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A Preliminary Report on the Effect of Forskolin on the Meiotic Progress of Germ Cells into Prophase I in Fetal Rabbit Ovaries Cultured *in vitro*

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Abstract The effects of forskolin, which is an adenylate-cyclase activator, on the progress into meiotic prophase I was investigated in fetal rabbit ovaries of 26 days of gestation, using organ culture (stainless grid-lens paper method, according to Fell and Weiss, 1965) for 12 days.

The explanted ovaries became flattened in their macroscopic shape, and necrosis in the deeper parts of the ovarian tissue was recognized in histology, caused by adhesion to the lens-paper, with the extra-ovarian connective tissue proliferating into the basic medium.

In order to estimate the degree of progress into meiotic prophase I, the percentages of oogonia (mitotic and resting stage) and oocytes (leptotene, zygotene, pachytene and diplotene) were compared for all germ cells counted in 3 groups of organ cultures-0 μM (*control*), 20 μM (*f20*) and 100 μM (*f100*) forskolin. Although oocytes in diplotene were never observed, germ cells transforming into oocytes were recognized in all explanted ovaries of each group, except for the control, in which no oocytes in pachytene were found. According to the comparisons of the ratio of total oocytes to all germ cells counted in *control* (4.0%), *f20* group (21.1%) and *f100* group (9.0%), the degree of progress of meiotic prophase I in *f100* group was not significantly different from that in *control,* although the degree of progress was somewhat higher. On the other hand, the degree of progress in *f20* group was significantly higher than in *control* ($p < 0.01$).

It is suggested that the addition of forskolin to the basic medium of organ culture is effective in promoting progress into meiotic prophase I.

INTRODUCTION

It is suggested that the number of mitochondria decreases and their function declines in germ cells transforming into oocytes (SASABE *et al.,* 1990, in press). These are synchronous with the decline in division-activity of the oocytes. According to the fact that the growth of oocytes lies dormant in the absence of follicular growth (MAULEON and MARIANA, 1977), the various enzyme activities regulating energy production and the formation of the mitotic and/or meiotic apparatus would be declining in this period. In general, the production of specific proteins is carried out through the interposition of various enzymatic reactions in functional cells, and most of the enzymatic reactions are activated by cAMP contained in

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cytoplasm, in accordance with the activation of adenylate-cyclase resulting from extracellular stimuli such as hormones (Beck, 1984).

Although the role of cAMP is different in each organ and each cell, it is generally accepted that cAMP is regarded as one of the important signals giving rise to a series of enzymatic reaction (Finean *et al.*, 1981). The effects cAMP on oocyte maturation have also been investigated (Tsafriri *et al.*, 1972; Schorderet-Slatkine and Baulieu, 1982; Ekholm *et al.*, 1984; Thibault *et al.*, 1987; Hubbard and Price, 1988) and the results suggest that cAMP is an oocyte-maturation inhibitor. Although the mechanism of the inhibition by cAMP has never been clarified, an adequate quantity of cAMP for inhibition of oocyte maturation seems to be transferred from the cumulus cells through the gap junction between the oocyte and cumulus cells (Thibault *et al.*, 1987).

The resumption of oocyte maturation *in vivo* results from a transient increase in intrafollicular content of cAMP by the stimulation of an LH surge. This fact is seemingly contradictory, however, cAMP is produced excessively in the cumulus and granulosa cells by LH surge or by the addition of forskolin, which is an adenylate-cyclase activator, and the cAMP content becomes high. Then excessive accumulation of hyaluronic acid among the cumulus cells causes destruction of the gap junctions. Thus, the resumption of meiotic division of oocytes follows this destruction (Yano *et al.*, 1987).

Byskov (1974; 1975), Challoner (1975) and Sasabe *et al.* (1989) have suggested that some chemical signals, supplied to the oogonia from the rete ovarii and/or the connective tissue derived from ovarian medulla, are responsible for transformation of oogonia into oocytes.

The purpose of the present study was to investigate the effects of forskolin on the meiotic progress of germ cells into prophase I in explanted ovaries from the fetal rabbit.

MATERIALS and METHODS

Animals and recovery of ovaries

Seventeen female fetuses of Japanese White rabbits at 26 days of gestation were collected aseptically, by abdominal incision, from pregnant uteri of 5 does. After sexing of the fetuses, ovaries of each female fetus were recovered aseptically, placed in prewarmed (38°C) phosphate buffer solution (PBS) containing 5% inactivated rabbit serum, and trimed by excision of extra fat and connective tissue. The ovaries were washed twice with PBS and once with basic medium and then cultured as follows.

Culture procedures

The washed ovaries were explanted in organ culture according to a technique described by Fell and Weiss (1965). In each small dish, fewer than five fetal ovaries were cultured on the lens-paper over the stainless grid placed just beneath the surface of the basic medium (approximately 3 mℓ) composed of 85% TCM 199 (Gibco, Grand Island, N. Y.), 15% inactivated rabbit serum, 10 units of penicillin/mℓ, and 10 mg of kanamycin/mℓ, at pH 7.4. Forskolin (Calbiochem, La Jolla, CA) was dissolved in ethanol (2 mM) and stored at −83°C until use. Three series of organ culture were carried out with no forskolin (*control, f0*), 20 μM (*f20*) and 100 μM (*f100*) forskolin. The concentration of ethanol was standardized at 1.5% in each culture medium. Ovaries in the dish were cultured for 12 successive days at 38.5°C in an atmosphere of 5% CO_2 and 95% air. At the end of culturing, the ex-

planted ovaries were separated from the lens-paper by 0.05% trypsin.

Histological procedures

The cultured ovaries were fixed in Bouin's fixative, dehydrated with graded ethnol, embedded in paraffin wax for serial sections at 6 μm, and stained with hematoxylin-eosin (H. E.).

To evaluate the effect of forskolin on the onset of meiosis, all germ cells in 8 microscopic fields ($\times$400) selected randomly in each ovarian specimen were counted in all serial sections of the ovaries. Frequencies of oogonia (mitotic stage and resting stage) and of oocytes in four stages of meiotic prophase I (leptotene, zygotene, pachytene and diplotene) were estimated as proportions of each stage to total germ cells counted in the ovarian tissue.

Statistical analysis

Data were transformed to arcsin $\sqrt{\%}$ and then examined by analysis of variance. Differences among means of proportion of oocytes in the experimental series were tested by Duncan's multiple range test.

RESULTS

Macroscopic findings on explanted ovaries

Although the sizes of explanted ovaries were similar to those of fetal ovaries at 26 days of gestation, their shapes were slightly flattened because of adhesion of cells derived from the proliferating ovarian connective tissue to the lens-paper.

Histological observations on explanted ovaries

The microscopic ovarian structure was changed considerably due to occurrence of excessive adhesion of ovarian tissue to the lens-paper. Disordered layers of cells with condensed nuclei were recognized in deep sites of the ovarian tissue (the innermost part of the ovarian cortex); moreover, extensive necrosis was recognized in the inner part (the ovarian medulla) (Figs. 1 & 2).

In order to estimate the degree of progression into meiotic prophase I, distributions of oogonia and oocytes in the different stages, in the explanted ovaries in three different culture media, are compared in Table 1. There were no oocytes in diplotene nor any follicles in any of the explanted ovaries.

In *control* ovaries (Fig. 3), the average proportion of oogonia in resting stage was very high (approximately 90% of all germ cells counted), and ratios of oogonia in mitotic stage and oocytes in leptotene and zygotene were approximately 6.4%, 3.7% and 0.3%, respectively. No oocytes in pachytene were observed.

In ovaries of *f20* group (Fig. 4), the proportion of oogonia in mitotic stage was approximately 2.7% (range 1.9-3.7%). The progress into meiotic prophase I was further advanced in all ovaries in *f20* group (leptotene, 8.0%; zygotene, 9.3%) than in those of the other groups; and oocytes in pachytene constituted 3.8%, even though there were none (*control*) or very few (*f100*) oocytes in pachytene in the other two groups.

In ovaries of *f100* group, the proportions of oogonia in mitotic and resting stages were approximately 3.3% and 87.7% on the average, respectively. The progress into meiotic prophase I had advanced more than in *control* group, though it was far below that of *f20*

Table 1. Distribution of germ cells in different stages of mitotic and/or meiotic division in the organ-cultured ovaries.

Group[1]	No. of rabbits	No. of germ cells[2]	Ratio of oogonia (%)		Ratio of oocytes (%)		
			mitotic	resting	leptotene	zygotene	pachytene
control	*f0*-1	368	7.9	85.1	6.2	0.8	0.0
	f0-2	209	7.3	89.3	2.9	0.5	0.0
	f0-3	278	9.7	83.5	5.8	0.4	0.0
	f0-4	271	8.8	90.5	0.7	0.0	0.0
	f0-5	179	2.2	97.8	0.0	0.0	0.0
	f0-6	306	9.2	88.5	2.3	0.0	0.0
	f0-7	421	0.7	94.7	5.1	0.2	0.0
	total	2032	6.4	89.6	3.7	0.3	0.0
f20	*f20*-1	201	1.9	71.6	10.1	13.9	3.5
	f20-2	196	3.3	53.8	13.7	22.6	6.6
	f20-3	309	3.7	69.7	7.8	10.4	8.4
	f20-4	259	2.8	91.4	3.9	1.9	0.0
	f20-5	279	2.0	88.3	6.8	2.5	0.4
	total	1244	2.7	76.2	8.0	9.3	3.8
f100	*f100*-1	233	3.9	91.4	4.7	0.0	0.0
	f100-2	364	3.3	84.0	6.3	5.8	0.6
	f100-3	168	4.2	93.4	1.8	0.6	0.0
	f100-4	297	2.7	83.3	6.3	6.7	1.0
	f100-5	270	3.0	90.7	2.6	3.7	0.0
	total	1332	3.3	87.7	4.7	3.9	0.4

[1] *Control, f20* and *f100* indicate, repectively, no addition of forskolin and additions of 20 μM and 100 μM forskolin in organ cultures.

[2] Total number of germ cells counted in eight microscopic fields (×400) in all serial sections.

group. In general, the degree of meiotic division in prophase I of *f100* group remained at values intermediate between *control* and *f20* group.

Ratios of oocytes, including different stages, to all germ cells counted in each of the 3 groups are given in Table 2. The ratio in *f20* group was significantly higher than in *control,* but the difference between *control* and *f100* groups was not significant.

DISCUSSION

It is generally accepted that necrosis is apt to occur in the deeper sites of ovarian tissue when ovaries are explanted in organ culture, because of a shortage of nutrient supply and O_2 in those sites (Challoner, 1975; Prépin *et al.,* 1985). According to Blandau and Odor (1972) and Mazur and YoungLai (1986), the rete ovarii and ovarian medulla in fetal mouse ovaries and neonatal rabbit ovaries degenerated immediately after explantation to organ culture. However, the tissue of the ovarian cortex adjacent to the surface was healthy, the proliferating germ cells were transforming into oocytes, and even folliculogenesis was recognized in some explanted ovaries. In the present results, although necrosis was found in the deeper parts of ovarian tissue, mainly in the medulla, advanced meiotic division into

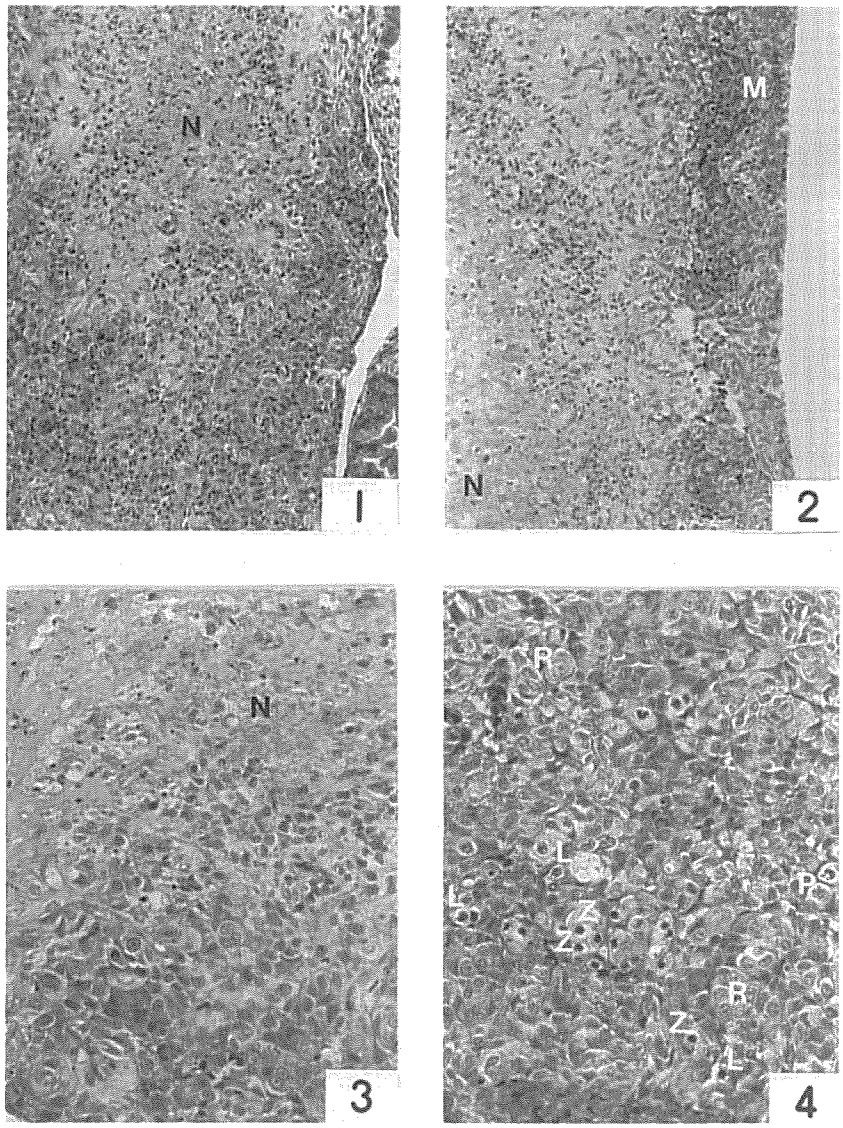

Fig. 1. A *control* explanted ovary. A region with necrosis (N) is recognized in the deeper site of ovarian tissue. The tissue of the ovarian cortex adjacent to the surface seems to be healthy.
H. E. ×100.

Fig. 2. An *f20* explanted ovary. A region with necrosis (N) is again recognized in the deeper site of ovarian tissue. The tissue of the ovarian cortex adjacent to the surface seems to be healthy, and germ cells transforming into oocytes (M) are observed.
H. E. ×100.

Fig. 3. Tissue of ovarian cortex of a *control* explanted ovary. Germ cells with no abnormalities histologically are recognized; however, most of them are oogonia in resting stage, and few oocytes are observed.
N, necrosis. H. E. ×200.

Fig. 4. Tissue of ovarian cortex of an *f20* explanted ovary. Many oocytes in leptotene (L) and zygotene (Z), and a few oocytes in pachytene (P), are observed.
R, oogonium in resting stage. H. E. ×200.

Table 2. The ratio of oocytes[1] to total number of germ cells counted.

Group	Ratio of oocytes
control	4.0%[a]
f20	21.1%[b]
f100	9.0%[ab]

[1] The ratios of germ cells transformed into oocytes (the numbers of oocytes in leptotene, zygotene and pachytene are combined).

prophase I, up to pachytene, was noted in all groups, especially in *f20* group. However, the number of oocytes transformed from oogonia was small, and the degree of progress to meiotic prophase I seemed to be lower than in ovaries *in vivo* at seven days of age (equivalent in time to fetal ovaries at 26 days of gestation cultured *in vitro* for 12 days) (Peters *et al.*, 1965; Sasabe *et al.*, 1989). According to Byskov (1974; 1975), Challoner (1975), and Sasabe *et al.* (1989), the rete ovarii and the connective tissue derived from the ovarian medulla seemed to play a role as an inducer to transform oogonia into oocytes, a process which might include some chemical signals. If so, the lower degree of progress of meiotic division into prophase I in explanted ovaries may be caused by a shortage of factors promoting the transformation from oogonia into oocytes in the ovarian cortex, because of degeneration of the ovarian medulla, where the rete ovarii and the connective tissue are located and might be secreting the promoting factors.

cAMP is regarded as one of the chemical factors in regulation of cellular function (Finean *et al.*, 1981; Beck, 1984), and effects of cAMP on the resumption of oocyte maturation have been investigated (rat—Tsafriri *et al.*, 1972; Ekholm *et al.*, 1984; *Xenopus*—Schorderet —Slatkine and Baulieu, 1982; rabbit—Thibault *et al.*, 1987; guinea pig—Hubbard and Price, 1988). According to most of these reports, cAMP is an oocyte-maturation inhibitor. Oocytes matured automatically when recovered from fully ripe follicles and cultured *in vitro* (Pincus and Enzmann, 1935; Edwards, 1965); however, this automatic maturation was inhibited by addition of cAMP and/or the inhibitor of phosphodiesterase. Yano (1989) showed that adenylate-cyclase involved in production of cAMP is located on the plasma membrane of the oocyte. An amount of cAMP adequate to inhibit maturation was supplied by not only the oocyte, itself, but also continuously by cumulus cells through the gap junction (Thibault *et al.*, 1985; Yano, 1989).

It is generally accepted that forskolin is an activator of adenylate-cyclase. Yano (1989) and Ekholm *et al.* (1984) investigated the effect of cAMP on the resumption of meiosis with forskolin. They found that the content of cAMP within explanted ovaries was increased by addition of forskolin, and that the degree of increase in cAMP was dose-dependent on forskolin. In the present study, the maturation rate of oocytes *in vitro* was promoted by addition of 20 μM forskolin, but not significantly by addition of 100 μM forskolin. No follicular cells were recognized around any oocytes in the present study. So it is suggested that there was no continuous supply of cAMP from oocyte-surrounding cells to the oocyte cytoplasm, and that forskolin probably affected the plasma membrane directly to cause ac-

tivation of adenylate-cyclase, thus inducing a change in the intra-oocyte content of cAMP.

In the present results, the degree of progress of meiotic division into prophase I of *f100* group was slightly higher than in *control* group, though a significant difference was not recognized between them. On the other hand, the degree of this progress in *f20* group was significantly higher than in *control* group. Degeneration of the deeper parts of ovarian tissue, which contained germ cells in further advanced stages of meiotic prophase I (SASABE *et al.*, 1989), was observed, so it is not possible to estimate exactly the degree of progress of meiotic division into prophase I. Although the optimum dose of forskolin to induce meiotic division has never been investigated sufficiently, it is suggested that forskolin is effective in promoting progress in meiotic division into prophase I.

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器官培養家兎胎子卵巣における第一減数分裂前期の進行に及ぼす forskolin の影響（予報）

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胎齢26日の家兎胎子より卵巣を摘出し，器官培養することによって adenylate-cyclase activator である forskolin が減数分裂の開始に影響を及ぼすかどうかについて検討した。

金網台ーレンズペーパー法を用いて連続12日間，卵巣を器官培養したところ，卵巣表面の結合組織が卵巣を被嚢する状態にまで増殖し，卵巣は扁平化し，組織の崩壊が認められた。卵巣中心部には広範な壊死が見られたが，卵巣表層部の組織は健全であり，対照区，forskolin 20 μM 添加 (*f20*) 区および 100 μM 添加 (*f100*) 区の全ての区で減数分裂に移行した卵母細胞が観察された。生殖細胞の減数分裂への移行の程度は対照区で4.0%，*f20* 区で21.1%，*f100* 区で9.0%であった。*f100* 区での減数分裂の進行程度は対照区のものと比較してやや早い傾向が認められたものの有意ではなく，*f20* 区で有意に早まった。

これらのことから，最適濃度は明確ではないが，forskolin の培養液への添加は生殖細胞の減数分裂開始時期の促進に有効であることが示唆された。