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The Steroidal Environment of Estradiol and Progesterone in Dominant Follicles does not Affect the Competence of Bovine Oocytes to Develop from Small Antral Follicles

Junichi Maeda[1, 2*], Fumikazu Kotsuji[1], Tsukasa Matsui[3], Kiyotaka Sasaki[2] and Toshiro Tominaga[1, 4]

[1]Department of Obstetrics and Gynecology, Fukui Medical School, Matsuoka-Cho, Fukui 910-1104,
[2]Fukui Prefectural Reinan Wagyu Experiment Station, Kaminaka-Cho, Fukui 919-1523,
[3]Fukui Prefectural Animal Experiment Station, Mikuni-Cho, Fukui 913-0004 and
[4]Tominaga Obstetrics and Gynecology Clinic, Fukui-City 910-0015, Japan

Abstract: The aim of this study was to examine the effect of the endocrine environment of dominant follicles on the competence of bovine oocytes to develop from small antral follicles 2–5 mm in diameter. Twenty-seven pairs of ovaries with mature corpora lutea were obtained from a local abattoir. The mean diameter and wet weight of the corpora lutea of individual cows were 23.3 ± 2.7 mm and 4.7 ± 1.0 g, respectively. Follicular fluid was aspirated from large follicles more than 6 mm in diameter, and estradiol (E_2) and progesterone (P_4) concentrations were determined by a RIA. Ovaries were classified into each group of E_2-active-dominant (E_2-A-dom; $E_2 \geqq 50$ ng/ml and E_2:P_4 ratio>1); 12 pairs of ovaries, E_2-dominant (E_2-dom; E_2<50 ng/ml and E_2:P_4 ratio>1); 6 pairs of ovaries or P_4-dominant (P_4-dom; E_2:P_4 ratio<1); 9 pairs of ovaries. The concentrations of E_2 and P_4 in E_2-A-dom were 132.5 ± 97.6 ng/ml and 8.4 ± 3.5 ng/ml, in E_2-dom were 27.7 ± 9.7 ng/ml and 9.2 ± 3.7 ng/ml, and in P_4-dom were 4.2 ± 4.7 ng/ml and 98.5 ± 208.4 ng/ml, respectively. The mean number of small antral follicles, the mean number of oocytes fertilized, the rate of cleavage and of blastocysts in E_2-A-dom were 44.4 ± 20.3, 24.2 ± 10.9, 68.2%, 26.0%, in E_2-dom were 48.5 ± 11.9, 28.7 ± 8.6, 70.4%, 20.2%, in P_4-dom were 41.7 ± 24.0, 23.0 ± 15.3, 70.9%, 20.0%, respectively. There were no significant differences in the number of small antral follicles, the number of oocytes fertilized and developmental capacity of the oocytes among the groups. Results of the present study indicate that in cattle E_2 and P_4 in a dominant follicle do not affect the competence of the development of oocytes derived from small antral follicles.

Key words: Bovine oocyte, Dominant follicle, Follicular fluid, Estradiol, Progesterone.

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*To whom correspondence should be addressed.

The induction of superovulation in cattle yields a highly variable number of transferable or total embryos [1, 2]. This large variation is a major factor limiting embryo transfer activities. Recently several groups of investigators mentioned that this variation was attributable to the presence of a dominant follicle before superovulation treatment [3–6].

In the case of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of bovine oocytes obtained from the abattoir also, individual variation in the yield of oocytes and the rate of embryonic development are reported [7–10]. One possible explanation for this large variation in developmental competence of the oocytes is the difference in the intrafollicular environment to which oocytes are exposed [11]. A few of follicles 6 mm or more in diameter exist in cyclic cattle, and steroidal environment of follicular fluid differ among those follicles [12–14]. Nonovulatory follicles 6 mm or more in diameter which develop after ovulation in heifers can be categorized into two classes depending on the steroidal environment. Estrogen-active follicles had higher concentrations of estradiol (E_2) than progesterone (P_4) and androgens, and had a low incidence of atresia. Estrogen-inactive follicles had higher concentrations of P_4 and/or androgens than E_2 and a high incidence of atresia [12].

In the present study, we examined the relationship

between the steroidal environment of dominant follicles and the developmental competence of oocytes, which were obtained from small antral follicles.

Materials and Methods

Collection of follicular fluid and oocytes

Twenty-seven pairs of bovine ovaries with functional corpus luteum [15] were collected from a local slaughter house. Cardiac blood samples were prepared within 30 min of slaughter and centrifuged (3,000 rpm, 10 min). The plasma samples were then kept frozen (−20°C) until E_2 and P_4 radioimmunoassay (RIA).

Ovaries were transported to the laboratory in saline at 37°C within 4 h after slaughter. The number of follicle(s) greater than 6 mm in diameter (large follicles) in the individual pairs of ovaries was counted (Each animal contains 1.6 ± 0.6 of large follicles). The follicular fluid of them was aspirated and centrifuged to remove debris, and then stored at −20°C until hormone assay. The number of small antral follicles (2 to 5 mm in diameter) on the ovarian surface of each pair of ovaries was also counted and cumulus-oocyte complexes were collected from each follicle as described previously [16]. In brief, the ovaries were washed several times in saline at 37°C. Each ovary obviated corpus luteum and the aspirated large follicle(s) was placed into a 90-mm petri dish with 15 to 20 ml Ringer's solution containing 1% inactivated bovine calf serum (BCS; Life Technologies, Inc., Grand Island, NY) and antibiotics (100 I.U./ml penicillin, 100 μg/ml streptomycin), and small visible follicles were cut with a disposable surgical blade. Ringer's solution containing the oocytes from a pair of ovaries was transferred into a 100-ml beaker. After a few minutes, the supernatant was discarded and 20 ml of the sediment was diluted with 60 ml Ringer's solution. The supernatant was discarded again, and the sediment was transferred into petri dishes with a grid. The total number of oocytes recovered was counted, and oocytes containing multilayered compact cumulus cells and showing an evenly granulated cytoplasm were selected [17, 18]. They were washed three times in Ringer's solution containing 1% BCS, and used for *in vitro* maturation culture.

In vitro maturation

The maturation medium used was M199 (25 mM Hepes buffered M199 with Earle's salts; Life Technologies, Inc., Grand Island, NY) supplemented with 5% BCS and 0.1 mg/ml FSH derived from swine (Denka, Kawasaki, Japan) and antibiotics.

Ooocytes derived from individual cows were placed separately into a 0.5-ml aliquot of the maturation medium under paraffin oil (Nacalai Tesque, Kyoto, Japan) and cultured for 20 to 22 h at 38.5°C in 3.5% CO_2 in air [16].

Sperm preparation and IVF

Commercially distributed frozen semen from one Japanese Black bull was used throughout this study. One straw of frozen semen was thawed at 37°C for 30 sec. The fertilization medium used was Gamete Preparation Medium (Laboratorios Serono, Madrid, Spain) supplemented with 5 mM caffeine (Sigma Chemical Company, St. Louis, MO) and 10 μg/ml heparin (Sigma) [9]. The thawed spermatozoa were washed twice with the fertilization medium by centrifugation at 500 × g for 5 min. The final pellet of spermatozoa was resuspended in the same medium, and the concentration of spermatozoa was then adjusted to 5×10^6/ml. Aliquots of 100 μl sperm suspension were placed under paraffin oil in 35-ml culture dishes. Five to twenty *in vitro*-matured oocytes were introduced into each aliquot of sperm suspension.

In vitro development

After 5 h of sperm/oocyte incubation, the oocytes were washed twice in M199 supplemented with 5% BCS, and were further cultured at 38.5°C for 43 to 47 h in M199 supplemented with 5% BCS and antibiotics on a monolayer that was grown from the cumulus cells surrounding the oocytes. Cleavage of embryos and development to the 2- to 7-cell stage and greater than 8-cell stage was recorded during the culture. Embryos were then cultured for seven days and development to the blastocyst stage was recorded.

Hormone assays

Concentrations of E_2 and P_4 in the fluid samples obtained from large follicles and in the blood samples were determined by a double-antibody RIA at previously described [19]. Each sample was reconstituted with 0.5 ml 0.01 mol/l phosphate buffer (pH 7.3) for assay. Intraassay coefficients of variation were less than 7% and 6% for E_2 and P_4, respectively.

From the results of follicular fluid hormone assay, each pair of ovaries were classified into 3 groups: E_2-active-dominant (E_2-A-dom; $E_2 \geqq 50$ ng/ml and E_2:P_4 ratio>1), or E_2-dominant (E_2-dom; $E_2 < 50$ ng/ml and E_2:P_4 ratio>1), P_4-dominant (P_4-dom E_2:P_4 ratio<1) [20–22]. In case the cow had more than two large follicles in a pair of ovaries, classification was done as follows. When the large follicles contained an E_2-active-dominant follicle,

the steroidal environment was classified as E_2-A-dom. When follicles contained both E_2-dom and P_4-dom, the steroidal environment was classified as E_2-dom.

Statistical analysis

The proportions of embryos that developed to various stages were analysed by chi-square test. Student's test was used for comparison of numbers of follicles, oocytes recovered and oocytes fertilized for each group. A value of $P<0.05$ was considered statistically significant.

Results

The diameter and wet weight of corpora lutea of individual cows were 23.3 ± 2.7 mm and 4.7 ± 1.0 g, respectively. The average diameter and volume of follicular fluid of large follicles (≧6 mm) were 10.7 ± 2.5 mm and 1.2 ± 0.6 ml, respectively. The concentration of P_4 of blood samples from cows with large follicles was 4.5 ± 1.4 ng/ml.

Twelve out of 27 pairs of the ovaries (44.4%) were categorized as E_2-A-dom. 6 cows pairs of the ovaries (22.2%) were categorized as E_2-dom. 9 pairs of the ovaries (33.3%) were categorized as P_4-dom. The mean concentrations of E_2 and P_4 in follicular fluid and mean diameter and volume of follicular fluid of the large follicles in these three groups are shown in Table 1.

The mean number of small antral follicles on the surface of a pair of ovaries, oocytes recovered, the mean number of oocytes fertilized, and embryos cleaving and developing to eight-cell and blastocyst stages are shown in Table 2. Among these three groups no significant differences were detected in numbers of follicles, oocytes recovered, oocytes fertilized or developmental capacity.

Table 1. Mean diameter and volume of the large follicles, and concentrations of E_2 and P_4 in follicular fluid of the large follicles (range in parentheses)

Dominant follicle	No. of cows	Mean diameter (mm)	Mean volume (ml)	E_2 (ng/ml)	P_4 (ng/ml)
E_2-A-dom	12	10.9 ± 2.5 (6–15)	1.2 ± 0.7 (0.5–3.2)	132.5 ± 97.6 (53.4–323.6)	8.4 ± 3.5 (4.8–14.1)
E_2-dom	6	12.0 ± 1.8 (10–15)	1.2 ± 0.5 (0.5–1.9)	27.7 ± 9.7 (17.0–42.6)	9.2 ± 3.7 (3.9–15.0)
P_4-dom	9	9.6 ± 2.5 (6–13)	1.0 ± 0.4 (0.4–1.8)	4.2 ± 4.7 (0.1–13.9)	98.5 ± 208.4 (7.0–740.0)

E_2-A-dom: $E_2 \geqq 50$ ng/ml, E_2: $P_4>1$, E_2-dom: $E_2<50$ ng/ml, E_2: $P_4>1$, P_4-dom: E_2: $P_4<1$.

Table 2. Mean numbers of small antral follicles and oocytes recovered and oocytes fertilized, and numbers of embryos that developed to cleavage and eight-cell stage by day 2 of culture, and to blastocyst stage by day 9 of culture, obtained from small antral follicles of cows with E_2-A-dom, E_2-dom or P_4-dom (percentage or range in parentheses)

Dominant follicle*	No. of small follicles	No. of oocytes recovered	No. of oocytes fertilized	No. of embryos developed to		
				2-cell	8-cell	Blastocyst
E_2-A-dom	44.4 ± 20.3	36.1 ± 17.0	24.2 ± 10.9 (67.0%)**	16.5 ± 7.4 (68.2%)***	8.7 ± 3.9 (36.0%)	6.3 ± 4.2 (26.0%)
	(11–78)	(10–71)	(6–50)	(4–26)	(2–14)	(0–15)
E_2-dom	48.5 ± 11.9	40.0 ± 8.4	28.7 ± 8.6 (71.8%)	20.2 ± 7.3 (70.4%)	9.0 ± 7.5 (31.4%)	5.8 ± 4.2 (20.2%)
	(34–65)	(30–55)	(18–40)	(12–28)	(0–14)	(0–12)
P_4-dom	41.7 ± 24.0	32.4 ± 22.5	23.0 ± 15.3 (71.0%)	16.3 ± 8.1 (70.9%)	6.8 ± 4.6 (29.6%)	4.6 ± 3.8 (20.0%)
	(18–88)	(15–78)	(5–49)	(5–29)	(0–13)	(0–12)

*Abbreviations explained in Table 1. **No. of oocytes fertilized/No. of oocytes recovered × 100. ***No. of embryos/No. of oocytes fertilized × 100.

Discussion

An ultrasonographic study revealed the existence of two or three waves of follicle development during the healthy estrous cycle in cattle [23, 24]. Takagi *et al.* [22] reported that most mature corpora lutea were 21 to 25 mm in diameter. Ireland *et al.* [15] reported that the diameter and wet weight of corpora lutea in the luteal phase were 16 to 20 mm and 3.4 to 3.6 g, respectively. Plasma concentrations of P_4 in the luteal phase reached about 2 to 8 ng/ml [25]. Savio *et al.* [23] showed that the numbers of follicles (≥5 mm) varied from 1 to 2 during most days of the estrous cycle, except at the beginning of the cycle, and that the range of maximum size of these follicles was 15 to 20 mm. Ireland and Roche [12] observed that the mean diameter of large follicles (≥6 mm) was 9.9 ± 0.6 mm or 10.0 ± 0.7 mm. In the present study, the diameter and wet weight of corpora lutea of individual cows were 18 to 30 mm (23.3 ± 2.7 mm) and 3.1 to 6.8 g (4.7 ± 1.0 g), respectively. The concentration of P_4 of blood samples was 3.0 to 8.9 ng/ml (4.5 ± 1.4 ng/ml). The number of large follicles (≥6 mm) per individual was 1 to 3 (1.6 ± 0.6). The diameter of large follicles was 6 to 16 mm (10.7 ± 2.5 mm). The volume of follicular fluid of large follicles ranged from 0.4 to 2.9 ml (1.2 ± 0.6 ml), indicating that ovaries used in our study were in the stage of the normal luteal phase of the healthy estrous cycle.

Numbers of E_2-A-dom, E_2-dom and P_4-dom were 12/27 (44.4%), 6/27 (22.2%) and 9/27 (33.3%), respectively, which were not in accordance with previous reports [13, 22]. Smith *et al.* reported that 7/15 (46.7%) of follicles in the early luteal phase of the cycle were P_4-dom, and Takagi *et al.* reported that 16/23 (69.6%) of follicles coexisting with a functional corpus luteum in the middle of the estrous cycle were P_4-dom, but they used follicles with a diameter ≧8 mm [13] and with a diameter ≧10 mm [22]. The difference in the percentages of E_2-A-dom, E_2-dom and P_4-dom may be due to the differences in the selection category of the large follicle.

No significant difference was detected in the number of small antral follicles and the number of oocytes fertilized among E_2-A-dom, E_2-dom and P_4-dom ($P>0.05$). Likewise, no significant difference in the developmental capacity of the fertilized oocytes was detected among these three groups ($P>0.05$). And individual variations in the yield of oocytes and the number of developmental capacity were observed in each group. Smith and colleagues [13] also reported that there were no differences in the developmental capacity of the oocytes, obtained from small follicles (2 to 7 mm in diameter), taken in the presence versus the absence of a large estrogen-active dominant follicle (≧8 mm in diameter) in accordance with our results.

In coclusion, results of the present study show that in cattle E_2 and P_4 in a dominant follicle do not affect the quality of oocytes in small antral follicles. Individual variations in the number of oocytes and the rate of embryonic development may be caused by hormone(s) other than E_2 and P_4, growth factors, unknown factor(s) or a more complex mechanism *in vivo*.

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