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# Calcium Uptake in Mouse Oocyte Matured in Vitro

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## 배양액 내에서 성숙한 생쥐 난자의 $Ca^{2+}$ Uptake

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### 국문초록

배양중인 생쥐 난자의 성숙에 미치는 배양액 내 calcim의 영향을 알아보기 위하여 1.71mM $Ca^{2+}$을 처리한 배양액과 $Ca^{2+}$이 존재하지 않는 배양액에서 난자를 배양하여 불꽃 원자 흡수 분광 광도계를 이용하여 배양된 난자의 $Ca^{2+}$농도를 측정하였다.

1) $Ca^{2+}$처리한 배양액에서 배양된 cumulus-cell이 제거된 (denuded oocyte)난자들은 시간이 지남에 따라 $Ca^{2+}$농도가 높게 나타났고, 2) $Ca^{2+}$처리하지 않은 배양액에서는 denuded난자는 3시간째 배양될때 부터 $Ca^{2+}$양이 줄어 들었다.

Cumulus-enclosed난자는 $Ca^{2+}$존재하에서는 GVBD가 일어났다고 생각되는 4시간까지 계속 증가를 보인 반면 $Ca^{2+}$-free에서는 배양되지 않은 난자와 거의 차이가 없게 나타났다.

3) 핵막붕괴가 일어난 후부터 15시간까지 배양시켰을 때에는 CEO와 denuded oocyte에서 공히 $Ca^{2+}$의 농도는 다시 증가된 상태로 계속 되었다.

이런 결과로 미루어 보아 난자 성숙시 부터 성숙과정이 끝날때가지 exteral $Ca^{2+}$이 요구되고 있음을 증명해 주고있다. 그러나 이러한 세포질내의 $Ca^{2+}$및 bound calcium이 난자 성숙시부터 어떤 역활을 하고 있는 기작에 대해서는 좀 더 연구가 있어야겠다.

## INTRODUCTION

In mammalian ovary, the process of meiosis is arrested at the diplotene stage of the first meiotic division. Meiotic resumption can be mediated by a hormonal stimulus in vivo or a suitable culture medium in vitro (Pincus and Enzman, 1935 ; Baker, 1972 ; Bae and Cho, 1982).

Calcium has been demonstrated to be essential for the nuclear maturation of oocytes of aquatic in vertebrates(Steinhardt et al., 1974 ; Hollinger et al., 1977 ; O' Connor et al., 1977 ; Baker and Whitaker, 1978 ; Cilkey et al., 1978). Also it has been implicated as the second messenger or dependent ion in the resumption of meiosis in oocytes from frogs(Wasserman et al., 1980 ; Wasserman and Smith, 1981), mammals (Tsafriri and Bar-ami, 1978 ; Batta and Kundsen, 1980 ; Paleos and Powers, 1981 ; Bae, 1981 ; Osborn, 1981 ; Jagiello et al., 1982). De Felici and Siracuse(1982) and Bae and Channing(1985) have shown that $Ca^{2+}$ is essential for survival in culture of fully grown mouse oocytes and oocytes isolated from medium-sized porcine follicles. An increase in intracellular calcium has been shown to have a role in the meiotic resumption in invertebrate and amphibian oocytes(Guerrier, Mo-

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reau and Doree, 1978 ; Kostellow and Morrill, 1980). In addition, the concentration of total calcium in cumulus-enclosed oocytes increased during matured in vivo after pregnant mares' serum gonadotropin (PMSG) treatment in the rat (Batta and Kundsen, 1980). On the other hand, Whitfield et al., (1979), Dulbecco and Elkington (1975) and Ralph (1983) showed that the level of intracellular calcium was critical to the regulation of somatic cell division.

However, there has been few paper to measure the influx or uptake of $Ca^{2+}$ in mammalian oocytes in vitro system (Bae et al., unpublished). To elucidate the involvement of calcium in oocyte maturation, the present study was undertaken to measure the level of intracellular calcium of both denuded and cumulus-enclosed mouse oocytes during culture in vitro.

## MATERIALS AND METHODS

Oocytes were obtained from the ovaries of ICR strain mice (3-4 weeks old). Those female mice were killed by cervical dislocation. Ovaries were dissected out and placed in the culture dish containing equilibrated washing media, modified Hank's Balanced Salt Solution (MHBS) containing 0.1% bovine serum albumin (BSA, Sigma). The ovarian follicles were puntured with a fine needle under a dissecting microscope (Wild M5A). Healthy-looking cumulus-enclosed oocytes with germinal vesicle(GV) were harvested by using a mouth operated pasteur pipette within 10 -25 min after follicle rupture. Denudation and selection of healthy oocytes were carried out in $Ca^{2+}$-free MHBS.

Denudation of cumulus cells were carried out after selection of healthy-looking cumulus-enclosed oocytes. Different diameter of finely drawn glass pipettes were used to denude cumulus cells layers from the oocytes. Careful aspiration and expelling were required to achieve denudation without exerting visible and univisible physical damage. Cumulus-enclosed oocytes(CEO) in the present study were just uniformly 3-4 layers of cumulus cell enclosed oocytes, and all were healthy looking oocytes with GV.

Washing ( $Ca^{2+}$-free ) or culture media was modified Hank's Balanced Salt Solution (MHBS) (Bae and Channing, 1985). MHBS culture media consisted of followings ; Nacl(140.73 mM), KCl(5.3655 mM), $MgSO_4$(0.8116 mM), $Na_2HPO_4$(0.3358 mM), $KH_2PO_4$(0.4408 mM), $NaHCO_3$(4.166 mM), $CaCl_2$(1.711 mM), glucose (5.55 mM), Na-pyruvate(0.3 mM), Na- lactate (2.5 mM), Penicillin(10,000i$\mu$/ml), Streptomycin(50mg/ml) and BSA(4mg/1). This medium was filtered through the Millipore membrane (Millipore Co.) with 0.45 um pore size after adjustment to pH 7.2.

Oocyte culture was carried out in a culture medium drop in Falcon plastic dishes under paraffin oil at 37.5℃ in 100% humidified atmosphere of 5% $CO_2$ in air.

After time-sequenced culture, the oocyte was washed 3 times with $Ca^{2+}$-free MHBS to remove calcium from the culture medium. After that, collected oocytes were placed in plastic tube containing 1ml calcium-and phenol red-free MHBS, and homogenated for 1 minute with sonicater (Fisher-sonic dismembrator Model 300).

Homogenated samples were analyzed with flame atomic absorption spectrophotometer (Perkin-Elmer, Model 5000). The samples injected through capillary tube were atomized by flame, and passed optical light emitted from hollow cathode lamp. Fuels in this method were $N_2O$ gas and $C_2H_2$ gas. Absorption was measured at 422.7nm (Brinley et al., 1979 ; Skoog and West, 1971).

There were 4-11 replicates experiment for each group. Significancy of the difference between the data were examined by a student t-test.

## RESULTS

Table 1 shows the level of total intracellular calcium of denuded oocytes cultured in calcium containing-and-free-medium. Calcium concentration of denuded oocytes cultured in $Ca^{2+}$ containing medium increased up to 4 hr and calcium level was 50% increase after 4-hour cultured. In denuded oocytes cultured in calcium free medium the $Ca^{2+}$ concentration showed a plateau from 1 to 2-hour culture and then, thereafter, it decreased. There was no difference until 2hr culture in denuded oocytes cultured in calcium-free medium, whereas thereafter, calcium uptake decreased.

Table 2 shows $Ca^{2+}$ uptake of cumulus-enclosed oocytes cultured for 1-4 hr. Calcium uptake increase was 54.2-64.1% in comparison of those of denuded oocytes from the control and experimental groups.

Table 3 shows culture period from 5 hr to 15 hr which is assumed to be a period after GVBD. The $Ca^{2+}$ concentration of long cultured groups(5-15 hr) increased with time, whereas the relative increase rate was not as high as in short time culture (Table 1 and 2).

However, there was not much increase of calcium uptake in calcium-free cultured group even though 2hr cultured in calcium free showed a peak during 4 hr culture. In con-

**Table 1.** Intracellular calcium concentration of denuded oocytes cultured for 1-4 hours in vitro

| Cultured time | Denuded oocytes (ppm) | | $\frac{\text{Cultured hr-control}}{\text{control}} \times 100$ | | P |
|---|---|---|---|---|---|
| | $Ca^{2+}$(1.71mM) | $Ca^{2+}$-free | $Ca^{2+}$ (1.71mM) | $Ca^{2+}$-free | |
| 0 (zero) (control) | 0.026±0.002 (58) | 0.031±0.007 (81) | | | |
| 1hr | 0.031±0.005 (52) | 0.033±0.014 (97) | 19.23% | 6.45% | p<0.01** |
| 2hr | 0.033±0.001 (56) | 0.033±0.004 (86) | 21.21% | 6.45% | p<0.01** |
| 3hr | 0.034±0.011 (47) | 0.025±0.010 (93) | 30.76% | -19.35% | p<0.01** |
| 4hr | 0.041±0.020 | 0.028±0.011 | 40.42% | -9.67% | p<0.01** |

ppm : Parts per million
Mean±S.E.M.
Number in parenthesis means number of oocytes cultured.
** : Significantly different from control value, p<0.01.

**Table 2.** Intracellular calcium concentration of cumulus-enclsed oocytes(CEO) cultured for 4 hours in vitro

| Cultured Time | Cumulus-enclosed oocytes (ppm) | | $\frac{\text{Cultured hr-control}}{\text{control}} \times 100$ | | P |
|---|---|---|---|---|---|
| | $Ca^{2+}$ (1.7mM) | $Ca^{2+}$-free | $Ca^{2+}$ (1.7mM) | $Ca^{2+}$-free | |
| 0(zero) (control) | 0.048±0.010 (99) | 0.043±0.005 (119) | | | |
| 1hr | 0.053±0.017 (73) | 0.047±0.006 ( 99) | 10.41% | 9.3% | p<0.01** |
| 2hr | 0.056±0.005 (79) | 0.049±0.004 (101) | 16.67% | 13.95% | p<0.01** |
| 3hr | 0.057±0.005 (82) | 0.045±0.004 (105) | 18.75% | 4.65% | p<0.01** |
| 4hr | 0.064±0.016 (67) | 0.047±0.007 (101) | 33.33% | 9.3% | p<0.01** |

ppm : Parts per million
Mean±S.E.M.
Number in parenthesis means number of oocytes cultured.
** : Significnatly different from control value, p<0.01.

trast, there was much difference of calcium uptake in the cumulus-enclosed oocytes between $Ca^{2+}$-present and-free culture media.

## DISCUSSION

The present study showed that intracellular uptake of calcium increased with time even in denuded oocytes cultured in $Ca^{2+}$-containing medium and relative increase rate is very high(Table 1). However, the $Ca^{2+}$ concentration of denuded oocytes cultured in $Ca^{2+}$-deficient medium decreased after 2 hour culture. Such a decrease seems to be caused by degeneration and death of these oocytes.

Calcium is required for the maturation process per se as well as for maintenance of overall oocyte viability (Powers and Paleos, 1982 ; Bae and Channing, 1985). The rapid necrosis of mouse oocytes in $Ca^{2+}$-free medium (Paleos and Powers, 1981) as well as in the denuded oocytes cultured in $Ca^{2+}$-deficient seems to be caused by the removal of the cumulus cells of the oocytes(Table 2). Cumulus cells may act to protect against degeneration of the oocytes by supplying $Ca^{2+}$to the oocytes through gap junction or $Ca^{2+}$-channel, or the compact cumulus-enclosed oocytes of the diplotene stage may be very resistent to degeneration as long as cumulus cells keep an intact connection with the oocytes(Table 2). Surely, in the present study cumulus cells keep the oocytes viable up to 4 hr in $Ca^{2+}$-free medium (Table 2) whereas the denuded oocytes show degeneration in short hour(2hr) in the table 1. Such a result is very similar as in porcine oocytes(Bae & Channing, 1985).

According to De Felici and Siracusa(1982) total protection from $Ca^{2+}$-deficient death of the mouse oocytes in given by 1mM $La^{3+}$ or 10mM $Sr^{2+}$, while $Mg^{2+}$(12mM) only confers protection on one third of oocytes. Dedge et al.(1969) and De Mello(1975) interpreted that the protective effect of $Sr^{2+}$ was porbably due to its ability to replace $Ca^{2+}$ in a variety of internal cellular function.

However, this explanation may not be applied to $La^{3+}$, which dose not enter intact cells (Caswell, 1979). $La^{3+}$ binds to the outer aspect of the cell memrane and prevent $Ca^{2+}$ transmembrane movements. De Felici and Siracusa(1982) interpreted this as following ; the protective effect of $La^{3+}$ might result from sealing action and the prevention of $Ca^{2+}$ outflow through the oocyte membrane.

Such an assumption of detaching of $Ca^{2+}$ from the membrane and blocking of transmembrane movement have not been tested in the present study and further study is still required for the role of $Ca^{2+}$ in degeneration or death of oocytes. The absence of external calicum might bring about intracellular free calcium down to levels imcompatible with oocyte life, and such a lowered intracellular $Ca^{2+}$ concentration may be insufficient to meet metabolic requirments.

As pyruvate is the main and the only substrate for energy production in denuded oocyte, there must be a metabolic route for $Ca^{2+}$ to couple with pyruvate being used for energy prodution passway to Kreb's cycle. In this passway of pyruvate to Kreb's cycle, $Ca^{2+}$ seems to mediate the activity of pyruvate dehydrogenase complex.

Hansford(1987), Stadon & Hansford (1987), Hansford & Stadon (1987) verified that in the rat hepatocyte there was a relationship between changes in cytosolic $Ca^{2+}$ content and changes in pyruvate dehydrogenase activity and the former was a determinant of the latter. So activation of pyruvate dehydrogenase by $Ca^{2+}$ would be expected to increase the rate of provision of NADH to the respiratory chain and to produce ATP required for oocyte viability and maturation process.

As shown in Table 3, the requirement of $Ca^{2+}$ in the maturation of oocyte may be a long time even after GVBD as shown in the previous studies(Alexandre and Gerin, 1977 ; Jagiello et al., 1982 ; Bae and Channing, 1985) that is different from amphibian (Kostellow

**Table 3.** Intracellular calcium concentration of denuded and cumulus-enclosed oocytes (CEO) from 5 to 15 hours after GVBD in culture medium in vitro

| Cultured Time | $Ca^{2+}$ (1.71mM) | | $\frac{\text{Cultured hr-control}}{\text{control}} \times 100$ | | P |
|---|---|---|---|---|---|
| | Denuded 0. (ppm) | CEO(ppm) | Denuded 0. | CEO | |
| 0(zeri) (control) | 0.035±0.002 (76) | 0.041±0.003 (69) | | | |
| 5hr | 0.036±0.001 (73) | 0.042±0.004 (70) | 2.85% | 2.43% | p<0.01** |
| 10hr | 0.042±0.004 (65) | 0.053±0.007 (57) | 20% | 29.26% | p<0.01** |
| 15hr | 0.043±0.010 | 0.048±0.003 | 22.85% | 17.07% | p<0.01** |

ppm : Parts per million
Mean±S.E.M.
Number in parenthesis measn number of occytes cultured
** : Significantly different from control value, p<0.01.

and Morrill, 1980). Such an increase of $Ca^{2+}$ uptake in oocytes after GVBD in contrary to the results obtained by Cho and Lee(1985) and the in vivo study(Batta and Kundsen, 1980). Such a contrast result should be further studies in the future.

Comparing denuded oocytes with CEO cultured in calcium deficient medium it appeares that the level of calcium is higher in CEO than in deunded oocytes.

It is thought that calcium contained in the cumulus cells is added to the total $Ca^{2+}$ concentration of CEO, and calcium in cumulus cell per se may move to the oocyte by an intracellular or extracellular route. Cumulus cells are coupled to the oocyte by gap junctions(Amsterdam et al., 1976 ; Anderson and Albertini, 1976). These junction have been shown to be highly permeable to small molecules and to facilitate electrical coupling between cells (Gilula et al., 1978 ; Moor et al., 1980 ; Dekel et al., 1981).

It may be suggested that the extracellular calcium as well as the intracellular calcium is required for the spontaneous meiotic maturation. Further studies by using antagonist related to calcium is required in more correct understanding on movement, localization and uptake of calcium.

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