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The Effects of Follicular Fluid on in Vitro Maturation of Bovine Follicular Oocytes

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ABSTRACT

The present experiments were conducted to evaluate the effects of follicular fluid on maturation of bovine follicular oocytes. TC medium 199 seemed to be in adequate for this purpose since a high proportion (ranging 84.1. to 97.0%) of the oocytes were able to resume meiotic division in both media-with or without the addition of follicular fluid.

This implies a possible similarity between TC medium 199 and follicular fluid with regard to the components initiating meiosis. Actually, TC medium 199 contains amino acids, vitamins and carbohydrates most of which are also found in follicular fluid. For this reason, the effect of follicular fluid on the ovum maturation was investigated by applying Krebs-Ringer bicarbonate solution (SECM), which was mainly composed of the salts, pyruvate and lactate. When the oocytes were cultured in SECM without the addition of follicular fluid, only 7-14% of them resumed meiotic division within 30 hours. However, when follicular fluid was added, the proportion of oocytes undergoing maturation was sharply increased to about 70%. Among the groups cultured in media containing different concentrations of follicular fluid, the proportion of the oocytes in meiosis remained constant, In pure follicular fluid, 87-89% of the oocytes showed enhancement of meiotic division. The presence of the follicular fluid contributed to a decrease in the production of degenerative oocytes. As a consequence it has been noted that addition of follicular fluid to the culture medium provides a more beneficial environment for cow oocytes. Meiotic division is initiated and production of degenerative oocytes is inhibited.

INTRODUCTION

It is known that resumption of meiosis in meiotic arrested oocytes is initiated through the stimulation of luteinizing hormone in early estrus, or by liberation of this hormone from ovarian follicles (Brambell; 1956). Since the studies of Pincus and Enzmann (1935) who succeeded in inducing maturation of rabbit follicular oocytes in culture, a number of people have investigated maturation of the oocytes in various animals in vitro. They noted the fact that oocytes immediately resume meiotic division when placed in an appropriate medium. However, the fact that the mammalian oocytes persist in the dictyate stage for a long period until freed from the follicles suggests that maturation of the oocytes is controlled by some unknown factors which are present in the intra follicular environment. Edwards (1962) mentioned that follicular fluid might contain oocytic inhibiting factor (s), (antimeiogenic factor(s)), against the activation of oocytes. Thereafter, Foote and Thibault (1969) postulated that the inhibiting factor(s) is released by granulosa cells. Recently, however, Cho et al., (1971) found that human oocytes cultured in a medium containing homologous follicular fluid matured more frequently than those placed in a plain medium. Also Foote and Thibault, and Hunter et al. (1973) observed a higher incidence of bovine oocyte maturation in a medium containing follicular fluid. This implies that follicular fluid provides conditions beneficial for in vitro oocyte maturation. However, at the present time the role or effect of follicular fluid on oocyte maturation has not been proven.

To further understand the mechanism of the oocyte maturation it would be helpful to find the relationship between follicular fluid and oocyte behavior in vitro. The present studies have been conducted in vitro to evaluate the effects of follicular fluid on oocytes. A part of the results are discussed here.

MATERIALS AND METHODS

Bovine ovarian samples were obtained from a slaughter house. Collection of the oocytes and follicular fluid was completed within three hours after sacrifice of the animals. Follicular fluid was drawn from the follicles with a capillary pipette which ranged from 3 to 15 mm in diameter. The fluid was sterilized by filtration and stored by rapid freezing(−20°C) until needed. Under a dissecting microscope the oocytes were liberated by puncturing or cutting open the follicles with a sharp needle or scissors. Only the oocytes with intact germinal vesicles were collected for these studies. The basic culture media used were, firstly, TC medium 199 plus 0.4% bovine serum albumin (BSA, Sigma) and secondly a modified Krebs-Ringer bicarbonate solution (SECM, Biggers et al.; 1971) supplemented with 0.4% BSA or Ficoll (Sigma). A part of the medium was mixed with selected portions of follicular fluid which had been thawed just before use.

The oocytes were cultured by the methods described by Cho et al., (1971). At the end of cultivation, oocytes were recovered and

Table 1. Maturation of bovine follicular oocytes cultured in TC medium 199 plus 0.4% BSA with or without the addition of follicular fluid (F.F.)

Duration (hours)	F.F.	No. of ova cultured	Nuclear phases		No. of ova on meiosis / No. of ova cultured
			MI-TI	PII-MII	
22—24	absent	8	7 *(87.5)	— —	7/8 (87.5)
	present	32	24 (75.0)	4 (12.5)	28/32 (87.5)
30	absent	48	31 (64.6)	11 (22.9)	42/48 (87.5)
	present	185	123 (66.5)	46 (24.9)	169/185 (91.4)

* Figure in parenthesis means percent of meiotic oocytes.

Table 2. Maturation of bovine follicular oocytes cultured for 30 hours in different media, and media containing different proportions of follicular fluid (F.F.)

Proportion of F.F. (%)	SECM plus 0.4% Ficoll	SECM plus 0.4% BSA	TC medium 199 plus 0.4% BSA
0	*3/43 (7.0)	5/36 (13.9)	42/48 (87.5)
13	14/27 (51.8)	24/35 (68.6)	37/44 (84.1)
20	22/27 (81.7)	27/37 (73.0)	34/37 (91.9)
30	21/30 (70.0)	26/37 (70.3)	32/33 (97.0)
44	18/25 (72.0)	19/29 (65.5)	36/38 (94.7)
67	24/32 (75.0)	24/35 (68.5)	30/33 (90.9)
100	26/30 (86.6)	40/46 (87.0)	32/36 (88.9)

*No. of ova on meiosis/No. of ova cultured (%)

Significant test: SECM vs TC medium 199 (excluding number of the oocytes cultured in 100% F.F.)
$X^2=8.62$, DF=1, $p<0.01$
SECM with vs without presence of F.F. (Test was made by summing all results obtained in the media containing different proportinns (13 and 67%) of follicular fluid, and SECM was summarized. Two results were obtained in different supplements; Ficoll and BSA as one factor) $X_2=9.59$, DF=1, $p<0.01$

freed from the cumulus cells by shaking both vigorously in 2 ml of saline in a test tube (10×100 mm) or by exposing them to 1 ml of 0.25% pronase solution for a few minutes followed by washing them several times in fresh saline. Thereafter, the oocytes were fixed in acetic alcohol, stained with 0.5% acetolacmoid and examined in their nuclear phase through a phase contrast microscope.

RESULTS

Table I shows that about 90% of the bovine

Table 3. The effect of follicular fluid (F.F.) on the degeneration rate of the follicular oocytes cultured in different media for 30 hours.

Medium	Without addition of F.F.	With addition of F.F.	Whole (100%) F.F.
SECM plus 0.4% Ficoll	*34/43 (79.0)	39/141 (27.6)	—
SECM plus 0.4% BSA	26/36 (72.2)	49/183 (26.8)	—
TC med. 199 plus 0.4% BSA	3/48 (6.3)	13/185 (61.1)	—
Whole (100%) F.F.	—	—	9/112 (8.0)

*No. of ova degenerated ova/No. of ova cultured. Number in parenthesis shows percent of the degenerative oocytes. Significant test: SECM plus 0.4% Ficoll and BSA without vs with addition of F.F.: $X^2=8.07$, DF=1, $P<0.01$ in TC med. 199 plus 0.4% BSA without Insignificant.

oocytes resumed meiotic division in the TC medium 199 supplemented with 0.4% BSA, either with, or without the addition of follicular fluid. The meiotic resumption was similar in the two different media. More metaphase II oocytes were found in the medium containing follicular fluid than in the plain medium, and in the group of cells cultured for 30 hours than in that cultured for 22-24 hours.

Table 2 shows the results obtained by culturing the oocyte in different kinds of media which contained differing proportions of follicular fluid. It was found that the presence of BSA and Ficoll in the medium of SECM had no effection meiotic initiation. TC medium 199 was more effective than SECM in producing maturation of the ovum. When the oocytes were cultured in plain SECM, only 10% of the oocytes resumed meiotic division. When follicular fluid was added to the SECM, the number of the oocytes undergoing maturation increased abruptly. About 70% of the oocytes resumed meiosis in the presence of follicular fluid. Among those resuming meiotic division, 17% had completed the maturation process by extruding the polar body. The proportions of the oocytes in the maturation process were nearly constant regardless of the concentration of follicular fluid in the medium.

In TC medium 199, either with or without follicular fluid, 84.1 to 97.0% of the oocytes resumed meiotic division. In othe words in our culture system follicular fluid had no significant effect on meiosis of oocytes in the TC medium 199. About 87% of the oocytes were able to grow even at 100% concentration of follicular fluid.

Follicular fluid seemed to decrease the production of degenerated oocytes (Table 3). When the oocytes were cultured in SECM, the majority of the oocytes (75%) underwent degeneration. However in the presence of follicular fluid, the proportion decreased to 27%.

Thirty-five abnormal oocytes, such as binuclei (5), multipolar (25) and those in nondisjunction (5) were observed throughout the experiments.

DISCUSSION

Recently, the value of the follicular fluid for animal ovum maturation has been emphasized (Foote and Thibalt; 1969, Cho et al.; 1971). Follicular fluid affects the sperm. (Gwatkin and Anderson: 1969, Iwamatsu and Chang; 1969, Yanagimachi; 1969). It has been assumed that follicular fluid would contain an antimeiogenic factor for the oocytes at the dictyate stage in the ovarian follicles. They are arrested at that stage until freed from the follicles (Edwards; 1962). It has been confirmed that a high proportion of the human oocytes resume meiotic division in a medium containing homologous follicular fluid (Cho et al.; 1971). This finding suggests that follicular fluid, which is removed from the follicle, would possess follicle, would possess factors which initiate meiosis of the oocytes.

Foote and Thibault (1969) induced the bovine oocyte maturation *in vitro* in TC metium 199. However, TC medium 199 seemed to be inadequate for studies of the effects of follicular fluid on oocyte behaviour. In TC medium 199, without addition of follicular fluid, a high proporation of bovine oocytes at the diplotene stage resumed meiosis. This implies the plain TC medium 199 may provide conditions beneficial to oocytes because the medium is composed of most of the available nutrients (such as amino acids, steroids and vitamins) which are also found in follicular fluid (Edwards; 1974). Such a similarity in composition between follicular fluid and TC medium 199 caused us, for the purpose of the studies to seek another simple medium which has no source of nitrogen (such as amino acids or bovine serum albumin), and containg steroids and vitamins. Hence, a modified Krebs-Ringer bicarbonate solution (Standard Egg culture medium; SECM) which contains only lactate and pyruvate as the energy sources was adopted for the present experiment. Ficoll was added to the SECM instead of bovine serum albumin which has been believed to provide nitrogen to the early mouse embryo. The finding (Brinster; 1971) that both Ficoll and BSA do not play any role on oocyte maturation was conformed in the present experiment. The role of BSA has been described as a factor in the control of the physicochemical environment within the medium, rather than itself being a nutrient (Brinster; 1971).

Biggers et al. (1967) postulated that pyruvate is essential for mouse oocytes during meiotic division and early cleavage. They emphasized that pyruvate could be used as an energy source. However, for the bovine oocytes, pyruvate seems not to be essential for initiating meiotic division because the oocytes were able to undergo meiotic division in pyruvate free TC medium 199. The present results suggest that maturation of cow oocytes is dependent on the presence of nitrogen sources derived from proteins or from amino acids for metabolism.

The addition of follicular fluid to the SECM provided more beneficial conditions for the oocytes. The proportion of oocytes already in maturation division increased in the medium containing follicular fluid. The rate was as high as that found in the plain TC medium 199. This strongly indicates that there are beneficial substances both common follicular fluid and TC medium 199.

Our recent experiments showed that the dialysed fraction of follicular fluid produced more oocytes undergoing meiosis than use in the non-dialysed fraction (unpublished). This suggests that micromolecules which are present in the dialysed fraction of follicular fluid act efficiently in inducing meiotic division. Our finding is inconsistent with the results of Cross (1973) who has reported that the nondialysable fraction (macromolecules) of serum provided conditions more beneficial to mouse oocyte maturation than the dialysable fraction (micromolecules). Such inconsistencies are assumed to be due to species specific nutrient requirements for oocyte maturation.

Maturation of bovine oocytes was seen in a nearly constant proportion in the SECM contain media which various concentrations of follicular fluid (13% to 100%). The results suggest that even the presence of a small amount of follicular fluid (13%) in the medium might provide enough factors to initiate meiotic division. When the oocytes were cultured in pure follicular fluid (100%), a high proportion of the oocytes were able to undertake meiotic division. These findings suggest two possibilities: one that follicular fluid does not contain any of the antimeiogenic factors; secondly that such factors, even though they existed initially in follicular fluid, would be denatured or altered immediately after removed from the intact follicle.

At the present time, little is known about the real role of follicular fluid, either in initiating or inhibiting oocyte maturation. More-detail investigations on the relationship between follicular fluid and oocytic maturation will be carried out.

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Explanation of Plates

Fig. 1. The oocyte just after recovery from the follicle. It shows a dictyate nucleus. Adhering cumulus cells were removed by exposing them to 0.25% pronase before fixing and staining. ×1100.

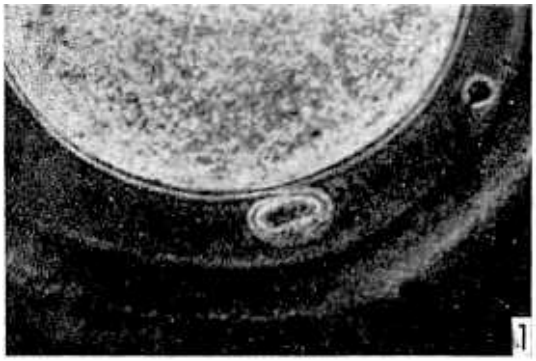
Fig. 2. The oocyte showing the prophase stage. Culture media was TC medium 199 plus 0.4% BSA containing 44% follicular fluid for 22 hours. The nucleus shows condensed chromatin. ×1100.

Fig. 3. The nucleus showing Metaphase I chromosomes. The oocyte was cultured for 24 hours in TC medium 199 plus 0.4% BSA containing 67% follicular fluid. ×650.

Fig. 4. The oocyte matured in 30 hours to the Telophase stage in SECM plus 0.4% Ficoll containing 44% follicular fluid. ×1100.

Fig. 5. Metaphase Ⅱ stage of the oocyte cultured for 30 hours in SECM plus 0.4% Ficoll containg 20% follicular fluid. The oocyte shows the Metaphase Ⅱ chromosomes and the chromatin mass of the first polar body. ×1100.

Fig. 6. The first polar body produced by an oocyte cultured for 30 hours in SECM plus 0.4% BSA containing 30% follicular fluid. Photograph was taken before fixation. ×1100.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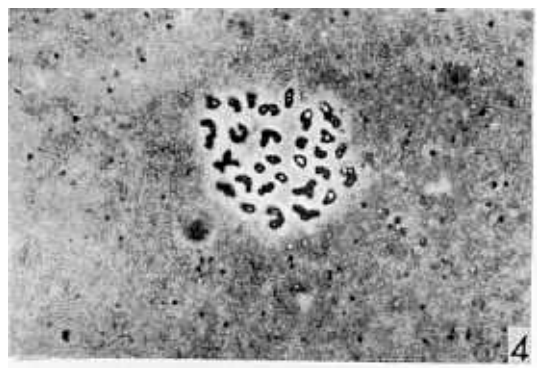
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