Studies on the Effects of Follicular Fluid and its Fractions on the Cow Oocyte Maturation *in Vitro*

Wan Kyoo Cho and Kwang Ja Lim

(Dept. of Zoology, Seoul National University)

濾胞液과 그의 分劃이 암소 濾胞卵子의
成熟에 미치는 영향에 관한 연구

趙 完 圭 · 林 光 子

(서울大 自然大 動物學科)

적 요

여포액이 여포안에서는 난자의 성숙을 억제하지만 여포액을 일단 여포 밖으로 내어 배양액을 섞거나 혹은 여포액만으로 난자를 배양할 때 난자는 성숙분열을 일으키는 것을 보아 왔다. 저자들은 소의 여포액 내의 성분중 어떤 것 들이 난자 성숙에 유효한 가를 밝히기 위하여 본 실험을 행하였다. 그 결과는 다음과 같다.

첫째, 여포액을 투석하여서 얻은 dialysable fraction을 이용하여 난자를 배양 하였을 때 높은 성숙율을 얻었다. 반대로 non-dialysable fraction를 포함하는 기본배양액 내에서는 난자의 성숙율은 극히 낮았으며 퇴화하는 경향이 있었다.

둘째, paper chromatography의 방법으로 dialysable fraction이 포함하는 free amino acid를 조사한 결과 8가지의 amino acid가 동정되었다. 이 amino acid를 기본배양액에 섞어 배양액으로 만들어 난자를 배양했을 때 난자성숙율이 90% 이상이었고 제2차 중기까지 도달한 난자는 30시간 이내에 10% 정도였다.

셋째, 상기의 amino acid가 들어있는 배양액에 다시 biotin를 첨가하여 배양 하면 48시간내에 제2차 분열 중기까지 도달하는 난자가 60%에 이르렀다.

위 결과로 보아 여포액은 *in vitro*인 경우 난자의 성숙을 유발하며 성숙을 유 발하는 주요 성분은 여포액내에 포함되어 있는 몇 가지 amino acid와 biotin 과 같은 vitamine B가 될 것이라는 결론을 얻었다.

INTRODUCTION

It has been generally accepted that the mammalian oocytes initiate their meiotic resumption in the medium immediately after liberation from the ovarian follicles

(Edwards, 1962, 1965; Cho, 1971; Foote and Thibault, 1969). Edwards (1962) has claimed that follicular fluid might contain unknown factor(s); anti-meiogenic substances which suppress the meiosis of the oocytes unless they are freed from such circumstance. Foote and Thibault (1969) and Foote *et al.* (1970) have assumed that the granulosa cells produce anti-meiogenic factors after the *in vitro* observation of cultivating the oocytes in the presence of the granulosa cells.

However, Cho *et al.* (1971) found that the presence of the human follicular fluid in the culture medium raised the proportion of the matured human oocytes, and they presumed that the follicular fluid would provide a better environment to the oocytes when the fluid is present *in vitro*. The same results were found by Hunter *et al.* (1972) and Kim *et al.* (1974) who investigated the effects of cow follicular fluid *in vitro* on the maturation of the cow oocytes. Thus, the action of the follicular fluid is different with regard to the meiotic resumption; inhibition *in vivo* and stimulation *in vitro*.

Until today, it has not been well explained why the follicular fluid acts in different way *in vivo* and *in vitro*. Based upon the previous studies, which showed that the *in vitro* addition of the homologous follicular fluid provides better environment for the occyte maturation, further investigation on the behaviour of the fluid is anticipated. At this situation, the present studies have been aimed at an attempt to find out the efficient factors contained in the follicular fluid for the oocyte, and in addition to this, the development of a suitable medium for the cow oocyte maturation was intended.

MATRIALS AND METHODS

1. Collection of the oocytes and method of cultivation

The oocytes were obtained from the cow ovarian pieces. The ovarian pieces were carried in a thermos filled with chilled 0.9% NaCl from the slaughter house. The oocytes were freed from the ovarian follicles (3~6 mm in diameter) in the medium by puncturing the follicles with a fine needle, and collection of the oocytes were performed within two hours after obtaining ovarian pieces from the slaughter house.

The basic medium for collection of the oocytes was a modified Krebs-Ringer bicarbonate solution (Biggers *et al.*, 1971). Throughout the experiments, the medium was supplemented with 0.1% Ficoll instead of bovine serum albumin (BSA). Only the oocyte intact with germinal vesicle was taken for the subject of the cultivation. The oocytes were washed twice in 1 m*l* of the basic medium in a watch glass covered with 1 m*l* of paraffin oil (Thomas, light) and transferred to a drop (50 μl) of the culture medium set on a plastic culture dish (Falcon, #3002) containing 15 m*l* of paraffin oil.

The culture medium in paraffin oil was previously equilibrated in an incubator supplied with 5% CO_2 in moistened air for 3~4 hours. After the cultivation, the cumulus cells attached to the egg surface were removed by sucking forth and back with a capillary pipette or by shaking in a test tube containing 1 ml of 0.9% NaCl with hand, and the oocytes were fixed with acetic alcohol for 12~24 hours and stained with 0.5% aceto-lacmoid for further observation of the nucleus.

2. Collection of follicular fluid

The follicular fluid was aspirated with a capillary pipette from the follicles of 7~15 mm in diameter. The fluid was kept frozen at −20°C until use.

3. Dialyzation

In order to obtain dialyzable or non-dialyzable fraction from the follicular fliuid, the cellophane tube (Thomas, ¼ inch, pore size 24 Å) was used. The frozen fluid was thawed and centrifuged at 1,500 rpm for 15 minutes, and the suspension was applied for dialysis. The cellophane tube (length: 12cm) containing 2 ml of follicular fluid was tied at both ends with string and put in a test tube (17×100 mm containing 2 ml of 0.9% NaCl), and left for 36 hours at 4°C. Non-dialyzable fraction was kept in a sterilized test tube at −20°C until use, and dialyzable fraction in 0.9% NaCl was filtered by Millipore and stored at −20°C in a sterilized test tube after concentrated two-fold at 60°C.

4. Chromatography

In order to find out the free amino acids, the dialyzable fraction was developed on a filter paper (Whatman No.1). For this purpose, the dialyzable fraction was obtained in distilled water instead of 0.9% NaCl. Thirty μl of dialyzable fraction was spotted on the paper. The primary solvent used was butyl alcohol: acetic acid: water (4 : 1 : 3) and the secondary one was phenol: water (7 : 3). The period of primary development was 8 hours and that of secondary 10 hours. After development, the paper was sprayed with 0.2% ninhydrin for the identification.

Nine spots were identified on the paper and each of specific spot was identified by comparing with standards.

5. Media

The composition of the basic medium applied throughout the experiments was a modified Krebs-Ringer bicarbonate solution (Biggers et al., 1971), supplemented with Ficoll instead of bovine serum albumin in order to eliminate any nitrogen source in the medium. Dialyzable fraction in 0.9% NaCl, non-dialyzable fraction dissolved in 0.9% NaCl, and the basic media supplemented with amino acids identified in the fluid and with biotin were used according to the experimental design. The components of the basic medium, amino acids and biotin were given in Table 1 and 2.

Table 1. Modified Krebs-Ringer bicarbonate solution
(Standard egg culture medium).

Components	mM
NaCl	94. 59
KCl	4. 78
KH$_2$PO$_4$	1. 19
MgSO$_4$·7H$_2$O	1. 19
Ca-lactate	1. 71
Na-pyruvate	0. 25
Na-lactate	21. 58
NaHCO$_3$	25. 07
Glucose	5. 59
Penicillin G	100 u/ml
Streptomycin	50 μg/ml
Ficoll	0. 1%

Table 2. Concentration of amino acids which were
added to the SECM.

Components	mM
DL-alanine	3×10^{-1}
DL-aspartic acid	5×10^{-2}
DL-glutamic acid	5×10^{-2}
Glycine	2
L-leucine	1×10^{-1}
L-methionine	3×10^{-1}
L-phenylalanine	3×10^{-1}
L-glutamine	2×10^{1}
Biotin	4.1×10^{-4}

SECM : Standard egg culture medium.

RESULTS

Table 3 shows the results obtained in the experiments cultivating the cow oocytes in dialyzable fraction and 10% non-dialyzable fraction dissolved in saline.

In the group cultured in simple dialyzable fraction, all oocytes resumed meiotic division and 35% of them reached metaphase II in 48 hours. However, non of the

Table 3. Nuclear phases of cow oocytes cultured in the dialyzable or in the non-dialyzable fraction of the follicular fluid.

Culture medium	No. of ova cultured	MI-TI[1]	MII	Other[2]	Deg[3].
100% dialyzable fraction	29	16 (55. 2)	10 (34. 5)	3 (10. 3)	
Non-dialyzable fraction in saline	20	9 (45. 0)			11 (55. 5)

1) MI-TI : Oocytes at metaphase I and through telophase I.
   MII : Oocytes at metaphase II.
2) Oocytes with activated or binuclei.
3) Degenerative oocytes.

oocytes underwent to metaphse II but 45% at metaphase-telophase I in the non-dialyzable fraction and the remainders had already shown degeneration.

In Table 4 are set the results obtained in the experiment of culture in the basic medium removed all sources of carbohydrates (glucose, pyruvate, and Ca-lactate but substituted by CaCl$_2$) but mixed with dialyzable fraction in order to evaluate the significance of the carbohydrates and their compensation in the dialyzable fraction.

Table 4. Comparison of the effect of carbohydrates in the SECM mixed with the dialyzable fraction on the meiotic maturation of the cow oocytes *in vitro*.

Culture medium	No. of ova cultured	MI-TI	MII	Deg.
SECM+dialyzable fraction+0.1% ficoll	28	23 (82.1)	2 (7.1)	3 (10.8)
SECM (−pyruvate, −lactate, −glucose)+dialyzable fraction +0.1% ficoll	20	6 (30.0)	1 (5.0)	13 (65.0)
SECM (−pyruvate, −lactate, −glucose)+0.1% ficoll	30	4 (13.3)	—	26 (86.7)

MI-TI : Oocytes at metaphase I and through telophase I.
MII : Oocytes at metaphase II, Deg. : Degenerative oocytes.

As shown in the table, most of the oocytes underwent degeneration(87%), and only 13% succeeded to resume meiotic division in the medium without addition of carbohydrate sources. If the oocytes were cultured in the mixture 0.4 m*l* dialyzable fraction and 9.6 m*l* carbohydrate free medium, the proportion of the degenerative oocytes decreased to 65% and that of the metaphase II oocytes contrarily increased to 30%. The medium mixed with the complete basic one and the dialyzable fraction produced the least proportion of the degenerative oocytes (11%), but the highest one of the metaphase I. Seven percent of them had already proceeded to the metaphase II.

Referring to the above two experiments, it became apparent that the dialyzable fraction acts beneficially for the initiation of the meiotic resumption of the cow oocytes. In order to find out the efficient factors in the dialyzable fraction, particular concern was made with the composition of the amino acids which might be contained in the fraction and such amino acids were identified by paper chromatography, and they were added to the basic medium to examine their significance in the initiation of the oocyte maturation. The results are shown in Table 5. If all amino acids previously identified were added to the medium, the maturality of the oocytes was progressed gradually as the culture period was extended from 18 to 30 hours (Figs. 1~5). When the oocytes were cultured in the basic medium, the production of the degenerative oocytes markedly increased (79~84%) and the rest arrested at the early stage of the meiosis. Most of the degenerative oocytes showed chromatin condensation or heteropycnosis in the heterologous cytoplasm with vacuoles.

Table 5. Nuclear maturation of the cow oocytes in the SECM added by eight amino acids which were identified in the follicular fluid.

Culture medium	Culture hours	No. of ova cultured	Dic.	Pro.	PMI	MI	AI	TI	MII	Other	Deg.
SECM+0.1% ficoll	18	16	1 (6.0)	1 (6.0)	2 (12.5)	4 (25.0)	1 (6.0)	—	—	—	7 (43.7)
	24	19	—	2 (10.6)	—	—	—	—	—	—	17 (89.4)
	30	14	—	3 (21.5)	—	—	—	—	—	—	11 (78.5)
SECM+0.1% ficoll+8 amino acids	18	20	1 (5.0)	—	8	8 (40.0)	1 (5.0)	—	—	—	2 (10.0)
	24	19	—	—	—	8 (42.1)	3 (15.8)	5 (26.3)	—	1 (5.3)	2 (10.5)
	30	20	2 (10.0)	—	—	2 (10.0)	—	9 (45.0)	2 (10.0)	3 (15.0)	2 (10.0)

Dic. : Oocytes at dictyate. Pro. : Oocytes at prophase I. P-MI : Oocytes at prophase I and through metaphase I. MI : Oocytes at metaphase I. AI. : Oocytes at anaphase I. TI. : Oocytes at telophase I. MII : Oocytes at metaphase II. Other : Oocytes in activated or binuclei. Deg. : Degenerative oocytes. Figures in parenthesis are per-cent to total number of oocytes cultured.

Table 6. Effect of biotin added to the SECM plus amino acids on the cow oocyte maturation *in vitro*.

Culture medium	Culture hours	No. of ova cultured	MI-TI	MII	Other	%MII
SECM+0.1% ficoll+8 amino acids	30	25	23	—	2	—
	42	18	15	3	—	16.6
	48	20	15	4	1	20.0
SECM+0.1% ficoll+8 amino acids+biotin	30	20	15	4	1	20.0
	42	20	11	7	2	35.0
	48	20	6	12	2	60.0

MI-TI : Oocytes at metaphase I and through telophase I, MII : Oocytes at metaphase II, Other : Oocytes in activated or binuclei.

As shown in the above table, only 10% of oocytes remained at the metaphase II stage in 30 hours. If biotin was added to the amino acid medium, the proportion of the metaphase II oocytes rapidly increased to 60% in 48 hours, while only 20% of the oocytes succeeded to reach at the metaphase II in 48 hours in the same medium but free of biotin (Table 6 and Figs. 6, 7). The oocytes grown in the biotin medium showed comparatively homogeneous cytoplasm.

DISCUSSION

Recently, many reports have been found regarding to the biological significance of the follicular fluid in particular connection with the oocyte maturation (Foote and Thibault, 1969; Cho *et al.*, 1971; Kim *et al.*, 1974) and with the sperm capacitation (Yanagimachi, 1969 a,b; Edwards, 1974; Mukherjee, 1974).

Their findings imply that the fluid acts favourably to the oocytes if it leaves

from the follicle. This means that the fluid keeps the inhibiting substance only inside the follicle. On the other hand, we also can make an assumption in this way: the ability of the meiotic resumption of the oocytes *in vitro* would not be due to the change of the follicular fluid but wholly due to the change of the oocyte itself regarding to the membrane behaviour. On this matter further investigation should be conducted.

Kim *et al.* (1974) succeeded to increase the proportion of the cow oocyte maturation in the medium mixed with follicular fluid in the ratio of 1 : 1. In the present studies, however, the dialyzable fraction in the saline provides better condition to the oocytes than the medium mixed with follicular fluid. This might imply that the dialyzable fraction which will contain most of micromolecules such as amino acids, carbohydrates and steroid hormones acts more preferentially to the oocytes than total fluid which contains macro- as well as micromolecules. Moreover, the oocytes tended to degenerate if they were cultured in the saline mixed with non-dialyzable fraction whose main components might be of the macromolecules such as proteins and polysaccharides.

Essential substances for the support of the oocyte maturation or early development of the embryos were studied in the various laboratory mammals. Finally it has been found that most of them required certain kinds of amino acids (Gwatkin and Haidri, 1973-hamster oocytes; Bae and Foote, 1975-rabbit oocytes; Daniel and Olson, 1968; Brinster 1970-rabbit embryos). As shown in the present studies, the cow oocytes also require amino acids for the oocyte maturation. The dialyzable fraction possibly contains such amino acids, and so the fraction acts beneficially for the oocytes. Actually, better results were obtained in the present studies by addition of amino acids identified in the dialyzable fraction into the basic medium than either addition of fraction or applying solely with the fraction.

Donahue (1968) found that the synthesis of protein takes place during maturation of the mouse oocytes even though they do not need any supply of exogeneous amino acids in the medium. Cho *et. al.* (1974) and Stern and Wassarman (1974) also confirmed that mouse oocytes even arrested by the inhibitory action of dbc-AMP carried out protein synthesis during culture in the amino acid free medium. Possibly, the mouse oocytes would utilize endogeneous amino acids in protein synthesis which is necessary for the maturation. However, the mammals other than the mouse generally require supply of the amino acids to keep enhancing protein synthesis by which finally the oocyte succeed to grow to the metaphase I.

The fact that maturation is less promoted by addition of whole follicular fluid than by addition of the dialyzable fraction would be related with the fact that the total fluid might contain some sorts of harmful substances. Actually, Cho *et al.* (1971) found that the addition of the heated follicular fluid into the medium

decreased the proportion of degenerative oocytes. This possibly means that the heat treatment of the fluid is enough to denature the toxic substances in the fluid.

In the present studies, the addition of biotin to the amino acid medium provided better environment to the cow oocytes, and the proportion of the metaphase II oocytes markedly increased. This possibly suggests that the treatment with biotin is enough to drive metaphase I oocytes to continue meiotic division and to form polar body, because biotin is known to mediate carboxylation associated with pyruvate carboxylase and finally produce oxaloacetate which is deeply related with the conversion of ATP to ADP by which energy is released. The significance of biotin in the cow oocyte maturation will be investigated further.

REFERENCES

Bae, I.H. and Foote, R.H., 1975. Carbohydrate and amino acid requirements and ammonia production. *Exptl. Cell Res.* **91** : 113.

Biggers, J.D., W.K. Whitten and D.G. Whittingham, 1971. The Culture of Mouse Embryos *in vitro. In:* Methods in Mammalian Embryology. (J.C. Daniel, Jr., editor). W.H. Freeman & Co. 86—116.

Brinster, R.L., 1970. Culture of two-cell rabbit embryos to morulae. *J. Reprod. Fert.* **21** : 17—22.

Cho, W.K., M.K. Kim and S.O. Chung, 1971. *In vitro* maturation of human follicular oocytes in the presence of follicular fluid. Intn. Cong. Ser. No. 278, *Fert. Steril. Proc. 7th World Cong. 1971, Excerpta Medica Amsterdam.* 706—708.

Daniel, J.C., Jr. and J.D. Olson, 1968. Amino acid requirement for cleavage of the ovum. *J. Reprod. Fert.* **15.** 453—455.

Donahue, R.P., 1968. Maturation of the mouse oocytes *in vitro.* ph.D. *Dissertation, Johns Hopkins University,* Baltimore, Md,. U.S.A.

Edwards, R.G., 1962. Meiosis in ovarian oocytes of adult mammals. *Nature* **196** : 446 —450.

Edwards, R.G., 1965. Maturation *in vitro* of mouse, sheep, cow, pig, Rhesus monkey and human ovarian oocytes. *Natue* **208** : 349.

Edwards, R.G., 1965. Maturation *in vitro* of human overian oocyte. *Lancet* **7419** : 926

Edwards, R.G., 1974. Follicular fluid. *J. Reprod. Fert.* **37** : 189—219.

Foote, W.D., D.L. Matley and F.D. Tibbits, 1970. Follicular inhibition of oocyte maturation *in vitro. Proc. Western Sect., Am. Soc. Anim. Sci.* **21** : 45.

Foote, W.D. and C. Thibault, 1969. Recherches experimentales sur la maturation *in vitro* des ovocytes de truie et de veau. *Ann. Biol. anim. Biochim. Biophys.* **9** (3) : 329—349.

Gwatkin, R.B.L. and A.A. Haidri, 1973. Requirements for the maturation of hamster oocytes *in vitro. Exptl. Cell Res.* **76** : 1—17.

Hunter, R.H., R.A.S. Lason and L.E.A. Rowson, 1972. Maturation, transplantation and

fertilization of cattle ovarian oocytes. *J. Reprod. Fert.* **30** : 325—328.

Kim, M.K., W.K. Cho, S.O. Chung and Y.H. Choi, 1974. The effects of follicular fluid on *in vitro* maturation of bovine follicular oocytes. *Yonsei Med. J.* in printing.

Mukherjee, A.B., 1974. *In vitro* embryogenesis: An experimental model for the udersta-nding of reproductive physiology and development in mammals. *Can. J. Genet. Cytol.* **16** : 11—30.

Thibault, C., 1971. *In vitro* maturation and fertilization of rabbit and cattle oocytes. *In*: The regulation of mammlian reproduction. (Segal, Crozier & Corfman, editors). Springfield, Illinois, C.C. Thomas; 231—240.

Yanagimachi, R., 1969a. *In vitro* capacitation of hamster spermatozoa by follicular fluid. *J. Reprod. Fert.* **18** : 275—286.

Yanagimachi, R., 1969b. *In vitro* acrosome reaction and capacitation of golden hamster spermatozoa by bovine follicular fluid and its fractions. *J. Exp. Zool.* **170** : 269—280.

EXPLANATION OF FIGURES

Fig. 1. Fresh cow oocytes showing germinal vesicle. A nucleolus is clearly seen inside the germinal vesicle (420X).

Fig. 2. Metaphase I chromosomes in the cow oocytes (840X).

Fig. 3. Anaphase I chromosomes (840X).

Fig. 4. Late anaphase I chromosomes (840X).

Fig. 5. Telophase I chromosomes (840X). The oocytes shown in Fig. 3 and through Fig. 5 were cultured in SECM supplemented with 8 amino acids.

Fig. 6. Metaphase II chromosomes and a polar body (840X).

Fig. 7. Same as Fig. 6 but metaphase II chromosomes are encircled by a vesicle like material which is often seen in the metaphase nucleus. The oocytes shown in Figs. 6 and 7 were cultured in SECM containing 8 amino acids and biotin for 48 hours (840X).