Effect of Dynein Inhibitor on Mouse Oocyte *in vitro* Maturation and Its Cyclin B1 mRNA Level[1]

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Objective To evaluate the effect of dynein inhibitor on mouse oocyte *in vitro* maturation and its cyclin B1 transcription level. **Methods** Immature mouse oocytes were cultured *in vitro* with a known dynein ATPase activity inhibitor-sodium orthovanadate (SOV) to detect the changes of maturation rate, and semi-quantitative RT-PCR and single cell RT-PCR were performed to detect the changes of cyclin B1 mRNA level. **Results** In dose-dependent experiments, the maturation rates of oocytes were significantly different between 5 μmol/L SOV and control groups ($P<0.05$), and decreased with SOV increasing doses. However, the elevation of cyclin B1 mRNA level of immatured oocytes cultured for 12 h depended on SOV concentrations ranging from 50 to 500 μmol/L. In incontinuity exposed SOV experiments, the maturation rates of oocytes markedly reduced after the first incubation with 400 μmol/L SOV at least for 1 h and were first cultured in SOV-free medium for 4 h or 8 h before exposure to SOV ($P<0.05$). In time-course experiment, the opposite changes of cyclin B1 mRNA level in oocytes between SOV and control groups were observed. **Conclusion** Dynein inhibitor might delay oocytes meiosis process, and cause ectopic expression of cyclin B1 in oocytes. Most Oocytes incubated with SOV blocked at germinal vesicles (GV) stage or M Ⅰ to anaphase transition due to dynein dysfunction and ectopic transcription level of cyclin B1.

Key words: Dynein; Oocytes; Cultured *in vitro* maturation (CVM); Cyclin B1; Sodium orthovanadate (SOV)

INTRODUCTION

In mice, oogenic meiosis begins in the embryo, but is arrested in the prophase of meiosis Ⅰ and resumes in response to progesterone while the animal reaches its sexual maturity. Progesterone triggers activation of maturation-promoting factor (MPF) and neosynthesis of Mos kinase (a germ cell-specific Ser/Thr protein kinase), responsible for mitogen-activated protein kinases (MAPK) activation. Both MPF and MAPK[1,2] activities are required for the success of meiotic maturation. Phosphorylation of Dynein intermediate light chains by MPF leads directly to the loss of membrane-associated dynein and an inhibition of organelle movement[3], suggesting that Cytoplasmic dynein function may be under

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cell cycle control.

Cytoplasmic dynein is a kind of microtubule-based related protein involved in many fundamental cellular processes including organelles transport[4], spindles assembly[5-8] and spindle checkpoint inactivity[9,10]. During prometaphase and metaphase, dynein is located in spindles and kinetochores correlated to chromosome movement[11,12]. Recent studies have shown that dynein promotes cell cycle checkpoint proteins (Mad2, Bub1R[9] and Mitosin/CENP-F[10]) deletion from kinetochores to spindle poles, resulting in cell proliferation from metaphase to anaphase. Thus, a major function of dynein in mitosis is in a kinetochores disassembly pathway that contributes to inactivation of the spindle checkpoint[6]. However, dynein inhibitor and dynein antibody could prevent dynein transport function[6,13] and caused cell cycle arrest[14].

To explore the related mechanism of meiosis arrest caused by dynein inhibitor, the study on the changes in maturation rate and the cyclin B1 mRNA level of oocytes cultured with dynein inhibitor, sodium orthovanadate (SOV) was performed.

MATERIALS AND METHODS

Oocytes Collection and Culture

Mature female BALB/C mice aged 4-6 weeks were induced to superovulate by intramuscular injection of 5-10 IU of pregnant mare serum gonadotrophin (PMSG, Ningbo Hormone Product Co., LTD. China) per mouse into the inguinal region and killed at 46-48 h after PMSG injection. Ovaries were removed and oocytes were released from antral follicles by puncturing the follicles with a needle in M2 medium. The oocytes surrounded with compacted cumulus cells (GV-intact oocytes) were collected and followed by rinsing three times in M2 medium, and then cultured in TYH medium containing 10 IU/mL PMSG and 10% heat-inactivated fetal calf serum at 37℃ for 12 h in a humidified atmosphere of 5% CO_2.

Oocytes Treatment

For dose-effect experiments, the GV-intact oocytes were randomly divided into six groups, exposed to 0, 5, 50, 250, 400, and 500 μmol/L SOV (Sigma-Aldrich, USA) in TYH medium respectively, and cultured at 37℃ for 12 h *in vitro*. The study on the effect of incontinuity exposure was performed as follows: 1) The GV-intact oocytes were randomly divided into four groups, first exposed to 400 μmol/L SOV for 0 h, 0.5 h, 1 h, and 2 h respectively, and were then put into SOV-free TYH to continue incubation up to 12 h. 2) The GV-intact oocytes were cultured in SOV-free TYH for 4 and 8 h respectively and then transferred into TYH containing 400 μmol/L SOV up to 12 h incubation. For time-course experiments, cyclin B1 mRNA level of immatured oocytes cultured with/without SOV was measured at 4 h, 8 h, and 12 h.

Oocytes cultured for 12 h were freed from cumulus cells by gentle pipetting in M2 medium containing 0.1% hyaluronidase (Sigma, USA) and counted for the immature ones (no first polar body) and the matured ones (extrusion of the first polar body) under microscope (×25). Cyclin B1 mRNA level of immatured oocytes was detected by a reverse transcription polymerase chain reaction (RT-PCR).

Semi-quantitative RT-PCR

The experimental procedures were referred to Wang *et al.*[15]. Briefly, total RNA was extracted from 20 immature oocytes using RNA isolation kit (TaKaRa, Japan), then reverse-transcribed into cDNA with 15 U/μL ThermoScrip^{TM}RT at 50℃ for 1 h (Invitrogen USA). The oligonucleotide primers are cyclin B1: 5′TATTGGGCGCCGGGTCACCA3′, 5′ACTTA-CTGTAGTTCTTCCACC3′, 417 bp, mGAPDH: 5′GTAATCCTTGCAGTGAG TGACG3′, 5′CATCTCCATCTGTCTGATCTGG3′, 746 bp. cyclin B1 and mGADPH fragments were amplified in a standard buffer containing 0.2 μmol/L of each pair of primer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP and 0.4 μL(5 U/μL) Platinum Taq DNA ploymerase (Invitrogen, USA) for 35 cycles. PCR products were analyzed on a 1.5% agarose gel. The density of the bands was quantified by UVP imaging and analysis system (Ultra-violet products Ltd. UK) and normalized by mGAPDH.

Single Cell RT-PCR

Oocytes cultured for 8 h were randomly collected from the control and 400 μmol/L SOV treated group. Cumulous cells were removed with 0.1% hyaluronidase. Oocytes were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered solution (DPBS, 2.6 mmol/L KCl, 1.4 mmol/L KH_2PO_4, 137 mmol/L NaCl, 8.0 mmol/L Na_2HPO_4,$7H_2O$), and then one oocyte (DPBS volume less than 1 μL) was put into 0.2 mL PCR tube using a glass pipes under microscope (×25), stored in −70℃ or used immediately to do one step RT-PCR (Invitrogene, USA) in 50 μL reaction mixture containing 1 μL one oocyte template, 25 μL 2× buffer containing 0.4 mmol/L dNTP, 2.4 mmol/L MgSO4, 1 μL each pair of primer (10 μmol/L), 1 μL SuperScript Ⅱ/Platinum *Taq* DNA Ploymerase Mix, 21 μL DEPC water. Samples were incubated in RTC-100 thermal cycler (MT Research Inc. USA), first for 30 min at 50℃ to reverse-transcribed RNA into cDNA, then 2 min for 94℃ to inactivate reverse transcriptase, followed by 40 cycles for 30 sec at 94℃, 30 sec at 55℃, 1 min at 68℃, and a final extension at 72℃ for 10 min. Negative controls were made by adding Platinum *Taq* DNA Ploymerase instead of SuperScript Ⅱ/Platinum *Taq* DNA Ploymerase Mix to RT-PCR reaction system. Take 2 μL of one-step RT-PCR products as template to reamplify cyclin B1 fragment by second around PCR amplification. Ten μL of each PCR reaction were analyzed by electrophoresis in TBE buffer with Ethidium Bromide (0.5 μg/mL) stained with 1.5% (W/V) agarose gel. The density of each band was visualized with the UVP imaging system (Labwork 3.0 Software).

Statistical Analysis

The data was statistically analyzed using the Emendation χ^2 test and Students' *t* test.

RESULTS

Dose-dependent Effect of SOV on Oocyte Maturation

Maturation rates of oocytes exposed to 5, 50, 250, 400, and 500 μmol/L SOV for 12 h were significantly different from those of the control ($P<0.05$, $P<0.01$, Emendation χ^2 test) and decreased with SOV increasing dose (Table 1).

TABLE 1

Dose-dependent Effect of SOV on Oocyte Maturation Rates

SOV (μmol/L)	Total No. of Oocytes	Mature Oocytes	Immature Oocytes	Maturation Rate (%)
0	141	73	68	51.8
5	94	34	60	36.2*
50	73	22	51	30.1**
250	204	28	176	13.7**
400	113	12	101	10.6**
500	242	28	214	11.6**

Note. *$P<0.05$, **$P<0.01$ *vs* Control group, Emendation χ^2 test.

Effect of Incontinuity Exposed SOV on Oocyte Maturation

1) Oocytes were first exposed to 400 μmol/L SOV for 0.5 h, 1 h, 2 h separately, and were then transferred into SOV-free TYH medium. Oocytes maturation rates of the 1 h and 2 h treated group were significantly decreased compared with those of the 0 h treated group ($P<0.01$, Emendation χ^2 test), but it failed to reach the lowest level (12 h treated group). There was no significant difference in oocytes maturation rate between the 0.5 h treated and the control groups (Fig. 1).

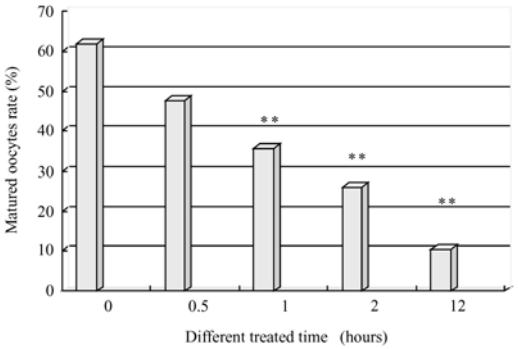

FIG. 1. Changes in oocytes maturation rate at different treated time with 400 μmol/L SOV (**$P<0.01$, *vs* 0 h group).

2) Oocytes were first cultured in SOV-free TYH medium for 4 and 8 h respectively and then transferred into TYH containing 400 μmol/L SOV up to 12 h incubation. The rates of mature oocytes in the 4 h and 8 h groups were 6/56 (10.7%) and 15/68 (22.1%) respectively. They were markedly reduced compared with the control ($P<0.05$). The oocytes maturation rate in the 4 h group was the same as that in the group continuously exposed to SOV (12/113, 10.6%). This data indicated that SOV significantly inhibited oocytes maturation within the first 4 h of cultivation *in vitro*.

Dose-dependent Effect of SOV on Cyclin B1 mRNA Level of Oocytes

The cyclin B1 mRNA level of oocytes in 5, 400 and 500 μmol/L SOV treated groups was obviously increased (P<0.05) compared with that of control groups (Figs. 2 and 3). This tendency of elevation presented a dose-dependent manner ranging from 50 μmol/L to 500 μmol/L.

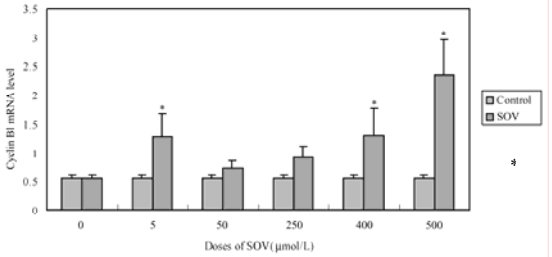

FIG. 2. Changes in cyclin B1 mRNA level of immature oocytes exposed to different concentrations of SOV at 12 h of culture *in vitro* (n=4, $^{*}P$<0.05, *vs* Control, Students' *t* test).

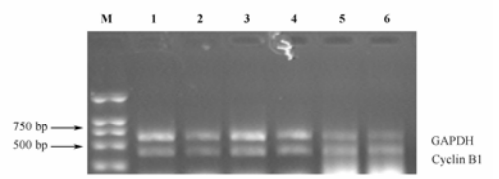

FIG. 3. RT-PCR amplification products of GAPDH and cyclin B1 gene, M: DNA marker, Lanes1-6: cyclin B1 and GAPDH expression in oocytes exposed to, 0, 5, 50, 250, 400, and 500 μmol/L SOV, respectively.

Time-course Effect of SOV on Cyclin B1 mRNA Level of Oocytes

In control groups, mRNA level of cyclin B1 of oocytes cultured for 4 h reached a peak, then it decreased in a time-dependent manner. It was significantly increased in the 4 h group,

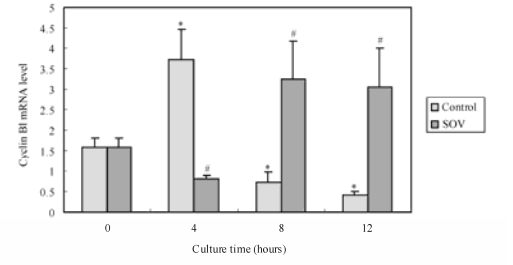

FIG. 4. Changes in cyclin B1 mRNA level in oocytes treated with 400 μmol/L SOV at different times of culture (n=3, $^{*}P$<0.05, *vs* 0 h group, $^{\#,*}P$<0.05, *vs* Control, Students' *t* test).

but markedly decreased in the 8 h and 12 h groups compared with the 0 h group (P<0.05). However, reverse changes in cyclin B1 mRNA level were observed when oocytes were treated with SOV. Cyclin B1 mRNA expression was still very low at 4 h, but the highest level was recorded at 8 h, and the high level remained at 12 h in the SOV treated group (Figs 4 and 5). There was significant difference in cyclin B1 level between the SOV treated group and the control group (P<0.05).

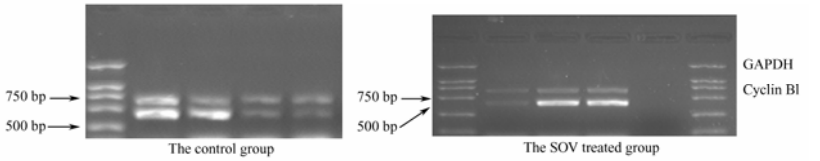

FIG. 5. RT-PCR amplification products of GAPDH and cyclin B1 gene in both the control and SOV the treated group at different times of culture, Lanes M: DNA marker, NC[#]= negative control.

Effect of SOV on Cyclin B1 mRNA Level of Single Oocytes

Oocytes were randomly collected from the control and the 400 μmol/L group. Cyclin B1 mRNA level of single oocytes cultured for 8 h was detected using single cell RT-PCR method. The mean density of cyclin B1/GAPDH band of the control and the 400 μmol/L SOV group was 20.8±7.26/8.8±0.45, and 43.3±8.45/7.7±0.81 (n=5), respectively (Fig. 6). Significant differences can be detected (P<0.05) in cyclin B1 transcription level between the SOV treated group and the control group.

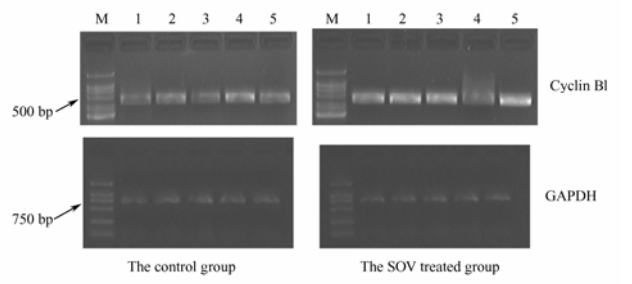

FIG. 6. Effect of SOV on cyclin B1 mRNA level of single oocytes, Lanes M: DNA marker, Lanes 1-5: Five single oocytes from the control and the SOV treated group.

DISCUSSION

Dyneins are massive molecular motor complexes of 1.2MDa that generate force towards the minus end of microtubules[16]. The mechanism of dynein movement is that Dynein binds to the microtubule and uses the energy in ATP molecules to move from the positive (+) end to the minus (-) end of microtubule[17,18]. Each step requires hydrolysis of one ATP molecule. SOV (Na_3VO_4) is a potent inhibitor of dynein ATPase activity and function. The kinetics of SOV inhibition show that it is due to SOV acting as a phosphate

analog and forming a relatively stable dynein · ADP · vanadate complex that acts as a dead-end block, with the vanadate presumably bound at the site normally occupied by the γ-phosphate of ATP[19]. Recently, Burgess SA and his colleague found that with both ADP and vanadate bound, dynein stem and stalk emerge from the head apart[18].

The primary oocytes retrieved from mouse ovary were arrested at diplonema in the prophase of the first meiotic division. After oocytes was cultured *in vitro* with hormones for 4 h to 6 h, they underwent germinal vesicle breakdown (GVBD), then extruded the first polar body and completed maturation from metaphase I to metaphase II at 12 h to 14 h cultured *in vitro*. However, the progression of meiosis was disturbed by SOV—a known dynein inhibitor, and, as a result, oocytes maturation rate was markedly decreased. Compared with the control, oocytes maturation relative rates in 5-500 μmol/L SOV groups were reduced to 69.9% (36.2/51.8), 58.1% (30.1/51.8), 27.5% (13.7/51.8), 20.5% (10.6/51.8) and 22.4% (11.6/51.8), respectively (Table 1). In incontinuity exposure SOV experiments, immatured oocytes rates were found to have significantly decreased after oocytes were first cultured with 400 μmol/L SOV for 1 h or 2 h and then transferred into SOV freee medium and after oocytes were first cultured with SOV free TYH medium for 4 h and 8 h and then transferred into SOV medium. The results suggested that SOV could prevent oocytes meiosis progression at different stages (GV, GVBD and MⅠ) and minimum effective time exposed to SOV was at least 1 h. Liu *et al.*[20] found that SOV as protein tyrosine phosphatase (PTPase) inhibitor also induced G2/M arrest in Raji lymphoblast-like cells. Zhang *et al.*[21] also reported that SOV caused lung epithelial cells G2/M phase arrest in time- and dose-dependent manner. In this experiment, SOV firmly blocked oocytes maturation. In order to explore the possible mechanism of meiosis block induced by SOV, cyclin B1 gene transcription level in oocytes was investigated.

CyclinB1 is a regulatory subunit of M-phase promoting factor (MPF). MPF activity is regulated by a translation-dependent mechanism that determines the level of cyclin synthesis[22,23]. Meiotic maturation of the mouse oocyte requires equilibrium between cyclinB synthesis and degradation, but only cyclinB1 mRNA-microinjected oocytes did not extrude their first polar body[24], this suggesting that cyclin B1 gene transcription level plays a crucial role in oocytes maturation.

In the normal case, the Cyclin B1 mRNA level reached an increasing peak when oocytes were cultured *in vitro* for 4 h. Cyclin B1 accumulation was responsible for MPF activity increase allowing oocytes to enter into metaphase Ⅰ (MⅠ). Its level rapidly degraded in anaphase, but met the secondly peak in metaphase Ⅱ (MⅡ)[22,25]. We also identified the same results (Figs. 4 and 5). However, when oocytes were incubated with SOV, the mRNA level of cyclin B1 in oocytes presented a different manner. Time-dependent effect showed that cyclin B1 mRNA level in the 4 h group was much lower than that in the 0 h group, but in the 8 h and 12 h group, cyclin B1 level remained at a higher level comparing with that in the control group. The high level of cyclin B1 regulated oocytes entering into MⅠ and also kept them in MⅠ. So oocytes met GVBD delay and could not pass anaphase to MⅡ. The results also confirmed an accordance with oocytes maturation and cyclin B1 level changes. In our experiment, decrease of cyclin B1 mRNA level of immature oocytes cultured for 4 h might be related to GVBD delay, while increase of cyclin B1 mRNA level of immature oocytes cultured for 12 h might be possibly linked to MI arrest. This suggested that dynein special inhibitor directly caused oocytes meiosis block and its mechanism might be related to cyclin B1 abnormal expression. We first time investigated cyclin B1 transcription level in oocytes using single cell RT-PCR method. The mRNA level of cyclin B1 of single oocyte cultured with 400 μmol/L SOV for 8 h was markedly elevated (P<0.05). This data was the

same with the findings of our measurement by semi quantitative RT-PCR. The positive rate (100%) of RT-PCR in single oocytes was higher than in somatic cells[26]. The reason is that oocytes are the biggest cell in the body, and high expression of cyclin B1 is present in oocytes. Zhang *et al.*[21] also reported that SOV caused lung epithelial cells G2/M phase arrest and a slight increase of cyclin B1 expression. However, Liu *et al.*[20] found that SOV as protein tyrosine phosphatase (PTPase) inhibitor induced G2/M arrest and down-regulated cyclin B1 expression of Raji lymphoblast-like cells. In oocytes following exposure to SOV, cyclin B1 decreased first, and then increased. This may help us to well understand the different results of cyclin B1 expression in G2/M arrest induced by SOV in somatic cells.

Addinall *et al.*[1] have found that release of dynein from membranes to kinetochores requires MPF activity that is active in meiosis. So, the possible mechanism of meiosis block induced by dynein inhibitor is regulated by cyclin B1: low cyclin B1 level causes MPF inactivity that prevents dynein release from membranes to kinetochors in early time cultured with SOV, and dynein function is destroyed in kinetochores by SOV even though high cyclin B1 level promotes dynein to accumulate at kinetochors in late stage culture with SOV. It is possible that dynein dysfunction in kinetochores and cyclin B1 ectopic expression result in oocytes maturation arrest.

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