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Further studies on factors affecting in-vitro maturation of extra-follicular bovine oocytes

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Abstract

Five experiments were conducted to investigate factors affecting in-vitro maturation of extra-follicular bovine oocytes. Oocytes recovered from follicles of 2–5 mm in diameter were cultured in Ham's F-12 based medium for 28 h. Among three culture methods examined, the culture in small test tubes kept in a CO_2 gas incubator with the condition of 5% CO_2 in air and 95% humidity resulted in the highest rate of oocyte maturation (60.6%), although there was no significant differences among the culture methods. The use of 0.2% trypan blue stain for viability of immature oocytes before culture was effective for elimination of abnormal or dead oocytes. However, it may be not necessary, because not only cumulus cell mass surrounding oocytes, but also zona pellucida which was partly stained could be harmful for post-culture viability. A fully expanded cumulus cell mass and a high stretchability (>5 mm) resulted in a high rate of oocyte maturation. It therefore indicates that evaulations of expansion and stretchability of cumulus cell mass in post-cultured oocytes is useful to estimate their maturity.

Introduction

It has been reported that in-vitro maturation of bovine oocytes is affected by many factors, such as medium (FUKUI et al., 1981), culture duration (FUKUI et al., 1985), the presence of cumulus cells (SATO et al., 1977; LEIBFRIED and FIRST, 1979; FUKUI and SAKUMA, 1980), and additional nutrients (FUKUI et al., 1981) or hormones (FUKUI et al., 1982; FUKUSHIMA and FUKUI, 1985) into medium. Besides the various culture methods mentioned above, a test tube culture system (WHITTEN, 1956; FUKUI and SAKUMA, 1980) and nonstatic culture method (STAIGMILLER and MOOR, 1984) have been successfully attempted to resume the meiosis of mammalian oocytes.

Viability of immature oocytes recovered from follicles is also one of the important factors. McGAUGHEY (1978) grossly classified

the porcine oocytes "good" to "poor", but the reality of live or dead in those oocytes was unknown. It has been demonstrated that the presence of a dense layer of cumulus cells around the oocyte is related to successful maturation in-vitro after culture (LEIBFRIED and FIRST, 1979; FUKUI and SAKUMA, 1980). It has been also argued that more than half the population of the follicles presented in ovaries are atretic, although this depends on their sizes and steroidgenesis (KRUIP and DIELEMAN, 1982; MOOR et al., 1984). FULKA et al. (1982) reported that a cytoplasmic substate responsible for male pronucleus formation after in-vitro fertilization is present in about 50% of randomly-selected bovine oocytes. Therefore, quality evaluation of oocytes and the careful elimination of obviously degenerate or denuded oocytes would be an initial step for high proportion of maturation after in-vitro culture (FUKUI et al., 1981). As an alternative mean for the evaluation, BALL et al. (1982) and LENZ et al. (1983) used a trypan blue stain and cultured only unstained, live cumulus-enclosed bovine oocytes.

Furthermore, not only the selection of viable oocytes before culture, but also the selection of truly matured oocytes after in-vitro culture is another important factor affecting subsequent fertilizability. At the present, all cultured oocytes have been utilized for studies of fertilization in-vitro or in-vivo resulting in variable results. Fertilization in-vitro or in-vivo of bovine oocytes could be improved if only matured oocytes were used following evaluation of cultured oocytes. From these respects, an accurate classification of matured oocytes following in-vitro culture has been strongly required in raw materials without fixation or staining procedures. In human oocytes, TESTART et al. (1983) used expansion and stretchability of cumulus cell mass as the criteria of maturity and obtained correlated results in in-vitro fertilization. Although the expansion status of cumulus cell mass after culture has been generally used as a criterion of maturity of bovine oocytes, there are few comparable studies of degrees of expansion and stretch-ability of cumulus cell mass and proportion of matured oocytes.

In the present study, five experiments were conducted to investigate further factors affecting oocyte maturation in cattle; culture methods (experiments 1 and 2), a trypan blue stain for evaluating the viability of immature oocytes before culture (experiments 2 and 3), and the maturity of cultured oocytes by a clasification of expansion (experiment 4) and stretchability (experiment 5) of cumulus coll mass.

Materials and Methods

General

Bovine ovaries were obtained from a local slaughter-house and returned to the laboratory in 0.9% (v/v) saline at 35–37°C within 2 h. Follicular oocytes were aspirated from follicles of 2–5 mm in diameter by 21-gauge needle. Only oocytes completely surrounded by cumulus cells were used for following experiments. The culture medium used was a Ham's F-12 containing 15% (v/v) fetal calf serum (FCS), 1 μg/ml follicle-stimulating hormone (FSH: NIAMOD-0 FSH-15, NIH, U.S.A.), 5 μg/ml luteinizing hormone (LH: NIADDK-0 LH-25, NIH, U.S.A.), 1 μg/m; esteadiol (Ova-hormone benzoate, Teikoku-zoki Co., Japan) and antibitotics (100 i.u./ml penicillin G and 50 μg/ml streptomycin). This has been described in more detail elsewhere (FUKUI and SAKUMA, 1980; FUKUSHIMA and FUKUI, 1985).

Experiment 1

Three culture systems were investigated. Method A was the same as in the previous reports (FUKUI and SAKUMA, 1980; FUKUSHIMA and FUKUI, 1985). In breif, 5–10 follicular

oocytes were placed into a small test tube (60 mm in length and 10 mm in diamter) containing 1 m*l* of medium. After gassing with 5% CO_2 in air for 1 min, the test tubes were tightly capped and kept in a normal incubator at 39°C for culture. In method B, the capped test tubes containing oocytes were set at a nonstatic, gentle agitated water bath at 39°C and each tube was connected by a polyethylene tube (o. d. 1.3 mm) with 22-gauge needles at both ends. A gasphase of 5% CO_2 in air was continously flowed into the tubes during culture. For method C, the test tubes loosely capped were placed in a CO_2 gas (5% CO_2 in air and 95% humidity) incubator (MCO-165, SANYO).

Experiment 2

Immediately after recovery from follicles, cumulus-enclosed oocytes were stained with 0.2% trypan blue solution for 2 min to determine their viability. The stained and unstained oocytes were washed twice in the medium and separately cultured by the three methods as in experiment 1.

Experiment 3

The present experiment was conducted to test the hypothesis that not staining, live cumulus-enclosed oocytes following 0.2% trypan blue stain results in a higher rate of maturation than that in control oocytes without the stain treatment, because low quality, dead oocytes can be eliminated for culture. Both unstained and control oocytes were cultured separately in the CO_2 gas incubator with the gasphase of 5% CO_2 in air.

Experiment 4

Degrees of expansion of cumulus cell mass following culture were classified into four groups; "+++" : > 80% of the cell mass is expanded, "++" : 40-60% is expanded, "+" : 10-30% is expanded, and "−" : small sized cumulus with < 10% of the cell mass is expanded. The maturation stages of all grouped thereafter.

Experiment 5

Stretchability of cumulus cell mass surrounding oocytes after culture was investigated. Each cumulus-enclosed oocyte was placed on a slide-glass with a small volume of medium. At observation under strep-microscopy, a part of cumulus cell mass was picked up gently with a 27-gauge needle to the maximum height, and the distance (stretch-ability) between the site of oocyte and the peak of stretch was measured. The stretchability of cumulus cell mass was then classified into four groups; A: > 10 mm, B: 5-10 mm, C: 1-5 mm, and D: < 1 mm.

Oocyte examination

After terminating the culture (experiments 1, 2, and 3) and classifying the morphyological types of cumulus cell mass (experiments 4 and 5), all oocytes were fixed with acetic alchohol (1 : 3) for 1-2 days and stained with 1% aceto-orcein. Stages of the meiotic division in each oocyte were determined by the phase-contrast microscopy following the methods of the previous studies (FUKUI and SAKUMA, 1980; FUKUI et al., 1985). Oocytes were considered matured when they reached metaphase II of meiosis. To analyze proportions of matured oocytes, a one-way analysis of variance was performed by chi-square test or Duncan's new multiple range test (STEEL & TORRIE, 1960).

Results

Experiment 1

A total of 541 oocytes were cultured by methods A, B and C, and the proportions of matured oocytes were 56.0% (102/182), 53.7% (107/195) and 54.9% (88/164), respectively. There were no significant differences in maturation rates among the three culture methods.

Experiment 2

The proportions of matured oocytes following the trypan blue stain were shown in Table 1.

The maturation rates in the unstained oocytes were significantly ($P < 0.01$) higher than those of stained oocytes in all culture methods. From the resulting extremely low maturation rates of stained oocytes (7.5–8.0%), it was shown that viability of follicular oocytes could be estimated by the trypan blue stain. Although there were no significant differences in maturation rates among the three culture methods, the culture in the CO_2 gas incubator resulted in the highest rate of maturation (60.6%).

Experiment 3

A total of 295 oocytes were cultured. The culture of unstained oocytes did not improve maturation rate as compared with that in control oocytes. The maturation rate of 131 unstained oocytes was rather lower than that in 164 control oocytes (49.6% and 53.7%, respectively).

Experiment 4

The results are shown in Table 2. A total of 260 cultured oocytes were classified into four groups by degrees of expansion of cumulus cell mass. Proportions of oocytes with "+++" to "−" of expansion statuses were 38.1%, 27.3%, 19.2% and 15.4%, respectively. The proportions of matured oocytes varied with the degrees of expansion of cumulus cell mass. The oocytes with highly expanded cumulus cell mass (+++ and ++) showed significantly ($P < 0.01$) higher maturation rates (59.1% and 50.7%) than those surrounded with "+" cumulus cells (36.0%) or non-expanded cells (12.5%). There was no significant difference in maturation rate between oocytes with "+++" and "++" cumulus cell mass.

Experiment 5

The results are shown in Table 3. A total of 127 cultured oocytes were classified into four groups by stretchability of cumulus cell mass. Number of oocytes with > 10 mm in stretchability was only 5 of 127 oocytes, but all oocytes had matured. The oocytes with the lowest stretchability (D) resulted in only 22.7% of maturation rate which was significantly ($P < 0.01$) lower than the other groups. Although the maturation rates in groups B and C were not significantly different, the oocytes with 5–10 mm in stretchability showed a higher rate of maturation than that in the oocytes with 1–5 mm in stretchability (62.8% and 52.6%).

Discussion

The present three culture methods did not affect oocyte maturation in-vitro; a CO_2 gas incubator seemed, however, to be superior to other methods. Among the factors investigated in the present study, it was shown that evaluation for viability of immature bovine follicular oocytes by the trypan blue stain is useful to eliminate abnormal or dead oocytes before culture. The effect of trypan blue stain agrees with other reports (BALL et al., 1982; LENZ et al., 1983). However, in the present study, unstained oocytes did not increase maturation rate as compared with those cultured without stain (control oocytes: experiment 3). It appears that the stain procedures with the trypan blue may be harmful for the subsequent maturity, although a lower concentration (0.2%) and shorter incubation time (2 min) were employed in the present study than the other reports (0.4% and 5 min: BALL et al., 1982; LENZ et al., 1983). Among the oocytes considered unstained, not only cumulus cells surrounding oocytes but also zonae pellucidae were partly stained. The concentration and the incubation time may be critical for trypan blue stain, so that more details should be investigated further for improved procedures because it could be applicated for not only the viability of immature oocytes, but also maturity of post-cultured oocytes (MARUSKA et al., 1984).

To achieve a constant level of oocyte matu-

ration in-vitro, the first step is the selection of normal oocytes surrounded with dense layer of cumulus cells at the pre-culture (MCGAUGHEY, 1978; FUKUI et al., 1981). Secondly, maturity of each oocyte following culture should be known without fixation or staining procedures. In mammals including cattle, FSH appears to be the primary gonadotropin responsible for expansion (BALL et al., 1980) and mucification (SZOLLOSI, 1980) of cumulus cells and cyclic 3', 5'-adenosine monophosphate (cAMP) is also the second messenger (BALL et al., 1982). In conditions of in-vivo, the expansion of cellular investments surrounding bovine oocytes are removed by oviductal components such as estrogen or progesterone, probably not by hyaluronidase, one of sperm acrosomal enzymes (LORTON and FIRST, 1979). The results in experiments 4 and 5 showed that degrees of expansion and stretchability of cumulus cell mass could be criteria to determine the maturity of cultured bovine oocytes. Especially, the evaluation of stretchability was encouraging. Of the oocytes with >5 mm in stretchability of cumulus cell mass, two-third of the oocytes had matured. TESTART et al. (1983) classified human oocytes by cumulus appearance, and reported that oocytes having a fully expanded cumulus cell mass and a stretchability of greater than 30 mm showed significantly higher incidence of in-vitro fertilization than those less expanded and having lower stretchability (<30 mm) of the cell mass (78.6% and 30.8%, respectively). In the present study with bovine oocytes, a stretchability of greater than 10 mm was only found in 5 of 127 oocytes examined. This was probably caused by different sources of oocytes and methods of measurement for stretchability. TESTART et al. (1983) used human oocytes recovered from preovulatory follicles following clomiphene citrate and gonadotropin treatments. In our study, a part of cumulus cell mass was picked up with the tip of the needle, and measured for maximum height. However, values of stretchability could be increased by a transverse pulling of the cumulus cell mass in oocytes placed on the slide-glass with a small volume of medium.

As concluded, pre-culture evaluation for quality of bovine follicular oocytes using a trypan blue stain appears to be time-consuming and the question of the harmfulness for subsequent maturity remains to be solved. Post-culture evaluation of expansion and stretchability in cumulus cell mass surrounding oocytes could be useful to determine the maturity of bovine oocytes.

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References

1) BALL, G. D., R. L. AX and N. L. FIRST (1980): Mucooly-saccharide synthesis accompanies expansion of bovine cumulus-oocyte complexes in vitro. In: Functional Correlates of Hormone Receptors in Reproduction, Eds. V. B. Mahesh, T. G. Muldoon, B. B. Saxena and W. A. Sadler, pp. 561–563, Elsvier/north-Holland, Amsterdam.

2) BALL, G. D., M. E. BELLIN, R. L. AX and N. L. FIRST (1982): Glycosaminoglycans in bovine cumulus-oocyte complexes: morphology and chemistry. Molec. Cell. Endocr. **28**: 113–122.

3) FUKUI, Y., M. FUKUSHIMA and H. ONO (1985): In vitro maturation of bovine oocytes recovered from follicles of various sizes. Res. Bull. Obihiro Univ., Ser. I **14**: 211–217.

4) FUKUI, Y., M. FUKUSHIMA, Y. TERAWAKI and H. ONO (1982): Effect of gonadotropins, steroids and culture media on bovine oocyte maturation in vitro. Theriogenology **18**: 161–175.

5) FUKUI, Y., S. ISHIDA, Y. TERAWAKI and H. ONO (1981): Effects of synthetic media and nutritional sources on in vitro maturation of bovine follicular oocytes. Res. Bull. Obihiro Univ., Ser. I **12**: 185–193.

6) FUKUI, Y. and Y. SAKUMA (1980): Maturation of bovine oocytes cultured in vitro: Relation to ovarian activity, follicular size and the presence or absence of cumulus cells. Biol. Reprod. **22**: 669–673.

7) FUKUSHIMA, M. and Y. FUKUI (1985): Effects of gonadotropins and steroids on the subsequent fertilizability of extrafollicular bovine oocytes cultured in vitro. Anim. Reprod. Sci. **9**: 323–332.

8) FULKA, J., Jr., A. PAVLOK and J. FULKA (1982): In-vitro fertilization of zona-free bovine oocytes matured in culture. J. Reprod. Fert. **64**: 495–499.

9) KRUIP, Th. A. M. and S. J. DIELEMAN (1982): Macroscopic classification of bovine follicles and its validation by micromorphological and steroid biochemical procedures. Reprod.Nutr. Dévelop. **22**: 465–473.

10) LEIBFRIED, L. and N. L. FIRST (1979): Characterization of bovine follicular oocytes and their ability to mature in vitro. J. Anim. Sci. **48**: 676–86.

11) LENZ, R. W., G. D. BALL, J. K. LOHSE, N. L. FIRST and R. L. AX (1983): Chondroitin sulfate facilities an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and in vitro fertilization. Biol. Reprod. **28**: 683–690.

12) LORTON, S. D. and N. L. FIRST (1979): Hyaluronidase does not disperse the cumulus oophorus surrounding bovine ova. Biol. Reprod. **21**: 301–308.

13) MARUSKA, D. V., M. L. LEIBFRIED and N. L. FIRST (1984): Role of calcium and the calcium-calmidulin complex in resumption of meiosis, cumulus expansion, viability and hyaluronidase sensitivity of bovine cumulus-oocyte complexes. Biol. Reprod. **31**: 1–6.

14) MCGAUGHEY, R. W. (1978): In vitro oocyte maturation. In: Methods in Mammalian Reproduction, Ed. J. C. Daniel, Jr., pp. 1–19, Academic Press, New York.

15) MOOR, R. M., Th. A. M. KRUIP and D. GREEN (1984): Intraovarian control of folliculogenesis: Limits to superovulation? Theriogenology **21**: 103–116.

16) SATO, E., A. IRITANI and Y. NISHIKAWA (1977): Factors involving in maturation of pig and cattle follicular oocytes cultured in vitro. Jpn. J. Anim. Reprod. **23**: 12–18.

17) STAIGMILLER, R. B. and R. M. MOOR (1984): Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicles. Gamete Res. **9**: 221–229.

18) STEEL, R. G. D. and J. H. TORRIE (1960): In: Principles and Procedures of Statistics. Eds. R. G. D. Steel and J. H. Torrie, McGraw-Hill Book Co., New York.

19) SZOLLOSI, D. (1980): Interaction between oocyte and follicle in vivo. Proc. 9th Int. Congr. Anim. Reprod. & A. I., Madrid **2**: 95–99.

20) TESTART, J., B. LASSALLE, R. FRYDMAN and J. C. BELAISCH (1983): A study of factors affecting the success of human fertilization in vitro. II. Influence of semen quality and oocyte maturity on fertilization and cleavage. Biol. Reprod. **28**: 425–431.

21) WHITTEN, W. K. (1956): Culture of tubal mouse ova. Nature, Lond. **177**: 96.

Table 1. In-vitro maturation rates of bovine oocytes stained by 0.2% trypan blue and cultured by three different methods

Culture method	% of matured oocytes	
	Un-stained	Stained
A	37.8[a] (37)*	7.5[b] (40)*
B	52.6[a] (19)	8.0[b] (25)
C	60.6[a] (30)	7.8[b] (51)

a, b: The figures with different superscripts in the same line are statistically significant ($P<0.01$).
*: The figures in parenthesis are numbers of oocytes examined.

Table 2. Relationships between degrees of expansion of cumulus cell mass and maturation rate after culture of bovine oocytes

Degrees of expansion of cumulus cell mass	No. of oocytes examined	No. of oocytes matured (%)
+++	99	59 (59.1)[a]
++	71	36 (50.7)[a]
+	50	18 (36.0)[b]
−	40	5 (12.5)[c]
Total	260	128 (49.2)

a, b, c: The figures with different superscripts are statistically significant ($P<0.01$).

Table 3. Relationships between stretchability of cumulus cell mass and maturation rate after culture of bovine oocytes

Ranks of stretchability of cumulus cell mass	No. of oocytes examined	No. of oocytes matured (%)
A (>10 mm)	5	5 (100)[a]
B (5–10 mm)	43	27 (62.8)[b]
C (1–5 mm)	57	30 (52.6)[b]
D (<1 mm)	22	5 (22.7)[c]
Total	127	67 (52.8)

a, b, c: The figures with different superscripts are statistically significant ($P<0.01$).

ウシ卵胞卵子の体外成熟に及ぼす種々の要因に関する研究

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摘　　要

ウシ卵胞卵子（第一次卵母細胞）の体外成熟に及ぼす種々の要因に関して5つの実験を行なった。直径2～5mmの卵胞から採取された卵子をHam F-12を基礎培地とする培養液で28時間培養した。

実験1では，3種の培養方法（A法：5～10個の卵子を1m*l*の培養液が入った小試験管に5% CO_2 in airを1分間注入し，ふ卵器内で培養，B法：同小試験管を37°Cの振とう恒水槽に設置し，5% CO_2 in airを常時通気して培養，C法：同小試験管を5% CO_2 in airに調整した炭酸ガス培養装置内で培養）を用いた。3種の方法による体外成熟率に有意差は認められなかった（実験1および2）が，方法3で最高の成熟率（60.6%）が得られた。実験2，3では，培養前の卵子の生存性を検討するため，0.2%トリパンブルー染色（2分間）を行なった。染色卵子の成熟率（7.5～8.0%）は不染卵子のそれ（37.8～60.6%）と比べて有意に（$P<0.01$）低かった。しかし，不染卵子のみと染色を行なわないで培養した卵子の成熟率（49.6%，53.7%）に有意差が認められなかった（実験3）ことから，不染（生存）卵子と判定された卵子は同染色により何らかの悪影響を受けたものと思われた。実験4，5では，培養後の卵子成熟度を判定するために卵丘細胞層の膨潤化（実験4）と伸長度（実験5）について検討した。両実験結果より，卵丘細胞層の膨潤化の程度が大きく（"++"以上），伸長度が＜5mmの卵子の成熟率はそれ以下のものより高かった。よって，卵丘細胞層の膨潤化と伸長度は培養後の卵子が成熟しているか否かを判定する指標になるものと考えられた。

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