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Effects of Puromycin and Actinomycin D on the HCG-Induced Expansion of Cumulus Oophorus *in vitro*.

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Puromycin과 Actinomycin D가 卵丘細胞의 分散에 미치는 影響

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摘 要

哺乳動物의 排卵時 濾胞卵子의 成熟再開와 더불어 卵子를 緻密하게 둘러싸고 있는 卵丘細胞들의 分散이 일어난다. 이 現象은 生殖巢刺戟호르몬의 促進을 받은 卵丘細胞들이 細胞間隔에 多量의 뮤코糖을 分泌함으로써 이루어지는데 이 때 cAMP가 第二 傳達者로 作用을 한다고 알려져 있다. 본 實驗에서는 卵子-卵丘 複合體를 培養하면서 HCG (10 IU/ml)에 의해 誘導된 卵丘細胞의 分散에 puromycin과 actinomycin D가 미치는 영향을 調査한 바 다음과 같은 結果를 얻었다.

1. Puromycin은 2 μg/ml의 濃度에서 卵丘細胞의 分散을 현저히 抑制하였으며 이 效果는 可逆的이었다.
2. Puromycin의 分散抑制效果는 HCG의 刺戟기간 (3시간) 뿐 아니라 뮤코糖의 合成時期 (3~18시간)에서도 나타났다.
3. Actinomycin D는 0.025 μg/ml의 濃度에서부터 卵丘細胞의 分散을 抑制하기 시작하였다.
4. Actinomycin D의 分散抑制效果는 부분적인 可逆性을 나타내었으며 0.1 μg/ml의 濃度에서는 非可逆的인 沮害效果를 나타내었다

위의 結果로부터 HCG의 卵丘細胞 分散誘導過程에는 蛋白質 내지 RNA의 合成過程이 관여하는 것으로 推定되며 따라서 cAMP는 轉寫 내지 解讀水準에서 卵丘細胞의 分散을 調節하는 것 같다.

INTRODUCTION

Mammalian oocyte in the antral follicle is surrounded by an investment of tightly packed

cumulus cells, which is called cumulus oophorus. A number of investigators have reported the presence of gap junctions in the areas where the cumulus cell processes contact the oolemma and suggested that these cells may supply nutrients or other substances to the oocyte through these junctions (Anderson and Albertini, 1976; Gilula *et al.*, 1978; Heller *et al.*, 1981). Prior to ovulation, concomitant with oocyte maturation, the cumulus structure becomes expanded as a result of the disposition of a hyaluronic acid matrix between the cumulus cells. In many mammals the matured ovum is ovulated while enclosed in this mucified mass of cumulus cells and only those oocytes with expanded cumulus can be penetrated by spermatozoa normally (Austin, 1961; Testart *et al.*, 1983). Cumulus expansion *in vivo* takes place following either the spontaneous surge of endogeneous LH or administration of exogenous human chorionic gonadotrophin (HCG) (Eppig, 1980; Hillensjö *et al.*, 1976; Schuetz and Schwartz, 1979). Expansion of cumulus *in vitro*, in isolated cumuli or follicles, can be induced by gonadotrophins, dibutyryl cyclic AMP (dbcAMP), phosphodiesterase inhibitors and cholera toxin. Therefore, it has been suggested that the effect of gonadotrophins on cumulus expansion is mediated by cAMP (Dekel and Kraicer, 1978; Dekel *et al.*, 1981; Eppig, 1979; Kwon, 1982; Phillips and Dekel, 1982). But there has been few reports about the way how cAMP acts on the cumulus cells and stimulates them to synthesize hyaluronic acids.

The present experiments were undertaken in order to know whether protein or RNA synthesis is required for the cumulus expansion stimulated by HCG *in vitro*. If the macromolecule synthesis is involved in the expansion, it would be able to assume that cAMP acts on the transcriptional or translational level in the cells.

MATERIALS AND METHODS

Four weeks old A-strain female mice were injected with 3 IU of pregnant mare serum (PMS). Forty-eight hours later the large Graafian follicles were punctured with needles and the oocyte-cumulus complexes were removed in TC medium 199. The complexes were washed by serially transferring with mouth-operated capillary pipettes three times through watch glasses containing 1 ml of the medium. The culture medium used were TC medium 199 with Hank's salts supplemented with 10% bovine serum (Difco). This medium is referred to as "plain medium" throughout this paper. As a cumulus expansion stimulators, HCG (10 IU/ml) were added and as metabolic inhibitors, puromycin and actinomycin D (Sigma) were added at the concentration of 0.5~4 μg/ml and 0.025~0.1 μg/ml, respectively. Stock solutions of puromycin and actinomycin D were prepared by dissolving them in 0.9 % NaCl solution at a concentration of 2.5 mg/ml and 0.1 mg/ml, respectively and were stored at −20°C in small aliquots.

The oocyte-cumulus complexes were incubated in a drop of the medium under paraffin oil using 50 mm disposable petri dish (Samwoo corp. Seoul) at 37°C overnight, in an

atmosphere of 5% CO_2 in fully moistened air (Brinster, 1963), and then examined for cumulus expansion under inverted microscope (Nikon). Some of the oocyte-cumulus complexes were fixed with acetic alcohol, and stained with aceto-orcein for further observation of the nuclear changes using a phase contrast microscope (Nikon Apophot).

RESULTS AND DISCUSSION

Effect of Puromycin on HCG-Stimulated Cumulus Expansion

When the oocyte-cumulus complexes were incubated for 18 hours in HCG containing medium, most of the cumuli oophori (94%) expanded fully in response to the hormone and the dispersed cumulus cells showed round and healthy normal shape (Table 1, Figs. 1, 5). But the expansion of the complexes incubated in puromycin-added medium was suppressed by the inhibitors in a dose dependent fashion (Table 1). When the complexes were cultured in the continuous presence of 2 μg/ml of puromycin, the expansion of the cumulus was markedly inhibited (26%) and at the same time the shape of some cumulus cells became abnormal and began to show the sign of degeneration (Fig. 7). When the above complexes were squashed and stained, some of them appeared to have a condensed and pyknotic nucleus (Fig. 8) instead of a normal round nucleus (Fig. 6). It was reported that the length of exposure to gonadotrophin (FSH) required to stimulate cumulus expansion *in vitro* was two hours and the peak hyaluronic acid synthesis occurred during 3~6 hour period of FSH stimulation (Eppig, 1980). It was found in this experiment that three hours was enough for the HCG to stimulate cumulus expansion *in vitro* (Table 2). To determine whether puromycin exerts its influence on the complexes during preincubation time with HCG or after HCG stimulation, the complexes were incubated for three hours in the medium containing both HCG and puromycin and transferred to plain medium and cultured for further 18 hours.

As shown in Table 2, puromycin markedly blocked HCG-stimulated cumulus expansion during the preincubation time. The complexes appeared remarkably similar to freshly isolated complexes (Fig. 2). The expansion was also suppressed when they were cultured in the puromycin medium after being stimulated by HCG for three hours. Therefore, puromycin seemed to affect the complexes throughout the HCG stimulating stage and hyaluronic acid synthesis stage.

In order to know whether puromycin in the medium gives an irreversible damage to the cumulus cells during the three hour culture, the complexes preincubated in the medium containting puromycin (2 or 4 μg/ml) were transferred to HCG medium and then cultured for further 18 hours. As shown in Table 2, those cumuli oophori which had been exposed to the inhibitors expanded normally and the nucleus of the cells appeared to be healthy and similar to those of control (Figs. 3, 4, 6). Thus, it is thought that puromycin did not give an irreversible damage to the cumulus cells during the preincubation time.

Table 1. The inhibitory effect of puromycin on the HCG-induced expansion of cumuli oophori isolated from mice *in vitro*.

Additions to plain medium*	Total	Number expanded	Percent expanded
HCG@	47	44	94
HCG+0.5 μg/ml puromycin	55	49	89
HCG+1 μg/ml puromycin	55	40	73
HCG+2 μg/ml puromycin	53	14	26
HCG+4 μg/ml puromycin	51	0	0

*Plain medium: TC 199, 90%+bovine serum, 10%
@HCG : 10 IU/ml
The oocyte-cumulus complexes were cultured for 18~20 hours in the continuous presence of HCG and puromycin.

Table 2. Effect of puromycin on the expansion of cumuli oophori induced by HCG in various culture conditions.

Preincubation medium* 3 hour culture	Incubation medium 18 hour culture	Total	Number expanded	Percent expanded	Number exps.
HCG@	plain medium	65	64	98	5
HCG+2 μg/ml puromycin	plain medium	60	10	17	5
HCG+4 μg/ml puromycin	plain medium	49	4	11	4
HCG	2 μg/ml puromycin	57	16	28	5
HCG	4 μg/ml puromycin	47	1	2	4
plain medium	HCG	34	32	94	3
2 μg/ml puromycin	HCG	40	34	85	3
4 μg/ml puromycin	HCG	41	34	83	3

*The oocyte-cumulus complexes were preincubated with or without puromycin for three hours and then transferred to the incubation medium and cultured for 18 hours in various conditions.
@HCG : 10 IU/ml

Effect of Actinomycin D on HCG-Stimulated Cumulus Expansion

Continuous presence of actinomycin D in the medium markedly suppressd the cumulus expansion from the concentration of 0.025 μg/ml and completely blocked it at the concentration of 0.05 μg/ml (Table 3). At the same time, some of the cumulus cells exposed to 0.05 or 0.1 μg/ml of actinomycin D for 18 hours showed the sign of degeneration. In fact, condensed and heavy pyknotic nuclei were observed among them when they were examined after staining as in the case of puromycin.

To determine whether actinomycin D affects the complexes during HCG stimulation period, the complexes preincubated in the medium containing both actinomycin D and HCG (10 IU) were cultured for further 18 hours in the plain medium. As shown in Table 4, the expansion of the cumulus was suppressed significantly (33%) by the inhibitors from the dose of 0.05 μg/ml. Therefore, it is thought that actinomycin D in the medium interfered with the stimulating process of HCG on the the cumuli oophori.

Table 3. The inhibitory effect of actinomycin D on the HCG-induced expansion of cumuli oophori *in vitro*.

Additions to plain medium*	Total	Number expanded	Percent expanded
HCG[@]	26	23	88
HCG+0.025 μg/ml actinomycin D	29	14	48
HCG+0.05 μg/ml actinomycin D	27	0	0
HCG+0.1 μg/ml actinomycin D	29	0	0

The oocyte-cumulus complexes were cultured for 18~20 hours in the continuous presence of HCG and actinomycin D.
*Plain medium : TC 199, 90%+Bovine serum, 10%
[@]HCG : 10 IU/ml

Table 4. Effect of actinomycin D on the expansion of cumuli oophori induced by HCG in various culture conditions.

Preincubation medium* 3 hour culture	Incubation medium 18 hour culture	Total	Number expanded	Percent expanded	Percent polar body formed
HCG[@]	plain medium	50	43	86	68
HCG+0.025 μg/ml actinomycin D	plain medium	40	33	83	69
HCG+0.05 μg/ml actinomycin D	plain medium	42	14	33	74
HCG+0.1 μg/ml actinomycin D	plain medium	55	1	2	60
plain medium	HCG	26	26	100	64
0.025 μg/ml actinomycin D	HCG	33	29	88	85
0.05 μg/ml actinomycin D	HCG	34	23	68	56
0.1 μg/ml actinomycin D	HCG	27	0	0	63

*The oocyte-cumulus complexes were preincubated with or without actinomycin D for three hours and then transferred to the incubation medium and cultured for 18 hours.
[@]HCG : 10 IU/ml

Expansion rate of the complexes exposed to actinomycin D for three hours and then transferred to plain medium was always higher than those of the complexes exposed to the drug for 18 hours continuously at the respective concentrations (Tables 3, 4).

In order to know whether actinomycin D caused an irreversible damage to the complexes during preincubation time, the complexes preincubated with the drug for 3 hours were transferred to HCG-containing medium and cultured for further 18 hours. 0.1 μg/ml of the drug appeared to give a fatal damage to the complexes, since few complexes were expanded in the following incubation (Table 4). But most of the complexes (68%) which had been exposed to 0.05 μg/ml of the drug expanded normally during the following culture time in HCG medium. So it seems likely that actinomycin D does not damage the cumulus cells irreversibly at this concentration.

From the results described above, it can be concluded that puromycin and actinomycin D inhibit the expansion of the cumuli oophori by suppressing the synthesis of protein or

RNA which is required for the expansion of the cumulus and therefore, the action of cAMP on the cumulus cells in inducing expansion seems to involve the process of macromolecule synthesis. However, the present experiments could not clearly exclude the possibility that the inhibition of cumulus expansion by the inhibitors is due to the general toxic effect of the inhibitors. In fact, the cumuli oophori exposed to the inhibitors for long time showed sign of degeneration as previously described. But from the fact that cumuli oophori incubated in the presence of the inhibitors for three hours and cultured further in HCG containing medium were morphologically indistinguishable from those incubated in control medium and the expansion of the cumuli occurred normally, it seems unlikely that the expansion of the cumulus was inhibited by the toxic effect of the inhibitors. In addition, spontaneous oocyte meiotic maturation did not appear to be affected by the inhibitors (Table 4).

There are a few reports about the regulation of cumulus expansion induced by gonadotrophin *in vivo* or *in vitro*. Eppig (1981 a, b) found that several sulfated glycosamino glycans (GAGS) inhibit FSH or dbcAMP stimulated cumulus expansion and hyaluronic acid synthesis by oocyte-cumulus complexes *in vitro*. He suggested that the sulfated GAGS may affect some process occurring after generation of cAMP and may function *in vivo* to block the response of the cumulus cells to FSH indigenous to the Graafian follicle prior to the preovulatory gonadotrophin surge.

At present, it is unknown whether the action of the GAGS on the cumulus cells has any relation to the process of macromolecule synthesis by the cells. But it is probable that the GAGS inhibits the cumulus expansion by suppressing the synthesis of some protein or RNA which is necessary for the expansion as the case of the inhibitors in this experiment. Further studies will be necessary to test this possibility and to elucidate the exact control mechanism of the cumulus expansion during ovulation.

SUMMARY

In order to know the mode of the action of gonadotrophic hormone on the expansion of cumuli oophori, oocyte-cumulus complexes isolated from Graafian follicles of mice were stimulated to expand *in vitro* with human chorionic gonadotrophin (HCG), and the effects of puromycin and actinomycin D on the expansion were examined. The complexes were cultured in medium TC 199 containing 10% bovine serum in the presence or absence of HCG and the inhibitors. Puromycin in the medium (0.5−4 μg/ml) suppressed the HCG-induced cumulus expansion dose dependently. This effect of puromycin was reversible. Puromycin affected the complexes throughout the HCG-stimulating stage (3 hours) and hyaluronic acid synthesis stage (3 18 hours). Actinomycin D also inhibited the expansion of the cumulus from the concentration of 0.025 μg/ml. But the effect of actinomycin D was not completely reversible and the drug appeared to give an irreversible damage to the

complexes at 0.1 μg/ml.

From the above results, it is suggested that RNA or protein synthesis is involved in the process in which HCG stimulates the cumulus cells to expand and therefore cAMP elevated by the gonadotrophin may control the expansion at the transcriptional or translational level.

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EXPLANATION OF FIGURES

Fig. 1. An oocyte-cumulus complex which had been preincubated with HCG (10 IU) for 3 hours and cultured for further 18 hours in plain medium. The cumulus is expanded. 300×

Fig. 2. A complex which had been preincubated with HCG and puromycin (2 μg/ml) for 3 hours and cultured for further 18 hours in plain medium. The cumulus is not expanded. 300×

Fig. 3. A complex which had been preincubated with puromycin (2 μg/ml) for 3 hours and then cultured further in HCG-containing medium for 18 hours. After culture, the complex was fixed and stained with aceto-orcein. The cumulus is expanded and shows normal round nuclei. 600×

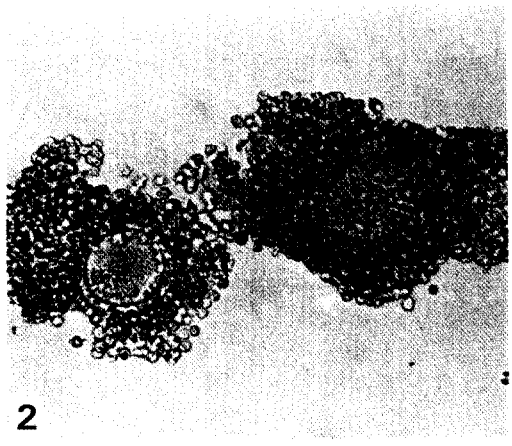
Fig. 4. An intact complex which was fixed and stained immediately after isolation. The cumulus is not expanded. 450×

Fig. 5. A squashed form of oocyte-cumulus complex expanded by stimulation of HCG for 3 hours. The shape of the cumulus cells looks healthy. 300×

Fig. 6. Cumulus cells expanded by HCG were fixed and stained. The dispersed cumulus cells show normal round nuclei. 900×

Fig. 7. A complex cultured in continuous presence of HCG and puromycin (2 μg/ml) for 18 hours. The cumulus is partially expanded but some of the cells show distinct sign of degeneration. 300×

Fig. 8. The same complex as Fig. 7. They were fixed and stained. Some of the cumulus cells have condensed and pyknotic nuclei. 900×



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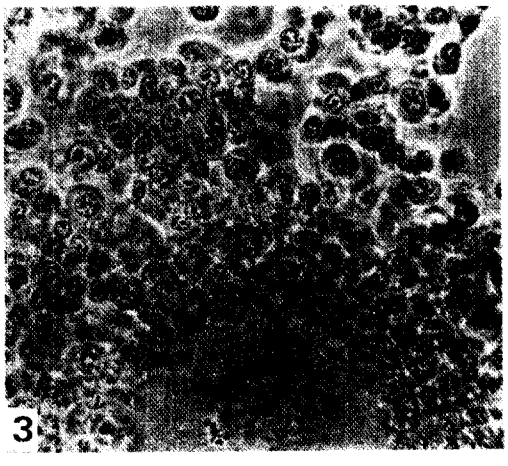

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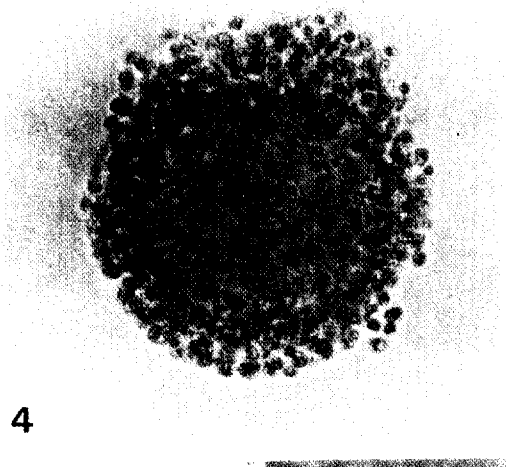

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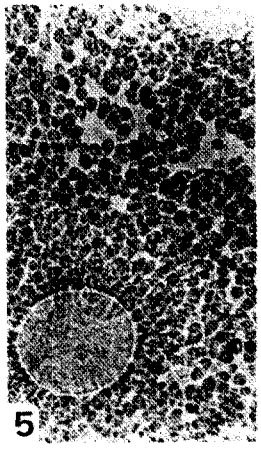

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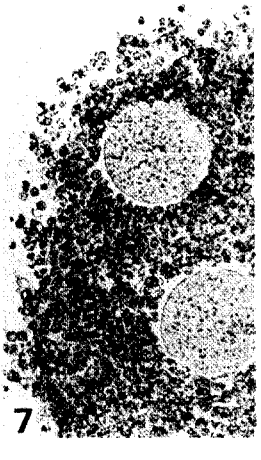

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