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Seiji YAMAMOTO[1], Hirotada TSUJII[2], Masao HOTTA[2],
Takanori OGINO[3], Tetsuya KOHSAKA[3],
Kazuyoshi HASHIZUME[4] and Shichiro SUGAWARA[5]

[1] *Vetetrinary Practitioner, Ina-shi, Nagano-ken 396*
[2] *Shinshu University, Faculty of Agriculture, Kamiina-gun, Nagano-ken 399-45*
[3] *Ibaraki University, Faculty of Agriculture, Inashiki-gun, Ibaraki-ken 300-03*
[4] *National Institute of Animal Industry, Tsukuba, Ibaraki-ken 305*
[5] *Faculty of Agtriculture, Tohoku University, Sendai-shi, Miyagiken 981, JAPAN*

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Summary

The development of follicles and ovulation are influenced by augmentation and suppression of FSH secretion from the pituitary. Inhibin is closely involved in the regulation of FSH secretion. As a clue to determine the mechanism of follicular development, the relationship between follicular size and inhibin and the localization of inhibin were investigated in this study. Bovine and porcine ovaries were collected immediately after slaughter. After measuring the outside diameters of follicles with slide calipers, follicular fluids were sampled by aspiration with a syringe. Using some of the ovaries collected, immunohistochemical detection of inhibin was carried out in 4% paraformaldehyde-fixed, paraffin-embedded sections. The contents of inhibin in the follicular fluids were measured by time-resolving fluorescent immunoassay (TR-FIA) using euporium. The inhibin contents in the follicular fluids were found to increase with an increase in follicular size. With bovine follicular size of 20 mm and porcine follicular size of 10 to 11 mm, the inhibin concentrations were found remarkably decreased. These sizes were presumed to constitute borderline sizes that divided follicles into ovulatory and anovulatory follicles. Immune response of inhibin was detected in granulosa cells, indicating that inhibin is produced in intrafollicular granulosa cell.

The development of follicles in cows begins early after parturition. Estrus-accompanied ovulation occurs approximately 51 to 66 d postpartum. In swine, ovulation-accompanied estrus is known to occur at approximately 1 wk after weaning.

The postpartum pattern of ovarian change in cows and sows is complicated. Dairy cows often develop ovarian cysts. These cysts can be divided into fol-

licular cysts and luteal cysts. It is evident that follicular cysts are associated with hypersecretion of follicle-stimulating hormone (FSH) and hyposecretion of luteinizing hormone (LH) (1). However, with the aimed of measuring FSH and LH in peripheral blood from dairy cows with follicular cysts several attempts were taken (2-5). They detected high LH and low FSH levels in many such cows.

Recently, inhibin in granulosa cells has been found to strongly inhibit the secretion of FSH, and its physiological role is being studied (6-8).

This study was designed to investigate the development of the follicle and changes in an intrafollicular inhibin-like substance in cows and sows.

Materials and Methods

Measurement of Inhibin Activity

Ovaries were collected from Holstein cows and Landrace sows immediately after slaughter at a meat center located in the neighborhood of our University. Ovaries for the measurement of intrafollicular inhibin content were immediately cooled with ice and brought back to Laboratory. Then, the diameters of the follicles were measured. Follicular fluid was aspirated with a syringe separately in accordance with follicular sizes and was frozen at $-40^{\circ}C$ after centrifugation at $3,000 \times g$ for 10 min. Assays of inhibin activity were carried out by time-resolving fluorescent immunoassay (TR-FIA), which is based on the principle of competitive binding using a solid-phased second antibody (8, 9).

Synthetic porcine inhibin was used for labeling antigen. Labeling was carried out using europium (Eu). Rabbit blood anti-serum (donated by Prof. Hasegawa of Kitasato University) was used as inhibin anti-serum. A sample and the first antibody were added to the plate of solid-phased second antibody, and the plate was incubated for 1.5 h. After incubation, Eu-labeled inhibin was added, and incubation was continued for 3.5 h. After completion of incubation, the plate was washed, and an enhancement reagent was added. Then, the fluorescence count was measured using DEL-FIA for 1 s/well.

Histological Examination

Ovaries of Holstein cows and Landrace sows were collected from slaughter house as described before. The size of follicles were measured with sliding calipers immediately after collection of ovaries, and the ovarian samples were fixed in 4% paraformaldehyde-PBS solution and brought back to Laboratory. Follicles were placed in 4% paraformaldehyde-PBS at 4°C and were fixed by exposure to microwave three times for 10 s in a microwave oven. After fixation, with 70% alcohol for 3 to 4 h, 90% alcohol for 3 h, anhydride alcohol 3 to 4 times for 1 h, xylene 3 times for 40 min, and xylene paraffin twice for 1.5 h, the samples were embedded in paraffin heated to 54 to 56°C. The localization of inhibin was

examined by immunohistochemical method (10). The paraffin-embedded follicle was cut into 7 μ-thick sections. After removed of paraffin, the follicle was allowed to react in a 100 dilution of inhibin anti-serum at 4°C for 12 h. Then, washed in PBS and was allowed to react with the second antibody at 25°C for 30 min. After washing in PBS, allowed to react with peroxides antiperoxides at 25°C for 45 min. Development was carried out with diaminobenzidine. The localization of inhibin was determined by microscopic examination of HE stained and compared to control section.

Results

Whether it was possible to determine the activity of inhibin in the follicular fluids used in this experiment was tested using calibration curves, and the result is shown in Figure 1.

Upon drawing a standard curve of inhibin using the TR-FIA method, a dose-response curve shown in Figure 1 was obtained. The sensitivity of this assay was 8.7 pg/well. The intra- and interassay coefficients of variation were 3.07% and 7.0%, respectively. Dilution curves for the bovine and porcine follicular fluids were prepared using this assay system. Bovine follicles were divided into those measuring less than 21 mm in diameter and those measuring more than 21 mm. Porcin follicles were divided into those measuring less than 10

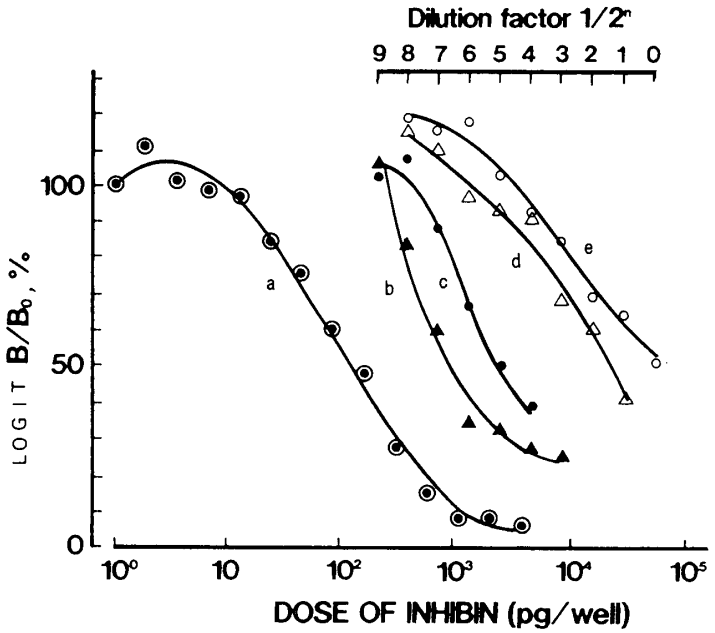

FIG. 1. Dilution curves produced by various inhibin preparations of (a) standard, (b) cow's follicular Fluid (1-20 mm), (c) sow's follicular fluid (1-10 mm), (d) cow's follicular fluid (20-50 mm), and (e) sow's follicular fluid (11-50 mm).

mm in diameter and those measuring more than 11 mm. At all serial dilution, dose-dependent responses were obtained. It was thus found possible to detect inhibin in both bovine and porcine follicular fluids by this assay system.

The concentrations of immunoreactive inhibin in the bovine and porcine follicular fluids were measured. The distribution patterns are shown logarithmically in Figure 2. Biphasic distributions were found to exist for the concentrations of inhibin. To express the relation between the biphasically distributed inhibin concentrations, normal follicles and follicular cysts, the concentrations of inhibin in the bovine and porcine follicular fluids are shown by follicular sizes.

Figures 3 and 4 shows the relation between the concentrations of inhibin in the bovine and porcine follicular fluids and the sizes of follicle. A positive correlation ($Y = 1.52x + 17.23$) was obtained in the cows; namely, the concentration of inhibin was increased with an increase in follicular size for follicles

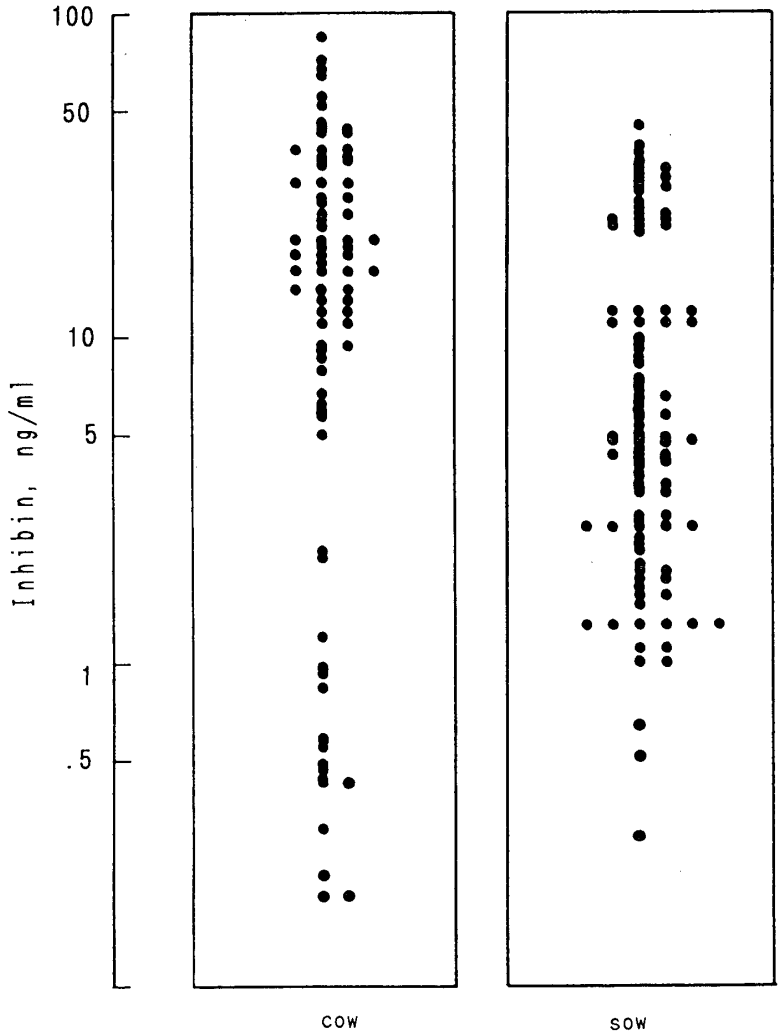

FIG. 2. The concentrations of immunoreactive inhibin in the cow and sow follicular fluids. The distributions patterns are shown logarithmically.

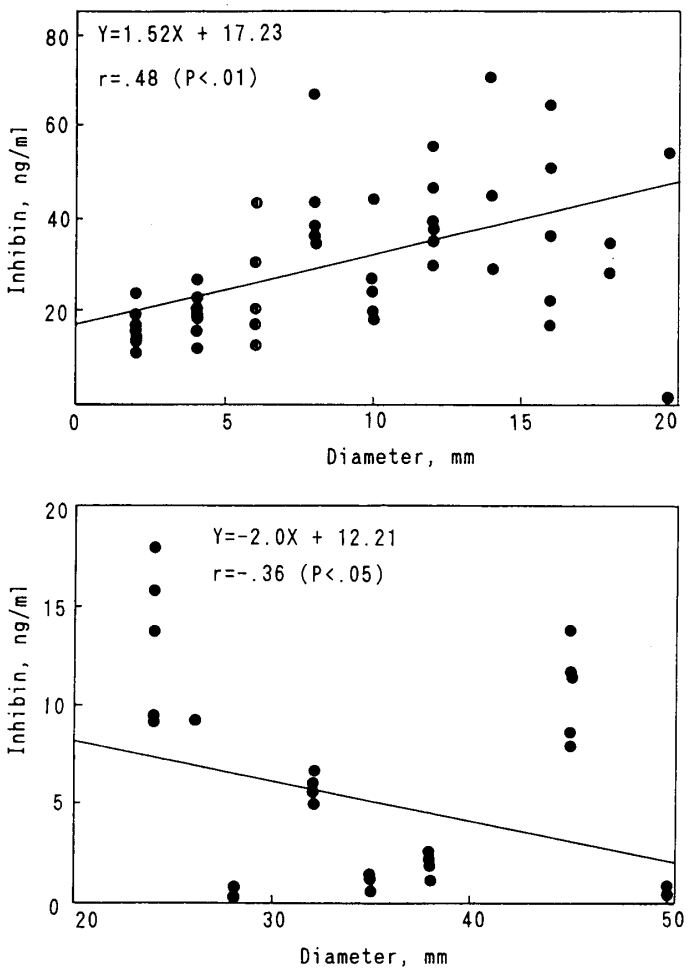

FIG. 3. Relationship between follicular size and concentration of follicular inhibin in cow.

measuring 2 to 20 mm in diameter. For follicles measuring 24 to 50 mm in diameter, conversely, there was a negative correlation ($Y = -.20x + 12.21$); namely, the concentration of inhibin was decreased with an increase in follicular size. A similar finding was obtained for the porcine follicles. For follicles measuring 2 to 10 mm in diameter, the concentration of inhibin and the follicular size were correlated positively ($Y = 1.34x + 16.95$). For those measuring 11 to 55 mm in diameter, these were negatively correlated ($Y = -.32x + 13.82$).

Figure 5 shows the immunohistochemical findings in bovine and porcine follicles. A substance that reacted with the two antibodies was found in granulosa layers of these animal species. The reacting substance seen in granulosa layers was not found in the immunostained control. This immune reaction was, therefore, confirmed to be specific. This demonstrates that inhibin is secreted from intrafollicular granulosa cells.

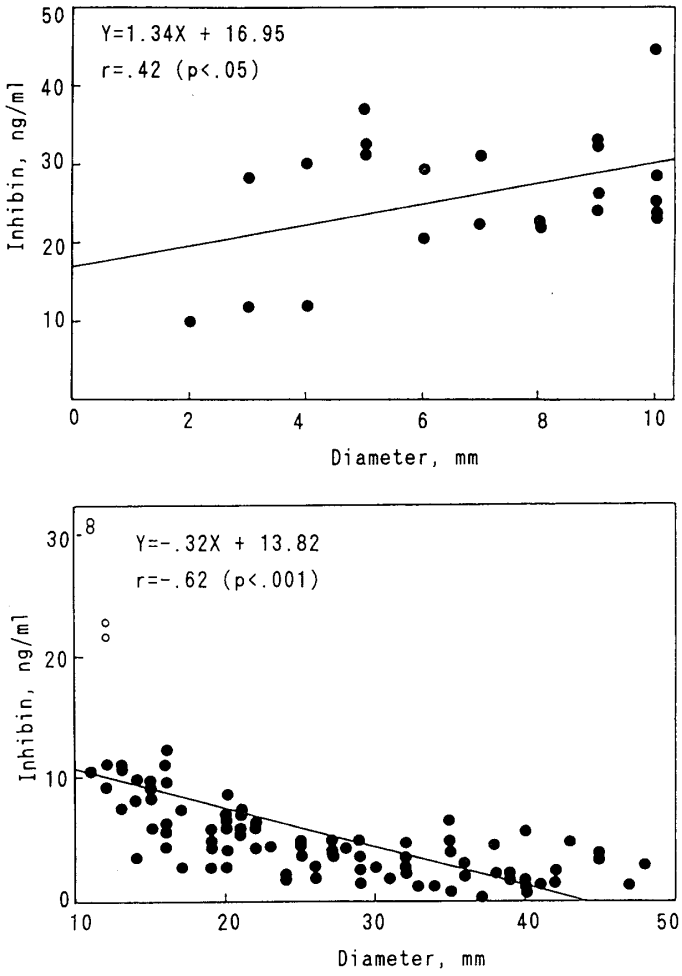

FIG. 4. Relationship between follicular size and concentration of follicular inhibin in sow.

Discussion

Follicle-stimulating hormone is directly related to follicular growth. The secretion of FSH is inhibited by inhibin. Inhibin inhibits the secretion of endogenous FSH and thereby suppresses the growth and function of the sexual gland. Earlier studies have suggested the production of inhibin in follicular granulosa cell layer (11) and the existence of inhibin in peripheral blood (12, 13). The inhibin activity is related to the size of the follicle (14-16). Apart from inhibin, an inhibitor of FSH binding to granulosa cells has been reported to exist in follicular fluid, and granulosa cell layer has been suggested to be the site of inhibin production (17-20).

In this experiment, the concentration of inhibin in bovine follicular fluid decreased with follicular sizes $\geqq$20 to 25 mm. This agreed with the reports of Henderson and Moon (21) and Henderson and Franchiman (22). In porcine

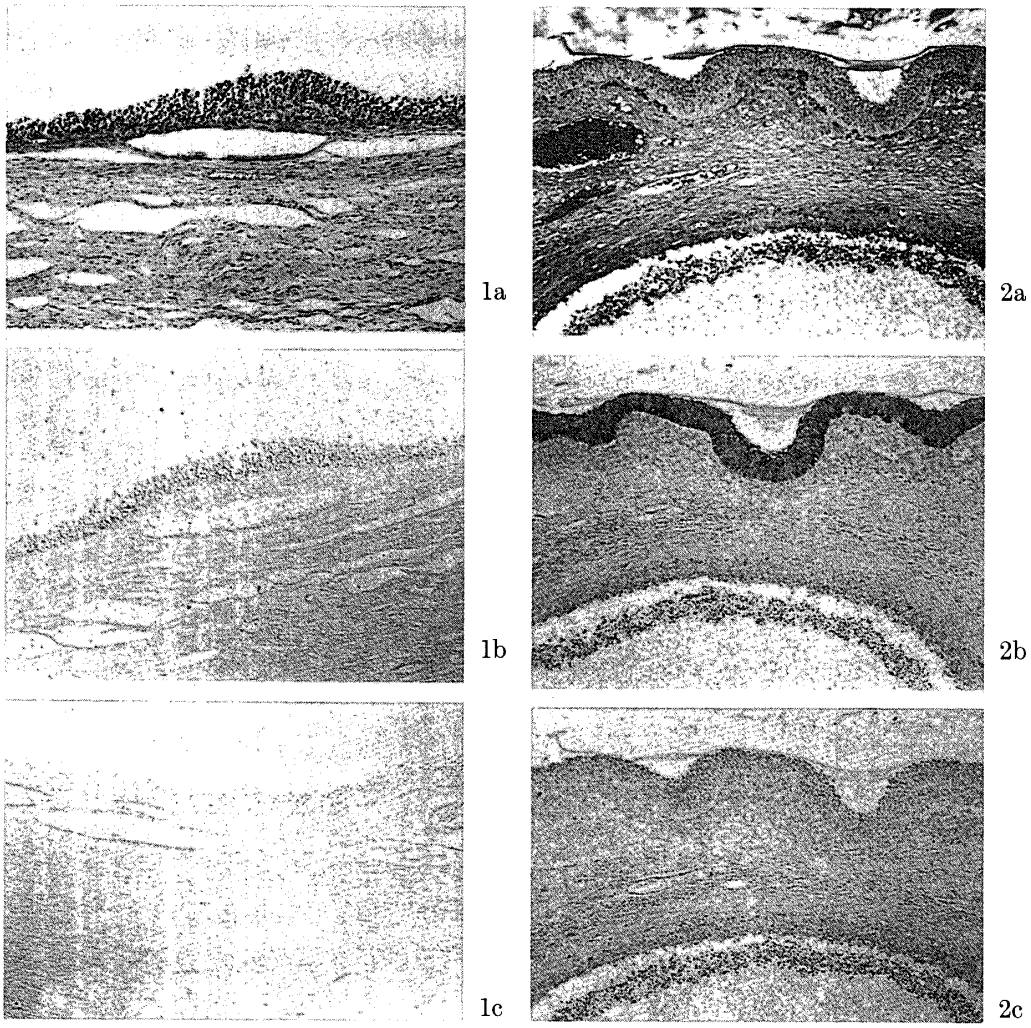

FIG. 5. Immunohistochemical photograph of (1) cow and (2) sow's follicles (a) HE dyeing, (b) antibody treated follicle, existing granulosa layer, (c) control follicle without treatment by antibody.

follicular fluid, the concentration of inhibin was likewise found to decrease with the follicular size $\geqq 10$ mm. Mizumachi *et al.* (7) studied the endocrinological condition of cows with follicular cysts by dividing into three categories according to the function of granulosa cells: (1) hyperfunction of follicle in accordance with increased inhibin activity and estrogen and testosterone concentrations, (2) decrease inhibin activity and estrogen concentration due to an incease in progesterone, and (3) almost complete loss of endocrine activity. Mizumachi et al., (7) assessed the hormonal levels in intracystic fluids and reported that the endocrine activity in follicular cysts showed a diversity of patterns ranging from almost the same level as or a higher level of activity than that in estrous follicles to the complete loss of follicular function. They attempted to categorize the follicular

function using the progesterone/estrogen ratio or testosterone/estrogen ratio (which have been conventionally used as indicators of follicular degeneration) and found that systematic categorization of follicular function into specific types was not possible due to large variations.

In requires further investigation to confirm whether the inhibin and endocrine activities in a follicle may reflect the hormone levels in peripheral blood. If the inhibin activity detected in follicular fluid can be interpreted as a factor which plays an important role as an inhibitor of FSH secretion from the pituitary gland, it is presumed that the blood FSH level in some of the cows with follicular cysts may be suppressed to the same level as or a lower level than that at estrus. Concentrations of FSH in peripheral blood have been found to be decreased in some cows with follicular cysts (2-5). Inhibin has been detected in follicular fluids from women with polycystic ovarian diseases, and the blood FSH levels in these cases have been found to be decreased than normal women (23).

In cystic follicles in which the follicular function is enhanced compared to normal follicles, the secretion of inhibin and estrogen may be diminished by regression of granulosa cells or by luteinization. Furthermore, the secretion of progesterone may be augmented to eventually result in the loss of endocrine activity. It was not possible to determine whether luteinization appeared during this process (until the loss of endocrine activity). Short (24) analyzed steroids in intracystic fluid and found that the progesterone level was elevated compared to that in normal follicles. In an extensive investigation of Yazaki *et al.* (25), progesterone levels were found to be higher in follicular cysts.

The production of inhibin has been studied using granulosa cells, the main inhibin-producing cells in females. It has been found from these studies that although the production of inhibin is primarily stimulated by FSH, it is largely influenced by the physiological condition of responding granulose cells. The production of inhibin by gonadotrophic hormone is accelerated by cAMP. Since the secretion of estrogen is augmented by the production of inhibin (8). Mizumachi *et al.* (7) found that the activity of inhibin and the concentrations of estrogen and testosterone were increased and the concentration of progesterone decreased in follicular fluid during the estrous stage. They detected almost no inhibin in luteal cysts as well as found a remarkably high level of progesterone and extremely low levels of estrogen and testosterone.

A large number of earlier studies have suggested that inhibin produced and secreted from the sexual gland reaches the pituitary via blood and inhibits the secretion of FSH or LH. The magnitude of inhibin-induced inhibition of FSH secretion from the pituitary is dependent on blood concentration of inhibin. Therefore, if the kinetics of inhibin in blood (which controls the secretion of FSH, a hormone most closely associated with the development of follicle) is studied, it might be possible to determine the mechanism that governs ovulation and

nonovulation from the size of follicle (20 mm for bovine follicles and 10 to 11 mm for porcine follicles as found in this experiment). In a study of the behavior of FSH secretion in blood during the sexual cycle in cows, Hasegawa (1988b) reported that the concentration of FSH that reached a relatively high level during the luteal phase was gradually decreased in the follicular phase and that the first peak of FSH occurred concomitant to the LH surge and accompained by a small but distinct second peak. This suggests that the increased production of inhibin from the luteal to follicular phase inhibits the secretion of FSH and that the blood inhibin level is transiently decreased by ovulation induced after the LH surge so that a small second peak of FSH is generated. The secretory behavior of inhibin in cows is characterized by the formation of its highest peak after the second peak of FSH. Inhibin is secreated at the early luteal phase and this secretion is gradually decreased with time. Hasegawa (9) also found that, a transient release of LH occurred in the follicular and luteal phases in cows, in addition to an ovulatory LH surge 2 d before ovulation. They also reported that at the preovulatory stage of LH surge, FSH showed no surge-like secretory behavior, and at the end of the luteal phase, the blood concentration of FSH was increased and began to decrease gradually in the follicular phase. This FSH decrease coincides with the inhibin increase, and the decrease in FSH is considered to result from the negative feedback of inhibin. When ovulation is induced by LH surge, the blood concentration of inhibin is rapidly decreased. Hasegawa (9) reported that the blood concentration of FSH was elevated with a decrease in inhibin. He observed that, unlike in sows, the secretion of inhibin in cows was accompanied by an increased secretion of estrogen. This suggests the development and regression of bovine follicles during the luteal phase. At ovulation, FSH is secreted by the release of GnRH from the pituitary. Follicles mature and develop in the presence of FSH. Ovulatory follicles in cows are determined 4 to 5 d before ovulation. At the early follicular phase, FSH is usually high and inhibin is low. When the follicle matures, the secretion of inhibin is augmented so that the secretion of FSH is suppressed. Granulosa cells in mature follicles react with LH, and this induces further secretion of inhibin. The secretion of inhibin from granulosa cells peaks during the preovulatory surge of gonadotrophic hormone, but a positive correlation is seen between the secretion of gonadotrophic hormone and that of inhibin, because the stimulatory effect of GnRH overpowers the inhibitory effect of inhibin. The preovulatory surge of gonadotrophic hormone is rapidly diminished due to the decreased stimulation by GnRH. The production and secretion of inhibin are also diminished. The blood concentration of inhibin is diminished by ovulation, and as a consequence, the formation of second FSH peak may appear in many mammalian species.

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