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Effect of Luteal Morphology of Donors on the Maturation and Subsequent Development *in Vitro* of Bovine Immature Oocytes

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ABSTRACT

The nuclear maturation and developmental competence of immature oocytes collected from donors at various morphology of corpus luteum (CL) and fertilized *in vitro* was investigated by comparing the meiotic activity and the yields of embryos. Ovaries were divided and classified into 4 groups as the following criteria: Group 1 ; ovaries showed evidence of recent ovulation (corpus hemorragicum). Group 2 ; apex of CL was red or brown. Vasculization was limited to periphery of CL. Group 3 ; apex of CL was orange or tan. Vasculization was covered over apex of CL. Group 4 ; CL was light yellow to white and firm in texture and the vascular network on the surface of CL had disappeared. Modified TCM 199 was used for maturation *in vitro* of immature oocytes and development was induced by using TLP-PVA as a basic medium. When oocytes collected from each group of donors had been matured for 4, 14, and 24 hours *in vitro*, the proportion of oocytes reaching metaphase I and metaphase II were not different among oocytes from 4 group of ovaries. Mature metaphase II stage of oocytes in each group was first observed at 14 hours, whereas completion of maturation of oocytes in each group was at 24 hours. Luteal morphology of ovaries had little effect on the proportion of embryos reached 2 cells and 8 cell stage. However, the proportion of embryos cleaved to morula and blastocyst stage was significantly higher in the oocytes obtained from group 1 and 3 than in the oocytes from group 2 and 4 ($p<0.05$). This data suggest that reproductive status of the donor significantly influences the yield of *in vitro* embryos.

(Key words: IVM, Luteal morphology, Ovary)

Ⅰ. INTRODUCTION

Oocyte maturation, characterized by germinal vesicle break-down (GVBD), formation of the first meiotic spindle (metaphase Ⅰ), expulsion of the polar body and arrest in metaphase of the second meiotic division (metaphase Ⅱ), occurs in preovulatory follicles in response to the surge of gonadotrophins and leads to an ovulated fertilizable oocyte. Mammalian oocytes released from follicular environment are able to undergo spontaneous nuclear maturation *in vitro* (Pincus and Enzmann, 1935). However, oocytes that reach metaphase Ⅱ *in vitro* are not necessarily competent to support normal fertilization and further embryonic development (Moor and Trounson, 1977). Many factors influencing the rate of success of producing bovine embryos *in vitro* have been investigated. Culture conditions used for *in vitro* maturation of bovine oocytes significantly influence *in vitro* fertilization and subsequent embryo development (Lonergan et

al., 1994). The use of a selected population of oocytes and culture media which support the resumption of nuclear and cytoplasmic maturation are essential for the attainment of advanced embryo stages (Hawk and Wall[a,b], 1994). It is possible that the intra-follicular environment to which oocytes are exposed is a major cause of the variability in developmental competence of the oocytes (Callesen et al., 1986). Healthy large antral follicles in cattle contain high oestradiol concentration, whereas atretic follicles contain higher progesterone or androgen concentration (Ireland and Roche, 1983) and dimeric inhibin (Hopko Ireland et al, 1994; Guibault et al, 1993). However, most oocytes collected for *in vitro* fertilization are recovered from small antral follicles. In cattle, ovaries obtained from local slaughter house constitute an economical source of oocytes, although the quality of these oocytes is highly variable. Ovaries are collected at random without any information on cow origin or on reproductive status.

The aim of the present study is to test the effect of ovarian luteal morphology on the meiotic and developmental capacity of oocytes *in vitro*.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation

The oocyte maturation medium was a glucose-free tissue culture medium (TCM)-199 (with Earle's salts) buffered with 25mM N-2-hydroxy- ethyl-piperazine-N-2-ethane salphonic acid (Hepes), and designated as modified (m) TCM-199. Cow ovaries from random breed were collected immediately post mortem at a local slaughterhouse and transported to the laboratory in 0.9% NaCl solution at 30 to 35℃ within 2 hours. Follicular oocytes were aspirated from small follicles (3～5 mm) using a 18-gauge hypodermic needle attached to a 10-ml disposable syringe, then pooled and allowed to settle in 10-ml test tube. The supernant was discarded and sediment were deposited to a little amount of a maturation medium on a watchglass. By use of a low-power (40×), only cumulus-intact oocytes with evenly granulated and pigmented cytoplasm were selected from follicular fluid with a fine-tip pipette. The oocyte-cumulus complexes (OCCs) were washed three time in maturation media. A group of 30 to 50 randomly selected oocytes were transferred into 50 μl maturation medium, mTCM-199 supplemented with 5.56mM glucose, 5mM hemicalcium lactate, 0.4mM sodium pyruvate, 10% (v/v) heat-treated (56℃, 30 min) fetal bovine serum (Sigma), 60 μg sodium penicillin G/ml and 100 μg streptomycin sulfate/ml. Oocytes were cultured for various time periods according to different experiment at 39℃ under an atmosphere of 5% CO_2－95% air with high humidity.

2. *In Vitro* Fertilization

The medium used for the treatment of spermatozoa and fertilization of oocytes was essentially the same as that used by Brackett and Oliphant (1975) for the fertilization of rabbit eggs *in vitro*, except that BSA and glucose were not added. The medium, designated as BO medium, was composed of 112.0 mM NaCl, 2.25 mM $CaCl_2$, 0.83 mM NaH_2PO_4, 0.52 mM $MgCl_2$, 37.0mM $NaHCO_3$, 1.25 mM sodium pyruvate and 31 μg sodium penicillin G/ml. The OCCs contained in each drop of maturation medium were removed, washed four times and introduced into 50 μl drop of BO medium supplemented with 20mg BSA (crystallized and lyophilized, essentially fatty acid-free, Sigma)/ml and 20 μg porcine intestinal mucosal heparin (181 USP units/mg; Sigma)/ml under paraffin oil in a polystyrene culture dish. The dishes were kept in a CO_2 incubator (5% CO_2 in air at 39℃) for about 30 minutes until spermatozoa were added.

For each experiment, a 0.5ml straw of frozen semen obtained from Holstein bulls was thawed in a water bath at 37℃ for 1 minute. Spermatozoa were washed twice by centrifugation at 833×g for 10 minutes each after dilution with BO medium supplemented with 10mM caffeine-benzoate (3.383 mg/ml, Sigma). The final sperm pellet was resuspended in the same medium as that used for washing to give a sperm concentration of $2\sim4\times10^6$ spermatozoa/ml. A 50 μl of the sperm suspension was introduced into 50 μl of the medium that included the OCCs for fertilization. The final concentration of the mixture was $1\sim2\times10^6$ spermatozoa/ml, 10mg BSA/ml, 10 μg heparin/ml, and 5mM caffeine and incubated at 39℃ in 5% CO_2 in air with high humidity for 8 hours.

3. *In Vitro* Embryo Development

A chemically defined medium was used for culture of embryos. The basic medium was a modified Tyrode's solution which comprised 110.0 mM NaCl, 3.2mM KCl, 2.0mM $CaCl_2$, 0.5mM $MgCl_2$, 25.0mM $NaHCO_3$, 10.0 mM sodium lactate, 0.5mM sodium pyruvate and 1mg polyvinyalcohol (PVA)/ml and designated as modified (m) TLP-PVA. At 8 hours post-insemination, the oocytes in each drop were stripped of cumulus cell by passing them through a fine-tip pipette and then were washed four times and 10~25 oocytes were placed into 100 μl culture medium under paraffin oil. The oocytes were cultured at 39℃ in an atmosphere of 5% CO_2-95% air with high humidity.

4. Experimental Studies

Experiment 1. the ability of oocytes to complete the first meiotic division *in vitro*.

The meiotic competence of oocytes from various luteal type of the ovaries was examined. Paired ovaries were recovered from cows of unknown reproductive history. Ovaries were divided into 4 groups as described by Ireland et al (1980) and Leibfried and First (1979). They were classified by the following criteria.

1) Group 1

Ovaries showed evidence of recent ovulation. The corpus albicantium of previous estrous cycle could be identified on one of the ovaries. Vasculature on surface of CL was not visible.

2) Group 2

A CL is fully formed with vasculature visible around its periphery. When the CL is bisected, the apex of CL was red or brown; while the remainder is orange or yellow. Diameter of CL was 1.6~2.0 cm.

3) Group 3

Apex of CL was orange or tan. Diameter of CL was same as that of group 2. Vasculization was covered over apex of CL.

4) Group 4

The ovary usually contains at least one large follicle (10 mm or more) and a regressed CL with no vasculature visible on its surface. CL was light yellow to white and firm in texture.

Aspirated oocytes from each group were incubated for 4, 14 and 24 hours. The time required for first polar body extrusion was examined in conjunction with luteal morphology.

Experiment 2. the ability of embryos to develop the bastocyst stage.

The ovaries were placed into 4 group of luteal morphology. Oocyte selection, maturation, fertilization and development were peformed as stated earlier in this section.

5. Statistical Analysis

Statistical analysis was performed with a stan-

dard computerized statistics program using χ^2. $P<.05$ was considered statistically significant.

Ⅲ. RESULTS

Maturational sequence of bovine oocytes from ovaries with various morphology of corpus lueum are shown in Table 1. In control group, 84.1% of oocytes was germinal vesicle breakdown and 15.9% remained germinal vesicle after 4 hours of culture. Percentage of GVBD oocytes in group 1, 2, 3 and 4 was 81.3%, 80.9%, 83.6% and 83.4%, respectively. After 14 hours of culture the proportion of oocytes reached metaphase I in control, group 1, 2, 3 and 4 was 72.9%, 75.0%, 72.3%, 67.5%, and 69.2%, respectively. Mature metaphase Ⅱ stage of oocytes in each group were first observed at 14 hours, whereas completion of maturation of oocytes in each group was at 24 hours. The proportion of oocytes reached metaphase Ⅱ was not significantly different by luteal morphology.

OCCs matured and fertilized *in vitro* were removed from cumulus using pipette of suitable diameter to determine whether luteal morphology of donor affect or not the *in vitro* development. Data for developmental competence by luteal morphology of donor is shown in Table 2. Embryos were examined for the proportion developing to 2 cells, 8 cell, morula and blastocyst after 48, 96, 144 and 192 hours of culture, respectively. When oocytes matured and fertilized *in vitro* were cultured, the proportion of embryos which reached blastocyst stage in control, group 1, 2, 3 and 4 at 192 hours after fertilization was 13.8, 19.5. 12.5, 22.1 and 8.1%, respectively. Although the proportion of embryos reached 2 cells and 8 cell was not significantly different by luteal morphology, proportion of embryos cleaved to morula and blastocyst stage were a significantly higher in the oocytes obtained from group 1 and 3 than in the

Table 1. Effect of luteal morphology of donor on the nuclear maturation *in vitro* of bovine oocytes collected from small follicle after various time of culture

Periods of culture (h)	Luteal morphology	No. of oocytes matured	No. (%) of oocytes			
			GV	GVBD	M Ⅰ	M Ⅱ
4 h	Control	63	10(15.9)	53(84.1)	0(0.0)	0(0.0)
	Group 1	48	9(18.7)	39(81.3)	0(0.0)	0(0.0)
	Group 2	42	8(19.0)	34(80.9)	0(0.0)	0(0.0)
	Group 3	49	8(16.3)	31(83.6)	0(0.0)	0(0.0)
	Group 4	42	7(16.7)	36(83.4)	0(0.0)	0(0.0)
14 h	Control	59	7(11.9)	5(8.4)	43(72.9)	4(6.8)
	Group 1	40	3(7.5)	3(7.5)	30(75.0)	4(10.0)
	Group 2	47	5(10.6)	5(10.6)	34(72.3)	3(6.4)
	Group 3	40	4(10.0)	6(15.0)	27(67.5)	3(7.5)
	Group 4	52	5(9.6)	6(11.5)	36(69.2)	5(9.6)
24 h	Control	61	6(9.8)	5(8.2)	6(9.8)	44(72.2)
	Group 1	49	4(8.2)	2(4.1)	7(14.3)	36(73.4)
	Group 2	47	5(10.6)	4(8.5)	3(6.4)	35(74.5)
	Group 3	55	6(10.9)	5(9.1)	6(10.9)	38(69.1)
	Group 4	67	7(10.4)	6(9.0)	8(11.9)	46(68.7)

Table 2. Effect of luteal morphology of donor on the *in vitro* development of *in vitro* matured/fertilized bovine oocytes collected from small follicles

Luteal morphology	No. of oocytes inseminated	No. (%) of embryos developed			
		2-cell [48]†	8-cell [96]†	Morula [144]†	Blastocyst [192]†
Control	152	106(69.7)	75(49.3)	49(32.2)^{a,b}	21(13.8)^{a}
Group 1	112	81(72.3)	62(55.4)	44(39.3)^{a}	22(19.5)^{b}
Group 2	125	84(67.2)	67(53.6)	36(28.8)^{b}	16(12.8)^{a}
Group 3	154	104(67.5)	88(57.1)	64(41.6)^{a}	34(22.1)^{b}
Group 4	132	83(62.9)	63(47.7)	26(19.7)^{c}	8(6.1)^{c}

†Numbers in this parenthesis indicate the time of examination (hours after insemination).
Values with different superscripts in the same column are significantly different($p<0.05$).

oocytes from group 2 and 4.

Ⅳ. DISCUSSION

The study presented here examined the maturational and subsequent developmental capacity of oocytes from small bovine follicles in relation to the luteal morphology of donor.

Although the size of oocytes remains unchanged during the growth of follicles between 2 to 7 mm, oocytes aspirated from follicles larger than 6 mm in diameter yield a significantly higher proportion of blastocysts than do those from smaller follicles. Larger follicles contain growth factors which enhance the morphological and functional status of the oocytes and the yield of embryos (Tan and Lu, 1990; Lonergan et al., 1994). Bovine large antral follicles have high concentration of oestradiol, whereas atretic follicles contain high progesterone or androgen concentration (Grimes et al., 1987; Spicer et al., 1987) and higher level of dimeric inhibin (Hopko Ireland et al., 1994). However, most oocytes used for *in vitro* fertilization are collected from small antral follicles (3~7mm). Small follicles obtained during the early luteal phase contain less testosterone than do follicles obtained during the late luteal phase (Kruip and Dielman, 1985), whereas small and medium follicles have higher oestradiol concentration in the early compared with the late luteal phase (Spicer and Zinn, 1987). Degeneration of follicles is accompanied by a decrease in oestradiol concentration and by an increase in progesterone concentration (Smith et al., 1996; Kruip and Dielman, 1985).

In agreement with a previous study (Leibfried-Rutledge et al., 1985), this study shows that there was no significant difference in the yield of MⅡ oocytes among oocytes collected from ovaries with different luteal morphology. Although the highest proportion of oocytes with degenerate features are found in the follicular stage, the ability to undergo nuclear maturation *in vitro* after removal from follicle is not dependent on either size of follicle or stage of cycle (Leibfried and First, 1979). Results showing that *in vitro* oocyte maturation was independent of the estrus cycle were also obtained by Fukui and Sakuma (1980). The results of this and previous studies indicate that the maturational competence *in vitro* of bovine oocytes collected from small follicles is independent of the luteal morphology. The initiation of maturation in cumulus-enclosed bovine oocytes is independent of exogenous amino acid, proteins and hormones as long as oocytes are cultured in the medium with

optimal concentration of energy substrate, inorganic ions, pH, osmolarity and 5% CO_2(Lim et al., 1997). However, hormonal supplementation, gonadotropin and estradiol-17β during bovine oocyte maturation *in vitro* enhance the subsequent fertilizability and developmental ability (Sakei et al., 1991).

Although there was no significant difference in the early cleavage rate of *in vitro* fertilized oocytes among oocytes collected from ovaries with different luteal morphology, the yield of morula and blastocyst was significantly higher in oocytes from group 1 and 3 ovaries than those from group 2 and 4 ovaries. It is important that earlier cleavage stages should not be used as a measure of normal maturation since many abnormalities induced during culture are not detectable until morula and blastulation.

According to Ireland et al. (1980), the point of rupture of a follicle following ovulation remain uncovered by epithelium for 4 to 5 days. After the point of rupture is closed, the apex of the CL remain red or brown for an additional 5 days. Then a vascular network is on the surface of CL, and the bisected CL appear bright orange or yellow. The length of this feature is 7 days. Now the vascular network on the surface of the CL has disappeared and ovary has a large dominant follicle. I consider that group 1, group 2, group 3, and group 4 of ovaries in this study represent day 1 through 4, days 5 to 10, day 11 through 17, and day 18 through 20 of an estrus cycle, respectively. This data suggest that developmental ability of *in vitro* matured and fertilized bovine oocytes was significantly higher in oocytes obtained from day 1 through 4 and/or day 11 through 17 of an estrus cycle than in those obtained from estrus cycle day 5 to 10 day and/or day 18 through 20. A similar finding that a proportion of oocytes developed into blastocyst and their developmental speed are significantly enhanced in oocytes obtained at the end of the luteal phase (day 14 to 16) has been reported (Machatkova et al., 1996). It has been also reported that the development rates decrease from cyclic with corpus luteum, cyclic without CL, prepubertal heifer to pregnant females (Thonon et al., 1993).

Follicular dynamics during the estrus cycle of the majority of cattle is characterized by the growth of 2 or 3 dominant follicles. In cycle with 2 or 3 dominant follicles the first dominant follicle is detected on average on Day 4, reach a maximum size on Day 6, go through a period of relative stability between Day 6 and 10, then begin to decrease in size. In cycle with 3 dominant follicles the second dominant follicle is detected by Day 12, reach maximum size on Day 16 and the third (ovulatory) follicle is identified on average by Day 16 and maximum size is reached on Day 21 (Savio et al., 1988). It is expected that after emergence of the dominant follicle, the small subordinate follicles would show signs of atresia. Small follicles obtained before emergence of the dominant follicle (days 3 of the estrous cycle) contain significantly more oestradiol than they do after emergence (days 6 and 7 of estrous cycle)(Smith et al, 1996). The number and growth of subordinate follicle are inversely proportional to the growth of the dominant follicle present on the ovary on estrus cycle day 7 to 9 and 18 to 2. Follicles in the growth phase are expected to be nonatretic and, therefore, produce high-quality oocytes with high developmental competence. The importance of timing hormonal treatment to minimize the suppressive influence of the dominant follicle on superstimulatory response and the yield of embryos has been reported. Ovarian response and embryo yield are significantly decreased when the superovulatory treatment is started in the presence of the dominant follicle (Adams et al., 1994).

However, these overall data are at variance with those of Ariotto et al. (1966) and Smith et al.

(1996), who suggested that the developmental competence of bovine oocytes from small antral follicles was not affected by the presence of a dominant follicle and by the stage of the estrus cycle, respectively. I consider that this difference may had been caused by the addition of oviductal epithelial cell, hormone and bovine serum into the medium for development, whereas the medium for development in this study was chemically defined, protein-free.

This study clearly shows that reproductive status of a donor significantly influences the rate of development of *in vitro* embryos. These data suggest that a better selection of collected donor in the slaughterhouse may increase the rates of success in embryo production *in vitro*.

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요 약

소 미성숙난자의 체외성숙과 배발생에 황체의 형태가 미치는 영향

김 병 기

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본 연구는 공여난소의 황체 형태가 소 미성숙난자의 체외 성숙과 발생에 미치는 영향을 감수분열과 수정란 생산을 비교하여 조사하였다. 공여난소를 황체의 형태에 따라 다음과 같이 4 group으로 분류하였다. Group 1; 최근에 배란한 흔적으로 보이는 혈포가 있는 난소. Group 2; 황체는 적색 또는 갈색이며, 혈관이 황체의 가장자리에 국한되어 분포하고 있는 난소. Group 3; 황체는 오렌지 또는 황갈색이며, 혈관이 황체의 정상부 까지 분포한 난소. Group 4; 황체는 엷은 황색 또는 백색이며, 황체 정상부의 혈관 분포가 사라진 난소. 미성숙난자의 체외성숙과 배발생을 위하여 각각 TCM 199과 TLP-PVA을 기본배지로 사용하였다. 각 group에서 채취한 미성숙 난자를 4, 14, 24시간 체외성숙시켰을 때 metaphase Ⅰ과 metaphase Ⅱ에 도달한 난자의 비율은 각 group 사이에 차이가 없었으며, 각 group에서 metaphase Ⅱ 난자는 체외성숙 후 14시간에 나타나기 시작하여 24시간에 성숙이 종료하였다. 체외 배발생 후 2세포기와 8 세포기까지의 발생에는 공여난소 황체의 형태가 영향을 미치지 않았으나, 상실배와 배반포로 발생한 수정란의 비율은 group 1과 3에서 채취한 난자에서 group 2와 4에서 채란한 난자에 비하여 유의적으로 증가하였다($p<0.05$). 이러한 결과는 공여난소의 번식상태가 체외 수정란생산에 유의적인 영향을 미치는 것으로 암시하고 있다.

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