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Studies on Synergistic Effect of dbcAMP and Progesterone on the Maturation of Mouse Oocytes *in vitro*

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배양중인 생쥐 난자의 성숙에 미치는 dbcAMP 및 Progesterone의 동시영향에 관한 연구

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요 약

생쥐난자의 성숙을 억제하는 것으로 알려진 dbcAMP와 progesterone을 동시에 처리하였을 때의 영향을 알아보기 위하여 본 실험을 행한 결과는 다음과 같다. 1) 10 μg/ml의 dbcAMP 혹은 2 μg/ml의 progesterone을 단독처리하였을 때에는 난자의 성숙을 억제하지 못했다. 2) 그러나 dbcAMP(10 μg/ml)와 progesterone(2 μg/ml)을 동시에 배양액에 처리하였을 때에는 난자의 성숙이 억제되었다. 3) 난자를 먼저 4시간동안 위의 두 억제제가 들어있는 배양액에서 배양한뒤 다시 정상배양액에 옮겨 계속 배양하면 난자들은 대조군에서와 같은 비율로 성숙을 하였다.

위의 결과로 보아 dbcAMP와 progesterone이 동시에 배양액에 존재할 때에는 각각 단독 존재하였을 때 영향이 없는 농도의 dbcAMP와 progesterone으로도 능히 난자의 성숙을 억제 시킬 수 있었으며 이 같은 억제효과는 가역적임을 알게 되었다.

INTRODUCTION

Since Cho *et al.* (1974a) had first found that dibutyryl cyclic AMP (dbcAMP) inhibited reversibly the meiotic resumption of the mouse oocytes *in vitro* at the germinal vesicle (GV) stage, similar results with rat (Magnusson and Hillensjo, 1977; Dekel and Beers,

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1978) and porcine oocytes (Rice and McGaughey, 1981) were observed. It has been well acknowledged that the effective dose of dbcAMP is about 100 μg per ml for mouse oocytes (Cho *et al.*, 1974a), 300~400 μg per ml for rat (Magnusson and Hillensjo, 1977; Dekel and Beers, 1978) or porcine oocytes (Rice and McGaughey, 1981) and more than 700 μg per ml for bovine, golden hamster or ewe oocytes (Jagiello *et al.*, 1981).

On the other hand, the effect of the steroid hormones on the oocyte maturation *in vitro* has been studied by a number of researchers such as Cho *et al.* (1974b), Eppig and Koide (1979) and Smith and Tenney (1980) for mouse, Robertson and Baker (1969) and Bae and Foote (1975) for rabbit, and McGaughey (1977), Richter and McGaughey (1979) and Rice and McGaughey (1981) for porcine oocytes. In mouse, the effective dose of progesterone was found to be about 60 μg per ml which is within the pharmacological range and it inhibits meiosis at any stage, even beyond the germinal vesicle break-down (GVBD). Progesterone also irreversibly induces the degeneration of oocytes if the exposure to the steroid is extended more than three hours (Cho *et al.*, 1974b).

Despite the studies on the oocytes with dbcAMP and steroid hormones have been carried out intensively, the answers on the role of those inhibitors have not become available yet, and the problem related with the reason why the inhibitors consistently work *in vitro* only with the high concentration is remained to be solved. Since the work on the porcine oocyte maturation by Rice and McGaughey (1981), it has been well documented that the effective dose of the agents become suboptimal when they are present together in the medium. Downs and Eppig (1984) also found that dbcAMP acts fairly as an inhibitor of meiotic division of the mouse oocytes in far lower concentrations if it is added to the medium with a fraction of the porcine follicular fluid that appears to contain various kinds of steroids.

In the present studies, therefore, we investigate the synergistic effect of dbcAMP and progesterone at each of its concentration known to be excessively high. We also aimed to study the role of those inhibitors combined to the oocytes, if it is true that they work at the suboptimal dose.

MATERIALS AND METHODS

Oocytes were obtained from the ovaries of ICR strain mice of 3 to 4 weeks old, bred randomly in the Experimental Animal Farm of Seoul National University. The mice were housed in 14 hours light and 10 hours dark with food and water *ad libitum*.

Ovaries were dissected out and placed in culture dish (Falcon Plastic, 60×15 mm) containing equilibrated medium. The ovarian follicles were punctured with a fine needle under a dissecting microscope (Wild), thereby forcing the oocytes out into the medium. Cumulus cells were removed by repeated expulsion from a finely drawn Pasteur pipet. Only healthy looking oocytes with germinal vesicle were collected and washed with fresh culture

medium for the experiment.

Culture of the oocytes was carried out mainly by a microtube culture method (Cho, 1974) which was developed for the culture of mammalian embryos with oil soluble agents such as steroids and prostaglandins. An oil drop method (Brinster, 1963) was also used when culture of the oocytes was performed in control or dbcAMP medium. The oocytes set in culture at 37°C in an atmosphere of 5% CO_2 in moistened air for a certain length of hours. Experiments for each group were repeated 3 to 5 times.

Washing or culture medium was Modified Hanks Balanced Salt Solution (MHBSS) consisted of the following components; NaCl(140.73 mM), KCl(5.37 mM), $MgSO_4$(0.81 mM), Na_2HPO_4(0.34 mM), KH_2PO_4(0.44 mM), $NaHCO_3$(4.17 mM), $CaCl_2$(1.71 mM), glucose (5.55 mM), Na-pyruvate(0.3 mM), Na-lactate(2.5 mM), penicillin(100 IU/ml) and streptomycin(50 μg/ml). Ficoll (Sigma, MW 400,000) at a concentration of 1mg/ml was added to the medium instead of bovine serum albumin (BSA) throughout all experiments. Medium was made up at each experiment from the stock solutions. Stock solutions of steroids (Sigma, 1 mg/ml) in ethanol and dbcAMP (Sigma, 2 mg/ml) in salt stock of MHBSS were stored at −20°C until use. All media were Millipore filtered before use, except medium containing steroid since Millipore filters avidly adsorb steroids. Final concentration of ethanol used for dissolving steroid was never exceeded 10 μl per ml. All glasswares and instruments were sterilized with a hot air sterilizer or an autoclave.

RESULTS

Table 1 shows the effect of dbcAMP on the oocyte maturation in the presence of progesterone. If the oocytes were exposed either to 10 μg per ml of dbcAMP or to 2 μg per ml of progesterone, they were not affected at all but proceeded into GVBD in the proportion as high as 71.1% in dbcAMP or 61.4% in progesterone medium. These values were very comparable to those of the control group. Thus, dbcAMP or progesterone at the given concentration did not work as the proper inhibitors to the meiotic division. However, if dbcAMP and progesterone at the same doses as above were given together into the medium, the GVBD was markedly inhibited and only about 8.5% to 18.6% of the oocytes were able to resume meiosis. These data show apparant synergistic effect of two inhibitors. As shown in Table 1, dbcAMP at the doses less than 10 μg per ml added with 2 μg per ml of progesterone, or progesterone at the doses lower than 2 μg per ml with 10 μg per ml of dbcAMP did not act as the potent inhibitors. Simultaneously, in the combination of 5 μg per ml of dbcAMP and 1 μg per ml of progesterone, its inhibiting potency was also not as great as in the other combination although the proportion of the GVBD was somewhat decreased.

Table 2 shows the reversible effect of combined agents on the GVBD of the mouse oocytes. It is clear that the oocytes whose GVBD were once suppressed by the complex of

Table 1. Synergistic effects of dbcAMP and progesterone on germinal vesicle break-down of mouse oocytes in culture for four hours.

(a) Concentration of dbcAMP was fixed at 10 μg/ml.

Treatment (μg/ml)	Progesterones					
	—[2]	—	0.25	0.5	1	2
No. of oocytes cultured	35	44	37	45	38	47
% of oocytes[1] undergone GVBD	77.1±3.7	61.4±9.0	64.9±5.0	47.8±4.4*	36.8±7.7*	3.5±6.7*

(b) Concentration of progesterone was fixed at 2 μg/ml.

Treatment(μg/ml)	dbcAMP					
	—[2]	—	1.25	2.5	5	10
No. of oocytes cultured	47	45	48	52	48	59
% of oocytes[2] undergone GVBD	72.3±0.9	71.1±9.7	52.1±6.7	57.9±2.8	43.8+7.3*	18.6±7.5*

(c) Various combinations of the concentration of dbcAMP and progesterone up to 10 μg and 2 μg per ml, respectively.

Treatment (μg/ml)		Progesterones			
	—	0.25	0.5	1	2
		dbcAMP			
	—	1.25	2.5	5	10
No. of oocytes cultured	61	63	69	61	62
% of oocytes[1] undergone GVBD	80.3±11.5	63.5±14.9	71.0±8.6	57.4±8.4	14.5±10.9*

1) mean±s.e.
2) No any agent was added into medium as the control.
* Significantly different from the control, $p<0.01$

Table 2. Maturation of mouse oocytes in the 16 hours culture in the plain medium after 4 hours preculture in the medium containing dbcAMP (10 μg/ml) and progesterone (2 μg/ml) to show the reversible effect of the inhibitors.

Treatment		No. of oocytes cultured	% GV oocytes	% MI-TI oocytes	MII oocytes	% Degenerative oocytes
4 hrs in	16 hrs in					
Plain med.	Plain med.	53	7.5±5.8	24.5±5.1	66.0±3.9	1.9±3.0
Prog. dbcAMP	Plain med.	53	13.2±5.0	17.0±5.2	69.8±9.5	0

MI: Metaphase I, TI: Telophase I, MII: Metaphase II.

progesterone and dbcAMP can resume their meiotic division immediately after removal of the inhibiting agents. The oocytes reached to metaphase II showed around 70% after been free from the inhibitors, and this data is very close to the control, 66%.

Table 3. Maturation of mouse oocytes cultured for 18 hours in the medium containing dbcAMP (10 μg/ml) and progesterone (2 μg/ml) after preculture in the medium for up to 4 hours.

Hours cultured in		No. of oocytes	% GV	% M I-T I	% M II	%
Plain med.	Agent med.	cultured	oocytes	oocytes	oocytes	Degenerative oocytes
18	—	60	13.3±3.0	28.3± 3.3	56.7± 2.7	1.7±1.9
—	18	56	82.1±3.3*	12.5± 3.5	5.4± 2.8*	0
0.5	17.5	51	72.5±0.5*	21.6± 2.1	5.9± 2.4*	0
1	17	57	42.1±7.7*	33.4±15.4	22.8±10.8	1.8±1.9
2	16	58	29.3±2.1*	29.2± 3.2	37.9± 4.5	3.4±3.8
4	14	54	16.7± 3.5	25.9± 1.8	57.4± 5.4	0

*Significantly different from the figures of the group cultured in the plain medium for 18 hours. $p<0.01$

In Table 3 are set the results of the experiments to show the effect of the combined agents on meiosis of mouse oocytes previously cultured in the plain medium for up to 240 min. As the table shows, the oocytes which have been cultured initially in the plain medium for up to 60 min, were greatly affected by the agents; most of the oocytes were remained at GV, and a small portion of them could undergo to metaphase II. However, when the oocytes were initially cultured in the plain medium for more than 120 min and then transferred into the dbcAMP-progesterone complex medium for the extended culture, the proportion of GV decreased while those grown to metaphase II increased as much as that of the control. This result demonstrates that the combined agents give effect to the oocytes only at the GV stage.

DISCUSSION

It has been well documented that dbcAMP *in vitro* mimics cAMP whose high level in the mammalian oocytes suppresses the meiotic resumption (Cho *et al.*, 1974a; Magnusson and Hillensjo, 1977; Dekel and Beers, 1978; Rice and McGaughey, 1981). The inhibition of meiotic division was obtained not only with addition of dbcAMP directly into the medium but also with addition of theophylline (Cho *et al.*, 1974a) or 3-isobutyl-1-methyl xanthine (IBMX, Schultz *et al.*, 1983) each of which is an inhibitor of phosphodiesterase activity. The inhibitory activity was also observed with addition of forskolin (Eppig *et al.*, 1983), follicle stimulating hormone (FSH, Eppig *et al.*, 1983) or cholera toxin (Schultz *et al.*, 1983). On the basis that these agents contribute to the elevation of cAMP by suppression of phosphodiesterase activity or by activation of adenylate cyclase activity, it is apparant that high level of intracellular cAMP is necessary to keep oocytes from the resumption of meiosis.

Along with the studies of dbcAMP, some of investigators have worked on the effect of

steroids on the oocyte maturation. It is well acknowledged by the researchers that progesterone is the most potent inhibitor among steroids of meiotic division (Nekola and Smith, 1974; Eppig and Koide, 1978; Kwon, 1979). As the preliminary experiment with steroids in the present studies, similar results to the previous one was obtained (data not shown).

In the previous studies by a number of investigators, it is commonly known that exogeneous agents such as dbcAMP or steroid hormones inhibit *in vitro* meiosis of the oocytes at the range of pathological doses; more than 80 μg per ml of dbcAMP (Cho *et al.*, 1974a) or more than 8 μg per ml of progesterone (data not shown). The reason why such excessive amount is required is still uncertain. Only the assumption that the vitelline membrane may be much resistable against exogeneous dbcAMP or steroid hormones.

Up to now many researchers have tried to explain the role of dbcAMP and steroid hormones to the oocyte maturation, but the definite mechanisms by them are still veiled. Cho and Yoo (1975) suggested that dbcAMP makes the oocytes unable to synthesize RNA which is detectable shortly before GVBD (Bloom and Mukherjee, 1972), and Schultz *et al.* (1983a) inferred that dbcAMP inhibits dephosphorylation of GVBD specific proteins. Cho *et al.* (unpublished) found that dbcAMP inhibits uptake of calcium ion into the oocytes, whose low concentration in the medium affects the oocyte maturation (Jagiello *et al.*, 1981; Bae and Channing, 1984). On the other hand, there are some of assumptions related with the role of steroid hormones. Daniel and Levy (1964) described that progesterone inhibits the uptake of nutrients by disturbing membrane transport system of the rabbit embryos, and then eventually stop their development. However, Kwon (1979) inferred that progesterone would inhibit syntheses of RNA and proteins which are essential for the oocyte maturation. Recently, Eppig *et al.* (1983) proposed a working model for the inhibitory function of cAMP and steroids to the oocyte maturation. They assumed the presence of inhibitor which is generated in the granulosa cells, reached and stored in the oocytes and finally activated with the aid of elevated cAMP and steroid hormones. They suggested that suppression of the oocyte maturation is more efficiently potentiated by the co-existance of dbcAMP and steroid hormones. Such synergistic effect by dbcAMP and testosterone was found in the pig oocytes (Rice and McGaughey, 1981). That is, GVBD of the pig oocytes was inhibited by addition of suboptimal concentration of two agents. The synergism between dbcAMP and a fraction of porcine follicular fluid (PFF) to the mouse oocytes has also been studied by Downs and Eppig (1984). When dbcAMP is added to the medium containing PFF fraction, suboptimal concentration of dbcAMP is required to suppress the oocyte maturation. Assuming that the fraction of PFF might contain various steroid hormones, these results are consistent with those of the previous studies. In fact, in our studies, it is required to suppress GVBD of mouse oocytes only with the concentration of one tenth of the optimal dose of dbcAMP (10 μg/ml) and almost one fifth of that of progesterone (2 μg/ml). Although it is clear that the far less optimal concentrations of

dbcAMP and progesterone present together in the medium is enough to inhibit meiotic division, no possible suggestion has been showed in regarding to the function of the agents in the oocytes concomitantly. Only Eppig *et al.* (1983) pointed out that the oocytes are a target for steroid potentiation of inhibition of maturation since steroids acted synergistically with a cAMP-dependent process in the oocytes, and steroid hormones may potentiate the action of the putative maturation inhibitor which is assumed to be present in oocytes in the collaborative with the elevated cAMP.

In the previous studies done by one of the present coauthors (Cho *et al.*, 1974a), it has been found that dbcAMP inhibits oocyte maturation only at the GV stage, and it does not give any effect to the oocyte beyond GVBD and allowing them to finish meiotic division without any disturbance. However, it has also been found that progesterone acts as an inhibitor to the oocytes at any stage of the meiotic division (Cho *et al.*, 1974b). In the present studies, as the results shown, when dbcAMP and progesterone are added together to the medium, only the oocytes at GV stage are affected as if dbcAMP alone is given to them. This means that the inhibiting function of dbcAMP is more potent than progesterone.

Since when dbcAMP was known as a potent inhibitor of the oocyte maturation *in vitro*, there have been a number of researches to investigate its mechanisms with the oocytes, and to clarify its role in the cells. Recently the effects of dbcAMP on the oocytes with steroid hormones have also been investigated actively by several laboratories. However, it would be necessary to carry out more intensive studies of the oocytes with dbcAMP to obtain accurate answers of its role.

SUMMARY

In order to investigate the synergistic effect of dbcAMP and progesterone which are known as the agents to inhibit maturation of mammalian oocytes *in vitro*, the present studies were done and the results were obtained as follow: 1) if 10 μg/ml of dbcAMP or 2 μg/ml of progesterone was given into the medium, each of the agents at the concentration above did not give any inhibitory effect on the maturation of the mouse oocytes *in vitro*; 2) however, when the two agents at the concentration shown above were given together into the medium, the mouse oocytes were arrested at GV stage; and 3) the oocytes, pre-cultured in the medium containing two agents at the same concentration as above for four hours, resumed their maturation division upon transfer to the plain medium for the extended culture.

Thus, it was found that dbcAMP and progesterone were capable to suppress the maturation of mouse oocytes at the suboptimal concentration when they were given together, and such inhibitory effect was reversible.

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