Co-treatment with Demecolcine and BMI-1026, a Potent cdk1 Inhibitor, Induces the Enucleation of Murine Oocytes

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ABSTRACT

Oocyte enucleation is essential for somatic cell nuclear transfer (SCNT) in the production of cloned animals or embryonic stem cells from adult somatic cells. Most studies of oocyte enucleation have been performed using micromanipulator-based techniques, which are technically demanding, time-consuming, and expensive. Several recent studies have used chemical-induced oocyte enucleation; however, each has been plagued by low efficiency and toxicity. In this study, I found that the co-treatment of murine oocytes with demecolcine and BMI-1026, a potent cdk1 inhibitor, resulted in a high enucleation rate (97%). This method is entirely independent of a micromanipulator and is suitable for the large-scale production of enucleated oocytes. This new method of enucleation will be useful in SCNT and in the development of handmade cloning techniques.

(Key words : Oocyte enucleation, Cdk1-inhibitor, Demecolcine)

INTRODUCTION

Somatic cell nuclear transfer (SCNT), which is essential for the production of animal clones and embryonic stem cells from adult somatic cells (Fulka *et al.*, 1998), involves two critical steps: oocyte enucleation for preparation of the recipient cytoplast, and the fusion of a somatic cell or its nucleus with the recipient cytoplast. To date, most SCNT experiments have been carried out using a micromanipulator-based technique; however, such procedures are technically demanding, time-consuming, and expensive. Thus, only a limited number of laboratories can conduct SCNT experiments. Recently, several studies aimed at creating a new method for SCNT that does not rely on the use of a micromanipulator (i.e., handmade cloning) have been conducted (Vajta *et al.*, 2005; Esheikh, 2007).

Oocyte enucleation is an essential step of somatic cell cloning that is used to produce a recipient oocyte. Most methods for oocyte enucleation rely on physical aspiration, using a micromanipulator (Wakayama *et al.*, 1998). However, several alternative methods using chemicals, such as etoposide (Fulka and Moor, 1993; Fulka *et al.*, 2004) and the microtubule-destabilizing agent demecolcine (Ibanez *et al.*, 2003; Hou *et al.*, 2006; Saraiva *et al.*, 2009), instead of micromanipulation, have been reported. Etoposide, a topoisomerase II blocker, restrains chromosome segregation at meiosis (Mailhes *et al.*, 1994). Oocytes activated in this way extrude the polar body, including a complete chromosome set, leading to the production of a chromosome-free cytoplast (Fulka and Moor, 1993). This method is effective for oocyte enucleation; however, problems with toxicity during the development of the reconstituted embryos remain to be solved (Fulka *et al.*, 2004).

In activated oocytes, spindle rotation before cytokinesis is essential for normal polar body extrusion. Exposure to demecolcine after oocyte activation inhibits spindle rotation, leading to one or two abnormal polar bodies with complete chromosome extrusion. Such methods result in reduced toxicity, compared with etoposide-induced enucleation; however, the efficiency of enucleation is reduced (Ibanez *et al.*, 2003). The reduced efficiency of demecolcine-induced enucleation seems to be connected with the failure of polar body extrusion from activated oocytes.

Previously, we found that BMI-1026, a potent cdk1 inhibitor, activated ovulated oocytes more efficiently and quickly than ethanol (Choi, 2007). In this study, I investigated the effects of activation by BMI-1026, instead of ethanol activation on demecolcine-induced oocyte enucleation.

MATERIALS AND METHODS

Collection of Ovulated Oocytes

Three-four-week-old ICR female mice were used. To

* This work was supported by research funds from Dankook University in 2008.

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obtain ovulated oocytes, the mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, MO, USA) and with 5 IU of human chorionic gonadotrophin (hCG; Sigma, St. Louis, MO, USA) 48 h later. Ovulated oocytes were collected from the ampullae of the oviducts 14~15 h after hCG injection. The oocytes were scripped from cumulus cells by incubation for 2~3 min in 0.1% hyaluronidase in M16 culture medium (Sigma, St. Louis, MO, USA) at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. The cumulus cell-free oocytes were rinsed three times and used in enucleation experiment. To accurately assess the degree of oocyte enucleation, the zona pellucida was removed by digestion with acidic Tyrode's solution (Sigma, St. Louis, MO, USA) and the polar bodies were detached by repeated gentle pipetting.

Induction of Oocyte Enucleation and Evaluation

For oocyte enucleation, demecolcine, a microtubule-depolymerizing agent, and BMI-1026, a cdk1 inhibitor, were used. To determine the optimum conditions for the experiment, the time of demecolcine treatment was varied (pre-, simultaneous-, or post-oocyte activation [treatment with BMI-1026]). For example, the oocytes in the Deme-10 group were treated with demecolcine 10 min before activation and then activated with BMI-1026 and cultured in medium including BMI-1026 and demecolcine for the indicated time. Similarly, the oocytes in the Deme+10 group were cultured with demecolcine and BMI-1026 10 min after activation with BMI1026. Ultimately, demecolcine was used at a final concentration of 0.4 μg/ml while BMI-1026 was used at a final concentration of 200 nM. To compare the results of this study with those from previous reports, some oocytes were activated using ethanol (Ibanez *et al.*, 2003) instead of BMI-1026. A total of 20 oocytes were tested at each time point, and the experiment was repeated at least three times.

Oocyte Fixation and DNA Staining

The oocytes were then fixed with 1.8% paraformaldehyde in PBS for 40 min and stained with 4',6-diamidino-phenylindol (DAPI) for visualization of the DNA. The stained oocytes were observed by Zeiss fluorescence microscopy (Carl Zeiss, Jena, Germany).

Classification of the Phenotypes of the Oocytes

The changes in oocyte structure induced by exposure to the chemicals were classified into four types as follows: Type A, oocytes not activated; Type B, activated oocytes; Type C, partial enucleation (protrusion oocytes); and Type D, enucleated oocytes (Fig. 1A).

Statistical Analysis

Each experiment was replicated at least 3 times. Statistical differences between groups were analyzed using one way ANOVA. Data shown as mean±SEM were calculated.

RESULTS

Classification of the Oocytes into Four Types

The oocytes were observed following DAPI staining after 2 h of exposure to their assigned culture conditions (Fig. 1B). Type A indicates oocytes that were not activated while Types B~D indicate activated oocytes. Of these, the Type C oocytes failed to completely extrude the polar body (cytoplasmic protrusion), while the Type D oocytes extruded a complete second polar body with a complete chromosome set (enucleated oocytes).

Influence of Treatment Time with Demecolcine Before and After Oocyte Activation on Oocyte Enucleation

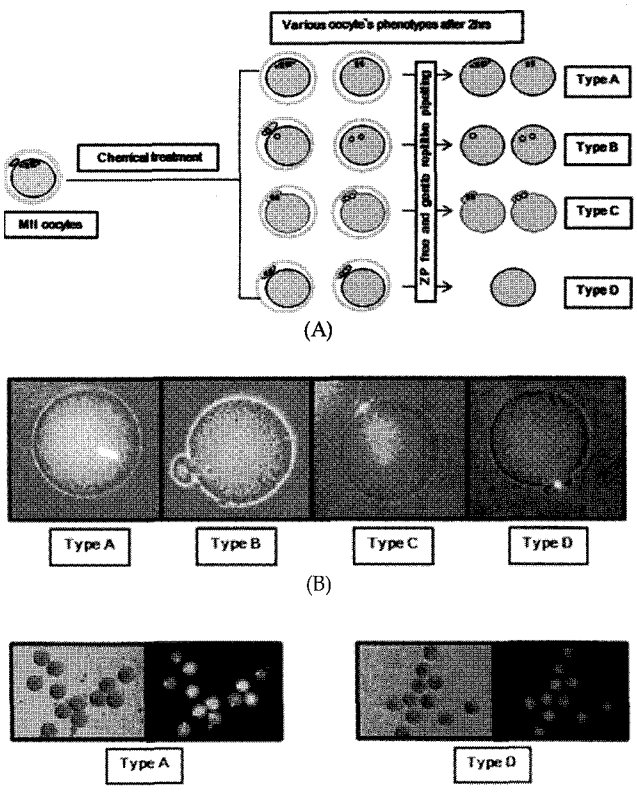

Fig. 1. The oocytes were classified into four types, based on their characteristics. (A) and their appearance following DAPI staining (B), (C) as follows: A, not activated; B, activated oocytes; C, activated and partially enucleated oocytes (protrusion); and D, activated and enucleated oocytes (extrusion). (B) Representative oocytes stained with DAPI. (C) type A and type D oocytes after gentle repetitive pipetting (DAPI staining images).

Table 1. Effect of demecolcine (Deme) and/or BMI-1026 on oocyte activation and enucleation.

	% of each type of oocyte (n)			
Time of Deme treatment	Type A	Type B	Type C	Type D
Untreated	98±3 (59/60)	2±3 (1/60)	0 (0/60)	0 (0/60)
BMI1026	0 (0/60)	100±0 (60/60)	0 (0/60)	0 (0/60)
Deme/Deme & BMI1026[a] (10 min pre-activation)	0 (0/60)	8±5 (5/60)	5±3 (3/60)	87±7 (52/60)
Deme/BMI1026 (simultaneous)[b]	0 (0/60)	3±3 (3/60)	0 (0/60)	97±3 (58/60)
BMI-1026/Deme & BMI-1026[c] (10 min post-activation)	0 (0/60)	85±5 (51/60)	2±2.8 (1/60)	13±6 (8/60)
EtOH	20±5 (12/60)	80±5 (48/60)	0 (0/60)	0 (0/60)
EtOH/Deme[d] (10 min post-activation)	15±9 (9/60)	30±13 (18/60)	33±8 (20/60)	22±10 (13/60)

[a] Oocytes were treated with Deme for 10 min then transferred to media containing Deme and BMI-1026. [b] Oocytes were cultured with BMI1026 and Deme (simultaneous processing). [c] Oocytes were activated with BMI-1026 for 10 min then transferred to media containing Deme and BMI-1026. [d] Oocytes were activated with ethanol for 10 min, then cultured in media containing Deme. EtOH: ethanol.

Previously, it was shown that the time between demecolcine treatment and ethanol activation was critical for oocyte enucleation (Ibanez *et al.*, 2003). In this study, I investigated whether the rate of oocyte enucleation varied according to pre-, simultaneous-, and post-treatment with demecolcine following activation by BMI-1026. Oocytes enucleated by demecolcine following ethanol activation were used as a source of comparison in this study (Table 1). BMI-1026 activated the oocytes regardless of demecolcine treatment. Those oocytes that were treated simultaneously with demecolcine and BMI-1026 were enucleated with the greatest efficiency (97%). The enucleation rate following co-treatment with demecolcine and BMI-1026 was much higher than that reported for oocytes treated with demecolcine and ethanol (97 vs. 22%).

These results show that it may be possible to achieve oocyte enucleation in a single step (simultaneous treatment with demecolcine and BMI-1026).

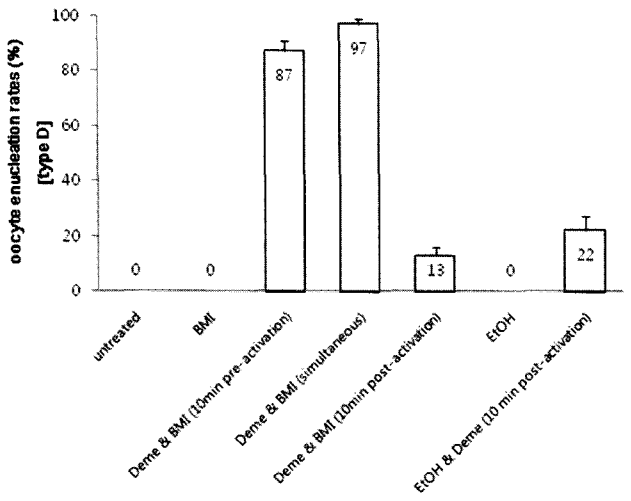

Fig. 2. Histogram of the data from Table 1 (selected type 4, enucleated oocyte rates). The numbers in the columns indicate the percentage of enucleated oocytes. The data given are the means ±SEM of triplicate experiments.

Time Course of Oocyte Enucleation Following Co-treatment with Demecolcine and BMI-1026

Oocytes were cultured with demecolcine and BMI-1026 for 0.5, 1, or 2 h then stained with DAPI and observed. After 0.5 h of treatment, 12% of the oocytes were enucleated; in comparison, after 1 and 2 h of

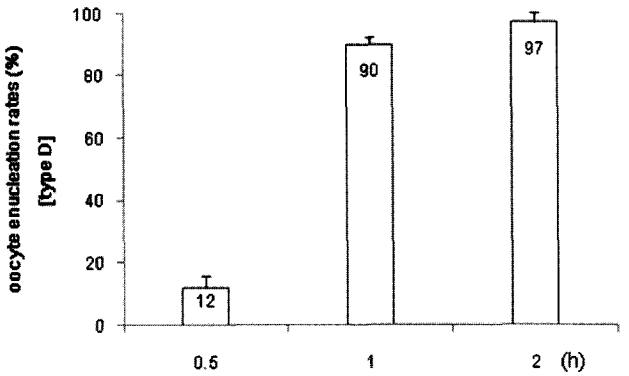

Fig. 3. Time course of oocyte enucleation following co-treatment with demecolcine (Deme) and BMI-1026. Oocytes were cultured with Deme and BMI-1026 for 0.5, 1, or 2 h, after which the zona pellucida and second polar bodies were removed. The numbers in the columns indicate the percentages. The data given are the means±SEM of triplicate experiments.

treatment, 90 and 97% of the oocytes were enucleated, respectively (Fig. 3). Although the rate of enucleation after 2 h of treatment was higher than that after 1 h, there was no significant difference ($p>0.05$) between the results obtained after 1 and 2 h of treatment.

DISCUSSION

In most cases, oocyte enucleation has been achieved by physical aspiration of the metaphase plate with or without DNA staining using a micromanipulator (Bell *et al.*, 1997; Hosaka *et al.*, 2000; Jisigami *et al.*, 2006). However, such methods are technically demanding, time-consuming, and expensive. Moreover, the development of the reconstituted embryos produced using these methods is retarded by mechanical damage caused during the enucleation procedure. Also, this method is unsuitable for the large-scale production of enucleated oocytes. In this study, I established a micromanipulator-independent method suitable for the large-scale production of enucleated oocytes.

In most mammalian species, ovulated oocytes are arrested at metaphase II. Fertilization or artificial activation releases this arrest, initiating meiosis II (MII) and extrusion of the second polar body. In these activated oocytes, chromosome segregation, spindle elongation, and rotation before cytokinesis are essential for normal second polar body extrusion. When MII oocytes were treated with demecolcine alone without oocyte activation, the oocytes formed cortical protrusions on their surfaces that contained complete chromosome sets (Yin *et al.*, 2002; Costa-Borges *et al.*, 2009). These protrusions could be readily aspirated using a micromanipulator. Additionally, demecolcine treatment after murine oocyte activation using ethanol effectively inhibits spindle rotation and results in impaired oocyte enucleation (Ibanez *et al.*, 2003). Low enucleation rates are caused by the suppression of polar body extrusion. It was previously shown that BMI-1026, a potent cdk1 inhibitor, could block germinal vesicle breakdown in mouse immature oocytes and could activate ovulated oocyes very efficiently. Compared with ethanol, BMI-1026 activates oocytes rapidly and efficiently (Choi, 2007). Oocytes activated by BMI-1026, instead of ethanol, can extrude their polar bodies, regardless of the presence of demecolcine, and are enucleated very efficiently when demecolcine is present.

In this study, spindle rotation was observed in most oocytes after 30 min of co-culture with demecolcine and BMI-1026 (data not shown). The protrusion induced by demecolcine and oocyte activation by BMI-1026 may result in a high rate of oocyte enucleation rather than the inhibition of spindle rotation caused by treatment with demecolcine.

Previous studies have shown BMI-1026 to be a potent cdk1 inhibitor [Ibanez, *et al.*, 2003; Seong, *et al.*, 2003]. In mouse oocytes, the effect of BMI-1026 appears to be reversible, based on results showing that GVBD occurred soon after the withdrawal of BMI-1026 (which suppressed GVBD) from immature oocytes. It is expected that BMI-1026 would produce a low level of toxicity. Although BMI1026 is a potent cdk1 inhibitor, it may inhibit another target(s) during oocyte enucleation. Although I tested other cdk1 inhibitors, including roscovitine and olomoucine (previously known), in place of BMI-1026, they did not effectively produce activated and enucleated oocytes (data not shown).

Several recent reports have attempted to develop methods for handmade cloning, which allows SCNT without the use of a micromanipulator. However, until now, the development of a method that is wholly micromanipulatorindependent has not been achieved (Vajta *et al.*, 2005; Vajta 2007). The novel method for enucleation presented in this study will be useful for SCNT and may be used in the development of handmade cloning techniques.

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(Received: 2 March 2010 / Accepted: 23 March 2010)