovulatory wave were classified as DFs rather than POFs (Fig. 2D in the 2nd/3rd waves). Ginther *et al.* [10] reported that follicles destined to ovulate reached 10–13 mm in diameter by the onset of luteolysis; therefore, the follicles classified as POFs were likely in the final stage of growth, approximately 1–3 days before ovulation. The significantly higher level of E2 found in the POFs, which is characteristic of preovulatory follicles under increasing LH stimulation, also supports this assumption.

In the present study, we classified large E-I follicles into three categories according to the timing of their appearance. Early atretic follicles (EAFs) appeared to be aged dominant follicles in the static or early regressing phase of the first or second follicular wave and to be rapidly losing the ability to ovulate or suppress FSH secretion [10, 12, 27] (Fig. 2 EA). We set the period of EAF occurrence between the time when the DF lost its E-A status and the time the next dominant follicle emerged, which is approximately equivalent to days 7–12 post-ovulation in animals with 2 follicular waves or days 7–11 and days 14–18 in animals with 3 waves [10, 12] (Fig. 2). Thus, EAFs were estimated to be atretic follicles up to 5 days old.

Around days 11–12 and day 18 post-ovulation, a new EDF/DF emerges. We classified large E-I follicles that accompanied these

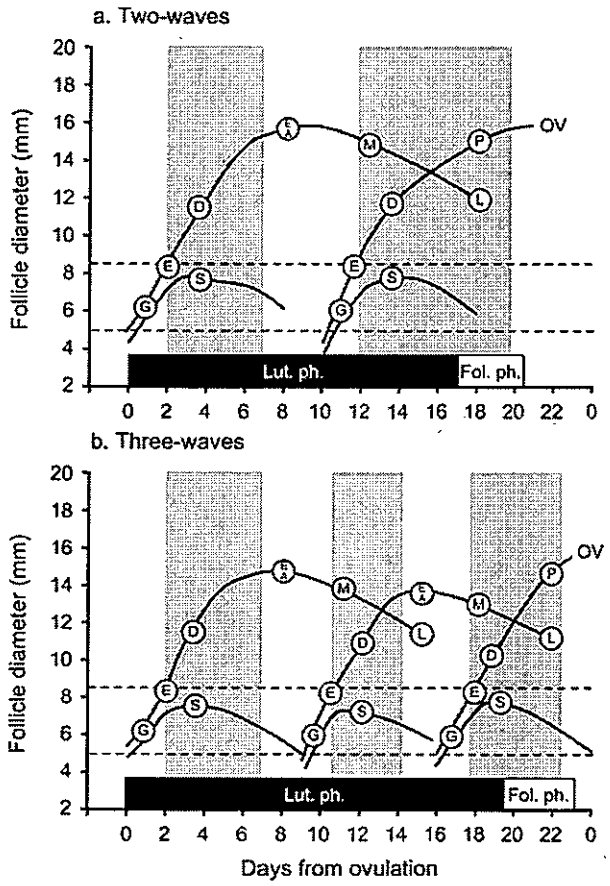

Fig. 2. Occurrence of follicular waves and types of follicles present during the bovine estrous cycle with 2 (a) or 3 (b) waves. This diagram was drawn based on the figure presented by Kulick *et al.* [12]. G: growing follicle. E: early dominant follicle. D: dominant follicle. P: preovulatory follicle. EA: early atretic follicle. M: mid atretic follicle. L: late atretic follicle. S: suppressed small follicle. Approximate time of follicular dominance (shadowed parts) and the lengths of the luteal phase (Lut. ph.) and follicular phase (Fol. ph.) are also shown.

dominant follicles as mid-atretic follicle (MAF). Biochemically, they were clearly distinguishable from EAFs, containing significantly higher levels of P4 and lower levels of E2. MAFs were estimated to be atretic follicles 5–10 days old.

Often, EAFs and POFs were accompanied by one or more E-I follicles. These E-I follicles contained much higher levels of P4 with negligible E2 and were easily distinguishable from EAFs. Classified as late atretic follicles (LAFs), they were likely aged atretic follicles of the previous follicular wave. Since the length of the growth phase varies among the waves within the estrous cycle, as well as between animals with 2 and 3 waves [10, 12], the age of the LAFs also varies (6–14 days). This variation makes it difficult to separate LAFs from MAFs by means of the present classification system. The follicular glucose concentration may be used as an auxiliary classification criterion to separate advanced atretic follicles from less advanced atretic follicles, and we discuss this possibility in a later section.

In the present study, small follicles accompanied with E-A follicles were classified as SSFs. Since no follicles of this size can survive in the presence of E-A follicles, we suggest this classification procedure is a simple and reliable means of identifying unselected subordinate follicles.

Unlike SSFs, small growing follicles (i.e., GFs) are difficult to identify. Follicles in this category only occur for about 2 days from the onset of each follicular wave [10, 12], which is equivalent to the latter half of the estimated EAF occurrence period. As a result, the GF group likely contains not only growing follicles but also aged SSFs derived from the previous follicular wave. SSFs and GFs are not mutually distinguishable with any of the criteria employed, so we attempted to identify real growing follicles as follows. We assumed that the concentration of E2 in the EAF gradually decreases as follicular atresia progresses. Meanwhile, follicles in the next wave start to grow and gradually increase E2 output, so we divided the follicles according to the ratio of E2 in the largest GF and the accompanied EAF in each ovarian pair (G/E E2 ratio). A highly significant negative correlation between the concentrations of E2 in the GF and accompanied EAF indicates that emergence of the next follicular wave coincides with loss of E2 output from the aging EAF. The follicles ~7 mm in diameter selected by this method contained significantly higher levels of E2, and they were likely future dominant follicles, as the first follicle to reach 7 mm generally becomes dominant [25].

Glucose is the indispensable energy substrate of ovarian activity [15, 16]. Despite its importance, our knowledge of glucose in ovarian physiology is fairly limited. The concentrations of glucose and lactate found in the present study were comparable to those reported previously in bovine FF [17, 28–30]. The present results clearly demonstrated that the FF concentrations of glucose and lactate correlate positively and negatively, respectively, with follicle size, regardless of the health status of the follicles. Highly significant negative correlations were also found between the concentrations of glucose and lactate in both healthy and atretic follicles. Similar results have been reported in cattle [18, 29, 30]; however, the size and physiological status of the follicles were not well-defined in those studies. Although the concentrations of glucose and lactate appear to be similar between healthy and atretic follicles, the mechanisms responsible for them are likely quite different. The concentrations of these substances in FF are in dynamic equilibrium essentially determined by the levels supplied, by cellular metabolism and by clearance [31]. In growing small follicles with a less developed vascular network, glucose supply and lactate clearance are low, whereas the metabolic activities in the theca and granulosa layers are increasing. In this situation, glucose would be rapidly metabolized to lactate, which would thereby accumulate in FF. As follicles grow, the vascular network also develops [32] and allows efficient glucose delivery and lactate clearance. The metabolic activity of follicles also increases [33], but the rate of increase in supply and clearance results in a shift in the L/G ratio more favorable to glucose. In atretic follicles, degeneration of the vascular network [32] would gradually impair glucose delivery and lactate clearance, and as follicular atresia progresses, the external supply of glucose eventually vanishes. At this point, FF would act

as a reservoir of glucose for the dying follicle. The present results indicate this situation occurs in the follicles classified as LAFs, for which a significant decrease in glucose concentration was observed. This implies that the decrease in glucose supply is not the cause of follicular atresia, although it and/or the increase in lactate in the FF could affect events associated with follicular demise, such as the onset of apoptosis.

These results indicate that neither glucose nor lactate is suitable as a general index for distinguishing healthy follicles from atretic follicles, at least on their own. However, together with other criteria, such as follicular size, the concentration of glucose can be used as a screening tool for follicular classification (e.g., follicular diameter >12 mm that contain <50 mg/dl glucose were all atretic in the present study; Fig. 1c). A significantly lower level of glucose and higher L/G ratio in LAFs compared with MAFs and EAFs also indicates that the follicular glucose concentration can be used as an index to identify large E-I follicles at the advanced stage of follicular atresia; we attempted this in a previous report [34] in which we used the glucose concentration to divide E-I follicles into early atretic (glucose ≥45 mg/dl) and late atretic (<45 mg/dl) follicles.

In conclusion, analysis of the follicular population in a pair of ovaries provides useful information about the physiological status of the follicles. Together with biochemical indices such as steroid and glucose concentrations in FF, this procedure would provide a simple and accurate means of bovine follicular classification.

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