Full Papers

Cooperative inhibitory effect of follicular fluid and cAMP on hamster oocyte maturation

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Summary. Porcine or human follicular fluid inhibited the spontaneous maturation of isolated hamster oocytes in vitro during the first 1.5 h of culture. Moreover, the presence of 50% follicular fluid combined with 100 μM dbcAMP cooperatively reduced the incidence of germinal vesicle breakdown. The addition of FSH also inhibited the resumption of meiosis, and the presence of LH did not overcome the inhibitory effects of follicular fluid and tended to impede isolated hamster oocyte maturation in vitro.
Key words. Oocyte; oocyte maturation inhibitor; follicular fluid; cAMP; gonadotropin.

Introduction

Meiosis in the follicular oocyte is arrested at the diplotene (dictyate) stage of the first meiotic division. Its resumption occurs in vivo just before ovulation. The preovulatory gonadotropin surge is thought to be involved, but the exact mechanism remains to be clarified.

Pincus and Enzmann[15] found that intact, immature mammalian oocytes resume meiosis spontaneously in the absence of any hormonal stimuli following removal from the follicular environment. This prompted the conclusion that there may be certain constituents in the antral follicle which inhibit oocyte maturation. This inhibition continues until stopped by the action of gonadotropins. Tsafriri and Channing[22] observed that porcine follicular fluid inhibited the spontaneous maturation of porcine oocytes in vitro. They assumed that porcine follicular fluid contains an oocyte maturation inhibitor (OMI) synthesized by granulosa cells and released into the follicular fluid. OMI activity has been found in the follicular fluid of various mammalian species by some investigators[7,9,21,27], while others failed to confirm its existence[11,16,18]. Therefore, the regulatory role of follicular fluid in oocyte maturation has been reinvestigated by us[22–25].

Maturation of isolated mouse oocytes has also been reported to be inhibited by cAMP derivatives or cyclic nucleotide phosphodiesterase inhibitors[17,20]. Downs and Eppig[5] found that cAMP and follicular fluid acted synergistically to inhibit mouse oocyte maturation. The present study was undertaken to confirm the presence of OMI activity in porcine and human follicular fluid, using hamster oocytes for the bioassay. We also assessed the effect of dibutyryl cAMP and gonadotropins on isolated hamster oocyte meiotic maturation.

Material and methods

Oocyte collection. Female golden hamsters, 8–12 weeks old, were injected i.p. with 25 IU pregnant mare serum gonadotropin (PMSG, Teikoku Zoki, Tokyo, Japan) during the metestrus period. This treatment led to follicular development and the ovaries were removed 48 h after the PMSG injection, but prior to the endogenous LH surge. The ovaries were placed in a watch glass containing culture medium supplemented with 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma, St. Louis, MO), a phosphodiesterase inhibitor. IBMX prevented spontaneous oocyte maturation during the isolation procedure[4,20]. Under a dissecting microscope, the mature follicles were punctured with a 26 gauge sterile needle to release the immature oocyte at the dictyate stage. Only oocytes with an intact, tightly adhering mass of cumulus cells were selected for examination. In some cases, cumulus cells were removed to obtain denuded oocytes by repeatedly pipetting the cumulus-oocyte complex with a mouth operated micropipette with an inner diameter slightly larger than that of the oocytes. The denuded and cumulus-enclosed oocytes were washed in IBMX free medium and transferred to the culture medium containing appropriate test substances.

Culture procedure. The culture medium was the GH-2 medium described by Haidri and Gwatkin[8]. It consisted of a balanced salt solution, supplemented with pyruvate, lactate, glucose, 12 amino acids, and 6 mg/ml crystalline bovine serum albumin (Sigma). The medium was adjusted to pH 7.3–7.4 with 10 mM Hepes (Sigma).

Follicular fluid was obtained from porcine and human ovaries. Porcine follicular fluid was collected from small (follicular diameter 1–2 mm), medium (3–5 mm) and large (6–10 mm) follicles respectively. Human follicular fluid was aspirated either from ovaries in situ or from isolated ovaries within 30 min after extirpation. The aspirated follicles were either small (follicular diameter < 5 mm) or large (> 15 mm). Cells and debris were removed from the fluid by centrifugation at 2000 × g for 10 min. The fluid was sterilized by filtration with a 0.22 μm membrane filter (Millipore, Bedford, MA) and kept frozen until used.

In the experiments, follicular fluid was thawed and ultrafiltrated with a PM-10 membrane (Amicon, Danvers, MA) to obtain a low molecular weight fraction (MW $< 10{,}000$)[5] and a mixture of 50% follicular fluid in GH-2 medium was used. Dibutyryl cAMP (dbcAMP, Sigma) was dissolved directly into the culture medium (control or 50% follicular fluid) at a concentration of 100 μM and the effects of dbcAMP alone or together with follicular fluid were assessed. LH (NIAMMD-bovine LH-4; 0.5

and 5 μg/ml) or FSH (F80001, Sigma; 2.5 μg/ml) was added to the culture medium (control or 50% follicular fluid) and their effects on oocyte maturation were also investigated. The oocytes were pipetted into droplets of test medium (0.1 ml) in a tissue culture dish (# 3001, Falcon, Oxnard, CA). Each droplet contained 15–20 oocytes and was covered with light paraffin oil. The oocytes were cultured for a specified period of time under 5% CO_2 in humidified air at 37 °C.

After the incubation, the oocytes were examined by Nomarski interference contrast microscopy. Oocyte maturation (resumption of meiosis) was expressed as the percentage of oocytes showing germinal vesicle breakdown (GVBD) to the total number of oocytes examined.

Statistical analysis. Significant statistical group differences were analyzed using χ^2 analysis with Yates' correction. A p-value less than 0.05 was considered significant.

Results

Effects of porcine follicular fluid on hamster oocyte maturation. Immature hamster oocytes with germinal vesicle (GV) were cultured in the GH-2 medium (control) or in 50% porcine follicular fluid from small or medium follicles. The cultured oocytes were scored for GVBD at specified times (fig. 1). The maturation of oocytes cultured in 50% follicular fluid was not completely inhibited and the percentage of GVBD gradually increased during oocyte maturation. GVBD after 1.5 h of culture was 90% in the control medium and 63% in 50% follicular fluid from small follicles (table 1). The same was noted in the experiment with denuded oocytes, although the extent of inhibition was less than that of the cumulus-enclosed oocytes. GVBD of denuded oocytes cultured for 1.5 h was 84% for the control and 70% for the 50% follicular fluid from small follicles. By the sixth hour of culture, in both the control and the 50% follicular fluid, oocytes had matured to the metaphase I stage without any significant differences.

By comparing the inhibitory effects of follicular fluid in relation to the original follicle size, the effect was more pronounced in fluid from small (63%) than from medium (79%) follicles (fig. 2).

Effect of human follicular fluid on hamster oocyte maturation. Hamster oocytes were cultured in 50% human follicular fluid aspirated from small or large follicles. The inhibitory effects were essentially the same as those of porcine follicular fluid. After 1.5 h of culture, only 45% of the oocytes resumed meiosis (GVBD) in human follicular fluid from small follicles and 60% in that form large follicles (control medium, 90%) (fig. 2).

Inhibitory effects of dbcAMP. The addition of 100 μM dbcAMP to the culture medium caused a significant inhibition of hamster oocyte maturation (80% of the cumulus-enclosed oocytes and 69% of the denuded oocytes matured) when compared to control oocytes 1.5 h after culture (table 1). The mixture of porcine follicular fluid and dbcAMP reduced GVBD significantly (45% of cumulus-enclosed and 44% of denuded oocytes matured).

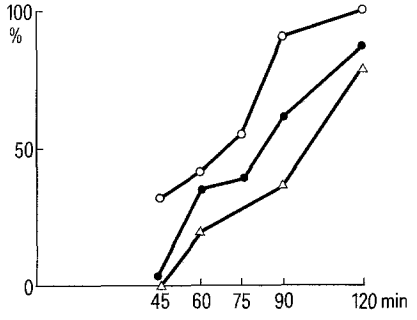

Figure 1. Maturation of hamster oocytes cultured in GH-2 (control), in 50% follicular fluid (porcine, small follicle), or in GH-2 with 5 μg/ml LH; the percentage of germinal vesicle breakdown is presented for various times. –○–, control; –●–, follicular fluid; –△–, LH 5 μg/ml.

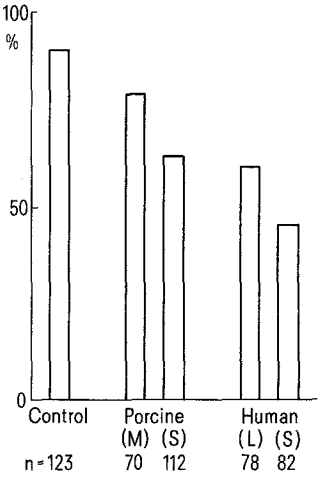

Figure 2. Inhibitory effects of porcine and human follicular fluid on cumulus-enclosed hamster oocyte maturation; the percentage of germinal vesicle breakdown after 1.5 h of culture.

Table 1. Effects of follicular fluid and dibutyryl cAMP on hamster oocyte maturation in vitro

Medium	No. of oocytes					
	Cumulus-enclosed			Denuded		
	Total	GV	GVBD (%)	Total	GV	GVBD (%)
Control	123	12	111 (90)	87	14	73 (84)
Porcine ffl (small)	112	42	70 (63)[a]	98	29	69 (70)[b]
Human ffl (small)	82	45	37 (45)[a]	–	–	–
DbcAMP (100 μM)	131	26	105 (80)[b]	93	29	64 (69)[b]
Porcine ffl + dbcAMP	66	36	30 (45)[c]	55	31	24 (44)[d]

GV, germinal vesicle; GVBD, germinal vesicle breakdown; ffl, follicular fluid; [a] $p < 0.01$, [b] $p < 0.05$, different from control; [c] $p < 0.05$, [d] $p < 0.01$, different from porcine ffl.

Table 2. Effects of gonadotropins on hamster oocyte maturation in vitro

Medium	No. of oocytes					
	Cumulus-enclosed			Denuded		
	Total	GV	GVBD (%)	Total	GV	GVBD (%)
Control	123	12	111 (90)	87	14	73 (84)
FSH (2.5 μg/ml)	80	69	11 (14)[a]	69	17	52 (75)[b]
LH (0.5 μg/ml)	99	30	69 (70)[a]	47	18	29 (62)[a]
LH (5 μg/ml)	151	97	54 (36)[a]	154	102	52 (34)[a]
LH (5 μg/ml) + porcine ffl	86	74	12 (14)[c]	–	–	–

[a] $p < 0.01$, different from control; [b] Not significant; [c] $p < 0.01$, different from LH (5 μg/ml).

Effects of gonadotropins on oocyte maturation in vitro. FSH (2.5 μg/ml in GH-2) significantly reduced GVBD (14%) after 1.5 h of culture and was more effective than 50% porcine or human follicular fluid. However, GVBD of denuded oocytes was not significantly altered in FSH medium (table 2). LH did not relieve the meiotic arrest due to porcine follicular fluid. LH at 0.5 and 5 μg/ml inhibited meiosis in both cumulus-enclosed and denuded oocytes (fig. 1 and table 2). Moreover, with 5 μg/ml LH plus 50% porcine follicular fluid, GVBD after 1.5 h of culture was significantly reduced (14% of cumulus-enclosed oocytes matured).

Discussion

Our data clearly show that porcine and human follicular fluid inhibit the maturation of isolated hamster oocytes. Oocytes cultured in 50% follicular fluid matured more slowly than in the control medium but with prolonged incubation, most of the oocytes resumed meiosis even in the presence of follicular fluid. This could possibly be due to differences in the in vitro and in vivo conditions or to some other factors yet unknown.

Cumulus cells are reported to participate in the transfer of OMI to oocytes and it was demonstrated that the maturation of denuded oocytes is not inhibited by follicular fluid[26]. Cumulus cells have cellular processes extending through the zona pellucida to connect directly with oocyte cytoplasma by gap junctions[1,6] assuring the exchange of various substances[10]. Therefore, OMI may also be transferred from cumulus cells to oocytes by gap junctions and inhibit oocyte maturation. In our experiments, however, follicular fluid also inhibited the maturation of denuded oocytes, although this effect was more pronounced in cumulus-enclosed oocytes. Thus, OMI secreted from granulosa cells into follicular fluid seems to affect oocytes directly, but the mediating role of cumulus cells may be important for the regulation of oocyte maturation.

Follicular diameter is an essential index of the maturation stage of follicles. Stone et al.[21] studied the inhibitory effects of porcine follicular fluid from small, medium, and large follicles and found in agreement with our results that the inhibitory effect was greatest when fluid from small follicles was used.

Since Cho et al.[2] reported that dbcAMP inhibits mouse oocyte maturation reversively, the role of cAMP on oocyte meiotic arrest has been investigated in many laboratories. Maintenance of the cAMP level in a cumulus-oocyte complex by a phosphodiesterase inhibitor (IBMX, theophilline), adenylate cyclase activator (cholera toxin, Forskolin), or FSH has been shown to inhibit oocyte maturation[17,20] and a decrease in cAMP in oocytes to promote its resumption[3,19]. Our results indicate that dbcAMP inhibits isolated hamster oocyte maturation and that this inhibition is more pronounced in cumulus-enclosed than in denuded oocytes.

FSH inhibits oocyte maturation transiently, and this inhibition correlates with elevated cAMP levels in cumulus-oocyte complexes[20]. According to some reports[3,4], increased cAMP in cumulus cells is transferred to oocytes by gap junctions to inhibit maturation. However, Schultz et al.[20] confirmed that cAMP in mouse cumulus cells is not transferred to oocyte cytoplasm and that adenylate cyclase is present in mouse oocytes. Therefore, with increased cAMP levels in cumulus cells, some mediator other than cAMP may be transferred to oocytes via gap junctions. Such a mediator would assist in maintaining the cAMP level in the oocyte and arrest meiosis. This mediator may possibly be OMI that is generated or activated by cAMP in cumulus cells.

Downs and Eppig[5] reported synergistic inhibition by follicular fluid and dbcAMP of mouse oocyte maturation. We observed a similar cooperative effect with 50% porcine follicular fluid and 100 μM dbcAMP on hamster oocyte maturation. The same was found in rat oocytes by Törnell et al.[28]. These findings confirm that cAMP and follicular fluid (OMI) cooperatively inhibit oocyte maturation.

LH is considered to be the meiosis-inducing hormone. It apparently decreases or inactivates OMI, enabling the oocytes in follicles to resume meiosis both in vivo and in vitro. It is reported that LH elevates the cAMP level in follicles and on the other hand, the cAMP level in maturing oocytes decreases[13,14,20]. LH has been believed to overcome the inhibitory effects of follicular fluid on the in vitro maturation of isolated oocytes. However, in our study, LH failed to show this effect and tended to inhibit the resumption of meiosis by acting directly on the isolated oocytes. Its inhibitory effect was greater when the oocytes were cultured in the medium containing both follicular fluid and LH. This may have been due to the increase in cAMP in the cumulus-oocyte complex by the direct effect of LH, which is similar to that of FSH. This shows that the direct effect of LH on isolated oocytes may differ from that on oocytes in follicles.

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Ultrastructural investigation of fetal rat brain hemisphere tissue in nonadherent stationary organ culture

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Summary. Fetal rat brain fragments grown in nonadherent stationary organ culture for 50 days have been investigated ultrastructurally. Synaptogenesis and myelin formation occurred at the same time as the corresponding time-dependent events in the developing brain in vivo. Intermediate junctions were observed between cellular processes lining a central cavity in the fragments and later associated with astrocytes at the surface. Gap junctions and tight junctions were also present. In some fragments cilia were observed in the central cavity. Subependymal basement membrane labyrinths were observed in all fragments after 10 days in culture. The ultrastructural characteristics and the tissue-like structure in general were preserved for at least 50 days in this tissue culture system. The brain fragments may therefore be a valuable supplement to existing culture methods for nervous tissue.

Key words. Nonadherent organ culture; rat brain tissue; ultrastructure.

Introduction

In the developing brain in vivo there is a differentation of primitive neuroectodermal cells into neurons and glial cells which together make up the nervous tissue. In recent years several tissue culture models have been elaborated to study this evolution in vitro. In these models it has been possible to investigate both morphological, biochemical and electrophysiological differentiation[3, 6, 8, 9, 11, 12, 14–16, 20, 21, 24, 27, 29–33, 37, 38]. An objection against these cultures as models for nervous tissue has been that the original histiotypic structure of the tissue is destroyed, either by dissociation or by reorganization due to adherence to a substratum. In order to avoid these problems we have recently described a method for the maintenance of fetal rat brain fragments in nonadherent stationary organ culture for 50 days[35]. Light microscopic and immunohistochemical investigations demonstrated good correlation between cellular differentiation and migration in the fragments and in vivo. The aim of the present report was to study development and organization of cells and ultrastructural characteristics of nervous tissue in the brain fragments as revealed by transmission electron microscopy.

Materials and methods

Animals. Rats of the inbred BD IX- strain were used[10].
Nonadherent organ culture. Whole brains were obtained from fetal rats at the 18th day of gestation. The brain lobes were dissected free, the meninges carefully removed and the cortical tissue cut into approximate cubes, measuring about 800 µm in all directions. Dulbecco's modification of Eagle's medium (DMEM, Flow Laboratories, Glasgow, Scotland) supplemented with 10% heat-inactivated newborn calf serum, four times the prescribed concentration of 100 × non-essential amino acids, L-glutamine (200 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) was used. The fragments were grown in multiwell dishes (Nuncleon, Nunc Plastic, Denmark) base-coated with 0.5 ml 0.75% semisolid agar (Agar Noble, Difco Laboratories, Detroit, U.S.A.). One fragment was transferred to each well and 1 ml medium was added. The fragments were maintained at 37 °C in 5% CO_2 in air with 100% relative humidity. The medium was changed every second day. The culture period was 50 days and the fixation times were days 0, 1, 2 and 5 and every 5th day thereafter until day 50.
Transmission electron microscopy (TEM). Fragments were fixed for 24 h in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, postfixed for 1 h in 1% osmium tetraoxide in cacodylate buffer and dehydrated in graded ethanol concentrations to 100%. After 2 × 10 min in propylenoxide and 12 h in a 1:1 mixture of propylenoxide/Epon 812, the fragments were embedded in pure Epon 812. The final polymerization was carried out at 60 °C for 3 days.