Effects of Artificial Stimulations on the Activation of Oocyte and the Expression of Cyclin B1 Protein in Mouse Oocytes

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쥐 난자의 활성화 처리가 난자의 활성화 및 Cyclin B1 단백질 발현에 미치는 영향

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요 약

본 연구는 쥐 난자에서 인위적 활성화 처리가 난자의 활성화, cyclin B1 단백질의 발현 및 난자의 활성화와 cyclin B1 단백질 발현간의 상관관계에 미치는 영향에 관하여 조사하고자 실시하였다. 난자의 활성화 처리는 7% ethanol(EtOH) or 10 µg/ml Ca-ionophore with or without 10 µg/ml cycloheximide (CH) 방법으로 단일 또는 복합처리 하였다. 난자의 활성화 비율은 단일처리($p<0.05$)와 복합처리($p<0.01$)한 난자가 무처리에 비하여 유의하게 높았다. Cyclin B1 단백질의 발현이 EtOH + CH 처리한 난자를 제외한 다른 처리군에서는 무처리에 비하여 유의하게 감소하였다($p<0.05$). 한편 EtOH + CH($r = -0.61$, $p<0.05$)와 Ca + CH($r = -0.86$, $p<0.01$) 처리그룹에서 cyclin B1 단백질의 발현과 난자의 활성화 간에 높은 역상관관계가 있음을 확인하였다. 하지만 단일처리 그룹에서는 두 요소간에 상관관계가 없음을 알 수 있었다. 따라서 단일(EtOH and Ca-ionophore) 또는 복합(EtOH + CH and Ca + CH) 활성화 처리가 난자의 활성화를 증가시키며, 이것은 난자의 활성화 처리에 따른 cyclin B1 단백질의 감소와 밀접한 연관이 있다고 사료된다.

(**Key words** : Artificial stimulation, Oocyte activation, Cyclin B1 protein, Mouse)

Ⅰ. INTRODUCTION

After the embryo reconstruction, their further development may be dependent upon a number of factors, including the synchronization of donor nucleus and recipient cytoplasm at fusion. Generally, the donor cells are likely in interphase while the recipient oocytes are arrested in meiotic metaphase II (MII). To control the synchronization, the enucleated MII oocytes are artificially stimulated prior to embryo reconstruction. The use of enucleated oocytes stimulated could increase the frequency of development to blastocyst in embryos reconstructed from unsynchronized donor blastomeres (Campbell et al., 1993). To activate the enucleated oocytes, several calcium-elevating agents, such as ethanol

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(Minamihashi et al., 1993) or calcium ionophore (Ware et al., 1989; Shi et al., 1993) have been used. Recently, protein synthesis inhibitors, like cycloheximide or 6-dimethylaminopurine (6-DMAP) have been used simultaneously with calcium-elevating agents to increase the higher oocyte activation and development rate (Presicce and Yang, 1994).

The cytoplast of MII oocytes contained high levels of M-phase promoting factor (MPF) that controls the onset of meiosis/mitosis. During the acquisition of meiotic competence, MPF activity increased gradually until the maximum level was achieved (Yang et al., 1993; Choi et al., 1991). The increase of MPF activity is strongly correlated with increased cyclin B1 synthesis. Destruction of cyclin B results in the inactivation of MPF and exits cells from M-phase of cell cycle (Verlhac et al., 1994; Chesnel and Eppig, 1995; Hample and Eppig, 1995 a,b; Winston, 1997).

This study was performed to investigate the effects of artificial stimulation on the increase of the oocyte activation, to evaluate the expression of cyclin B1 protein levels in enucleated mouse oocytes, and to investigate correlation between the oocyte activation and the cyclin B1 protein levels.

Ⅱ. MATERIALS AND METHODS

1. Collection of oocytes

B6C3F1 female mice, 4-5 weeks of age, were primed with 5IU pregnant mare's serum gonadotropin (PMSG) and 5IU of human chorionic gonadotropin (hCG) 48 hours later. The animals in each study were housed in an AAALAC-accredited animal facility incorporating a clean-dirty corridor system. Ovulated oocytes were collected from ampullar oviducts 15hr later hCG injection. The collected cumulus oocytes complexes (COC) were isolated and cultured in the medium containing 100μM 1-iosbutyl-3-methylxanthine(IBMX; Aldrich Chemical Co., USA) to maintain meiotic arrest. The COC were cultured at 37℃ in a humidified 5% CO_2 incubator. Resumption of meiosis was initiated by placing the oocytes into IBMX-free culture medium. The culture medium used for all experiments was Ham's F-10 supplemented with 10% FBS.

2. Enucleation procedure

The collected oocytes were freed of cumulus cells by vortexing in 500 μl of culture medium with 0.1% (w/v) hyaluronidase (H-4272, Sigma, USA) in an 1.5 ml tube for 2 to 3 min. The matured oocytes with an extruded first polar body were collected and then transferred into 20 μl culture medium drop supplemented with 7.5 μg/ml cytochalasin B (Sigma) under mineral oil. The zona pellucida was cut by cutting pipette and then approximately 20 to 30% of cytoplasm surrounding the first polar body was squeezed gently. The micromanipulation was performed using a Zeiss Diaphot inverted microscope (Zeiss, Germany) fitted with Hofman's contrast modulation optics (Modulation Optics Inc., Greenvale, NY, USA). After microsurgery, the confirmation of enucleation was proved by cytoplast staining with 5 μg/ml Hoechst 33342 for 15 min and then the oocytes were exposed briefly to UV irradiation to verify the absence of chromatin and complete enucleation.

3. Protocol of oocyte activation

The stimulation protocols were divided as follows: (1) 7% ethanol for 5 min (EtOH); (2) 10 μg/ml Ca ionophore for 5 min (Ca); (3) 7% EtOH for 5 min + 10 μg/ml CH (C-7698, Sigma, USA) for 3 hrs (EtOH + CH); (4) 10 μg/ml Ca-ionophore for 5 min + 10 μg/ml CH for 3 hrs

(Ca + CH). After stimulations, the oocytes were removed from CH solution and cultured in culture medium(Ham's F-10 containing 10% FBS). Three replications were performed at each group. Oocytes activation was determined by the presence of the 2_{nd} polar body extrusion or pronucleus formation.

4. Western blotting

The oocytes (40 oocytes/protocol) were lysed at 0, 3 and 6 hours in 0.5 ml of lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris pH 8.0) with freshly added 1mM PMSF (Sigma, Missouri, USA) for 30 min on ice. The lysates were centrifuged at 13,000 g for 15 min at 4℃ and the supernatants were stored at −70℃. Equal amounts of total protein were separated by SDS-PAGE and then transferred to supported Westran® PVDF (Schleicher & Schuell, USA) by applying 100V-1h with a plate electrode apparatus (Mini Trans-Blot Cell; Bio-Rad, USA). The blots were blocked for 1 hr in Tris-buffered saline-T (TBS-T) (0.2M NaCl, 0.1% Tween-20, 10 mM Tris, pH 7.4) containing 5% non-fat dry milk. The blots were incubated with antibody against cyclin B1 (1:500; Santa Cruz Biotechnology) in TBS-T and then the blots were also incubated in anti-rabbit IgG (1:2,000; Amersham Pharmacia Biotech, Germany) in TBS-T. The blots were washed several times with TBS-T after each step. The bound antibody was detected with an enhanced chemi-luminescence (ECL) system (Amersham Pharmacia Biotech).

The image was scanned with Gel Documentation System (Gel Doc 1000, Bio-Rad Hercules, CA, USA) and relative densities were analyzed using Multi-analyst fingerprinting program (version 1.1). The relative densities of the bands were expressed as arbitrary absorbance units per area.

5. Statistical analysis

The statistical analysis for activation rate was performed with Chi-square test. The statistical analysis for the differences of cyclin B1 protein expression among treatment groups was performed using the Kruskal-Wallis test. The correlation between cyclin B1 protein level measured by arbitrary unit and oocyte activation were tested by Pearson correlation coefficient using SAS program (Release 9.1. USA: Inst. Inc.; 2002). Statistical significance was given when P value was less than 0.05.

Ⅲ. RESULTS

The activation rate according to different stimulation protocols was shown in Table 1. The activation rate in single stimulation (EtOH and Ca) ($p<0.05$) and in combined stimulation (EtOH + CH and Ca + CH) ($p<0.01$) was significantly higher than compared to control group, respectively.

The expression of cyclin B1 protein in single stimulation (EtOH and Ca) was shown in Fig. 1. The intensity of cyclin B1 protein was not changed in control group during

Table 1. Activation rate of oocytes treated with single or combined stimulation

Stimulation	No. of oocytes	
	Stimulated	Activated (%)
Control	74	6 (8.1 ± 4)[a]
Ethanol	73	17 (23.3 ± 7.9)[b]
Ca-ionophore	74	22 (29.7 ± 6.7)[b]
EtOH + CH*	75	58 (77.3 ± 3.7)[c]
Ca + CH*	74	62 (83.8 ± 7.4)[c]

*The oocytes were treated with 10 µg/ml cycloheximide (CH) for 3 hr after single stimulation.
The results were expressed as mean ± standard deviation (SD).
[a] vs. [b]: $p<0.05$; [a, b] vs. [c]: $p<0.01$

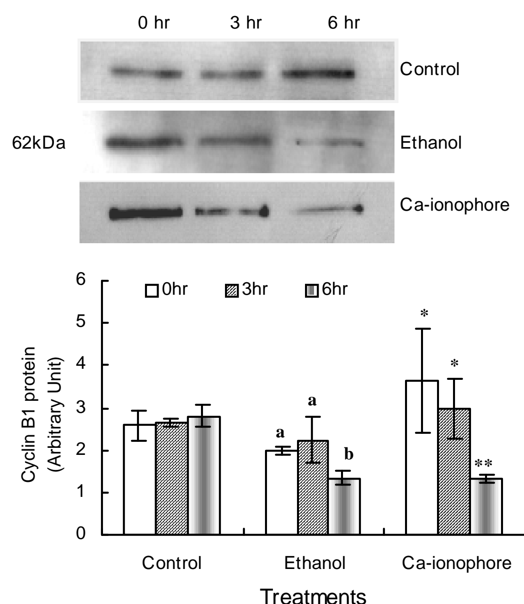

Fig. 1. The expression of cyclin B1 protein on single stimulation conditions. The protein was separated by SDS-PAGE. After immunoblotting, densitometry analysis was performed to analyze the intensity of the bands.

Control : no stimulation ; EtOH : 7% ethanol, for 5 min; Ca: 10 μg/ml Ca-ionophore, for 5 min. The results were expressed as mean ± standard deviation. Values in same graph with different superscripts differ significantly($p<0.05$).

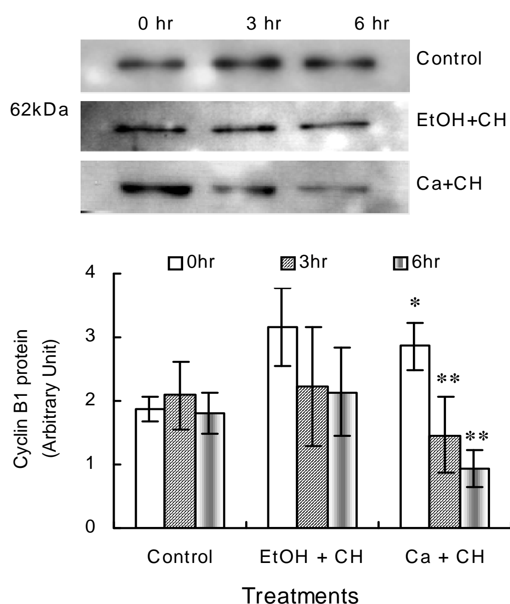

Fig. 2. The expression of cyclin B1 protein on combined stimulation conditions. The protein was separated by SDS-PAGE. After immunoblotting, densitometry analysis was performed to analyze the intensity of the bands.

Control: no stimulation; EtOH + CH : 7% ethanol, for 5 min followed by 10 μg/ml cycloheximide, for 3 hr; Ca + CH: 10 μg/ml Ca-ionophore, for 5 min followed by 10 μg/ml cycloheximide, for 3 hr. The results were expressed as mean ± standard deviation. Values in same graph with different superscripts differ significantly ($p<0.01$).

culture time. However, the cyclin B1 protein level of 7% EtOH and 10 μg/ml Ca-ionophore treatment group decreased significantly at 6 hr ($p<0.05$), respectively.

The intensity of cyclin B1 protein in EtOH + CH group showed a decreasing tendency during culture period, although the difference was not statistically significant. The cyclin B1 protein level was dramatically reduced after stimulation in Ca + CH group ($p<0.05$) (Fig. 2).

The correlation between activation rate and cyclin B1 protein level in single stimulation was shown in Fig. 3. The treated group showed a negative correlation, however, it was not statistically significant.

Unlike a single stimulation group, a higher negative correlation was shown in combined stimulation groups (Fig. 4). It showed a significantly negative correlation in EtOH + CH treated group ($r = -0.61$, $p<0.05$) and Ca + CH treated group ($r = -0.86$, $p<0.01$), respectively.

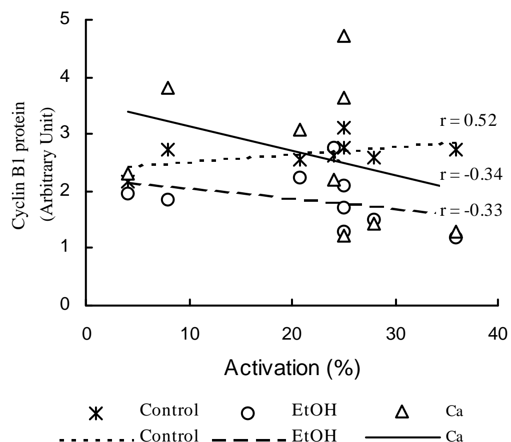

Fig. 3. Correlation between activation rate and cyclin B1 protein level on single stimulation conditions.
r = Pearson correlation coefficients. Abbreviations are as in Fig. 1.

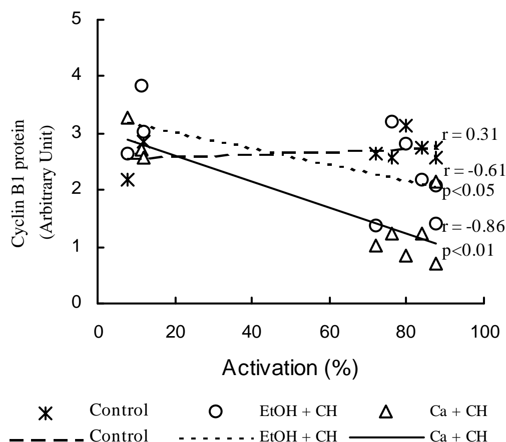

Fig. 4. Correlation between blastocyst development and cyclin B1 protein level on combined stimulation conditions.
r = Pearson correlation coefficients. Abbreviations are as in Fig. 2.

Ⅳ. DISCUSSION

It is generally accepted that the sperm induce oocyte activation but generate a transient increase in the intracellular calcium concentration which in mammals takes the form of a series of repetitive calcium transients that lasts for several hours (Polanski et al., 1998). Since the primary signal for activating the oocyte development program at fertilization seems to be the sperm-induced calcium transients, it can easily be accepted that applying the same stimulus oocyte development can be induced artificially. Several calcium elevating agents, like ethanol or Ca-ionophore, have been used to induce oocyte activation in a number of species and the activation rate was ranged from 20 to 40% (Yang et al., 1994; Loi et al., 1998; Balakier and Casper, 1993; Taylor and Braude, 1994). However, the use of combined stimulation protocols increased the cleavage and development of eggs (Precisse and Yang, 1994; Yi and Park, 2005).

Like previous reports, the results were similar to the present study. The activation rate was increased significantly in combined stimulation compared to a single stimulation. Although the activation rate in a single stimulation was higher than that of non-treated group, the activation efficiency was lower than that of combined stimulation groups.

Studies demonstrated that cyclin B1 degradation is essential for completion of meiosis (Glotzer et al., 1991; Holloway et al., 1993; Brandeis and Hunt, 1996). High levels of MPF are known to be essential for meiotic arrest of mammalian oocytes. The MPF is composed of cyclin B and $p34^{cdc2}$. Destruction of cyclin B results in the inactivation of MPF and exits cells from M-phase of cell cycle. However, the mechanism of cyclin degradation by artificial stimulation is poorly understood.

In oocytes treated with Ca-ionophore alone, cyclin B1 was destroyed within 1 hr after treatment and then increased from 4 hr to 15 hr. While the reduction of cyclin B1 was observed at 1 hr after treatment with Ca + CH, no increases

were observed until 15 hr in bovine oocyte (Lin and Yang, 1999). Fissore and Robl (1992) reported that single calcium peak induced by ethanol or Ca-ionophore would destroy the existing cytostatic factor (CSF), whereas the cycloheximide would prevent the renewal of CSF in the oocytes. The reduced level of CSF caused by complex stimulation would result in the degradation of cyclin B (Parrish et al., 1992).

In both fertilized and artificially stimulated eggs, the decrease in cdc2/cyclin B kinase activity is clearly due to the decrease in amount of cyclin B (Moos et al., 1996). Within 5 hr following the addition of cycloheximide to ovulated metaphase II-arrested mouse eggs the amount of cyclin B1 and histone H1 kinase activity decreased dramatically and reached levels similar to those observed following fertilization.

In the present study, the reduction of cyclin B1 protein in enucleated mouse oocyte was significant compared to control group. The expression pattern of cyclin B1 was similar to both single and combined stimulation. The levels of cyclin B1 protein were shown decreasing tendency in both single and combined stimulation groups. And the correlation between oocyte activation and the degradation of cyclin B1 protein was shown interesting results. Although the correlation in single stimulation groups showed negative pattern, the difference was not statistically significant. However, a higher negative correlation was shown in combined stimulation.

In conclusion, it can be suggested that single (EtOH and Ca-ionophore) and combined (EtOH + CH and Ca + CH) stimulation increases the oocyte activation, especially combined stimulation, because it induces the degradation of cyclin B1 protein after artificial stimulation treatments in mouse oocytes.

V. ABSTRACT

This study was performed to investigate the effects of artificial stimulation on the increase of the oocyte activation, to evaluate the expression of cyclin B1 protein levels in enucleated mouse oocytes, and to investigate correlation between the oocyte activation and the cyclin B1 protein levels. The oocyte activation was induced by 7% ethanol (EtOH) or 10 μg/ml Ca-ionophore with or without 10 μg/ml cycloheximide (CH). The activation rate was significantly higher in both single ($p<0.05$) and combined ($p<0.01$) stimulated groups compared to control group. The cyclin B1 protein level was significantly reduced in both stimulated groups ($p<0.05$), except for EtOH + CH treatment group. The expression of cyclin B1 protein showed a higher negative correlation with activation rate in EtOH + CH ($r = -0.61$, $p<0.05$) and Ca + CH ($r = -0.86$, $p<0.01$) stimulation groups, but not in a both single stimulation groups. Taken together, it can be suggested that single (EtOH and Ca-ionophore) and combined (EtOH + CH and Ca + CH) stimulation increases the oocyte activation, especially combined stimulation, because it induces the degradation of cyclin B1 protein after artificial stimulation treatments in mouse oocytes.

Key words: Artificial stimulation, Oocyte activation, Cyclin B1 protein, Mouse

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