ORIGINAL

Effects of activation with a Ca ionophore and roscovitine on the development of human oocytes that failed to fertilize after ICSI

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Abstract : Objective : The effects of oocyte activation with a Ca ionophore and roscovitine (Ca+R), a selective inhibitor of M-phase promoting factor, on unfertilized oocytes after intracytoplasmic sperm injection (ICSI) or testicular sperm extraction (TESE)-ICSI were evaluated. Method : Oocytes without pronuclei at 18 hours after ICSI were judged to be unfertilized and were exposed to the Ca ionophore A23187 (5 μM) with or without roscovitine (50 μM). The activation rate was measured 3, 7, and 18 hours later. Oocytes with two polar bodies and two pronuclei with a sperm tail were judged to have been activated. Results : At 18 hours, the activation rates in the control, Ca ionophore, and Ca+R groups were 3.5% (4/112), 26.9% (7/26), and 32.1% (17/53), respectively. The activation rate of the Ca+R group was significantly higher than that of the control and similar to that of the Ca ionophore group. Among the oocytes that remained unfertilized after TESE-ICSI, the activation rates of the Ca ionophore and Ca+R groups were 22.2% (2/9) and 43.8% (7/16), respectively. Conclusions : Sequential treatment with an Ca ionophore and roscovitine activates oocytes that remain unfertilized after ICSI. In TESE-ICSI, the activation rate tended to be increased by the co-administration of roscovitine with a Ca ionophore. J. Med. Invest. 70 : 321-324, August, 2023

Keywords **:** *Ca ionophore, roscovitine, ICSI, oocyte activation, TESE*

INTRODUCTION

Ovulated oocytes fall dormant in the middle of meiosis II, but fertilization causes meiosis to resume and a transition to somatic cell division. This is called oocyte activation. It is known that M-phase promoting factor (MPF) is closely involved in meiotic arrest and resumption. In ovulated oocytes, MPF activity is maintained at a high level, which results in meiotic arrest in the middle of meiosis II. When sperm enters an oocyte, a pulsatile increase in the calcium (Ca) concentration, called Ca oscillation, is observed ; MPF activity is reduced ; and the oocyte is activated.

In infertility treatment, intracytoplasmic sperm injection (ICSI) is used in cases of severe male infertility. Recently, however, it has been discovered that there are sperm that are unable to induce oocyte activation, and even if these sperm are treated with ICSI, fertilization cannot be completed. There have been many reports about the successful fertilization of such oocytes by using artificial activation methods in combination with ICSI, resulting in live births (1-6). We have tried to develop an artificial oocyte activation method using a Ca ionophore in combination with puromycin, a protein synthesis inhibitor (7, 8). This activation method has already been employed in clinical practice by Murase *et al*., resulting in live births (5). However, since puromycin is a protein synthesis inhibitor, there is concern that it may cause malformations or genetic abnormalities when used early in development. Thus, in this study we investigated the efficacy of the artificial activation of human oocytes using roscovitine, a selective inhibitor of MPF, instead of puromycin.

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MATERIALS AND METHODS

Ovarian Stimulation and ICSI Protocol

Oocytes were retrieved from 29 ICSI cycles in 27 couples at the Tokushima University Hospital. Twenty of the 27 couples had male infertility factors, i.e. low sperm concentration and/or motility, and they were recommended for ICSI. Among them, 6 couples had azoospermic infertility, and testicular sperm extraction (TESE)-ICSI was performed. Ovarian stimulation and ICSI were performed according to the method described as previously (1, 9). Briefly, ovarian stimulation was carried out with a gonadotropin-releasing hormone agonist long protocol, and oocytes were retrieved at 35 hours after the administration of human chorionic gonadotropin. If at least 2 glowing follicles were not developed, cycle was canceled. The retrieved oocytes were cultured for 4 to 5 hours. Then the cells of the cumulus and corona radiata were removed and the denuded oocytes were rinsed several times. Morphologically intact oocytes were selected for ICSI. Semen was produced by masturbation in most case and TESE was undertaken in azoospermic couples. Sperm was prepared and adjusted to appropriate concentration, thereafter ICSI was carried out. After the ICSI, the oocytes were incubated for 16 to 18 hours. Fertilization status was confirmed at 12 to 16 hours and 16 to 18 hours after the ICSI, and oocytes that did not exhibit any pronuclei without a second polar body by 18 hours were judged to be unfertilized (10). Unfertilized oocytes that exhibited a first polar body were used for this experiment. Informed consent was obtained from each patient and experimental protocol was approved by the institutional review board of the University of Tokushima School of Medicine and carried out

in accordance with the guidelines issued by the Japan Society of Obstetrics and Gynecology (11).

Oocyte Activation

A Ca ionophore (calcium ionophore A23187 mixed calcium magnesium salt C5149, Sigma-Aldrich, Tokyo) and roscovitine (R7772, Sigma, St. Louis) were dissolved in dimethyl sulfoxide (DMSO ; D4540, Sigma-Aldrich, Tokyo) at concentrations of 1 mg/mL and 10 mg/mL, respectively, and stored at -80°C until use. At the time of use, the Ca ionophore A23187 was diluted in m-HTF to a final concentration of 5 μM. This dose of Ca ionophore A23187 was determined by our previous study in which 5 μM of A23187 was enough to activate oocyte with no evidence of normal fertilization (7). Roscovitine was diluted in human tubal fluid containing 0.4% bovine serum albumin (HTF ; Cat. No. 9600-001, Nippon Medical & Chemical Instruments Co, Osaka, Japan) to a final concentration of 50 μM. This dose of roscovitine was determined by our preliminary examination with serial dilution experiments.

Unfertilized oocytes were activated by our method, which effectively produced haploid parthenogenones (7, 8). The procedure was as follows : After ICSI, unfertilized oocytes were transferred to HTF medium containing 4% human serum albumin (HSA ; Sigma) and were preincubated at 37°C in 5% CO_2 in air for 30 minutes. After preincubation, the oocytes were washed three times with m-HTF and exposed to 100 μL of m-HTF medium containing 5 μM of Ca ionophore A23187 (Sigma ; stored at 1 mg/mL in dimethyl sulfoxide at 20°C) under paraffin oil (Wako Pure Chemical) on a warm plate (37°C) for 5 minutes. The oocytes were rinsed several times with fresh HTF medium and then placed in HTF medium containing 10 mg/mL of roscovitine (Sigma ; stored at 10 mg/mL in DMSO at -20°C) with 4% HSA, before being incubated at 37°C in 5% CO_2 in air for 5 hours. Then, the oocytes were washed three times with fresh HTF medium and cultured in a 100-μL drop of HTF medium supplemented with 4% HSA at 37°C in 5% CO_2 in air under paraffin oil for 13 hours.

After being incubated, the oocytes were examined for the formation of pronuclei and the presence or absence of a second polar body. Activated oocytes were defined as oocytes that exhibited two polar bodies and two pronuclei with a sperm tail (2PN2PB) or oocytes that immediately cleaved, and the activation rate was calculated as follows : Number of activated oocytes / Number of treated oocytes × 100. Some of the oocytes that displayed two pronuclei and a second polar body were additionally incubated under the same conditions for 24 hours and then were examined to determine whether they had cleaved. Cleaved oocytes were scored for quality according to our embryo scoring system (12).

Statistics

Statistical analyses were performed using the chi-square test. Statistical analyses were performed by the software Statcel (v.4 ; OMS Publishing, Inc., Tokorozawa, Japan). *P*-values of < 0.05 were considered statistically significant.

RESULTS

The activation rates of the control, Ca ionophore, and Ca ionophore with roscovitine (Ca+R) groups were 3.5% (4/112), 26.9% (7/26), and 32.1% (17/53), respectively. Significant differences in the activation rate were observed between the control group and the Ca ionophore group, and between the control group and the Ca+R group (Table 1).

In the cases treated with TESE, the activation rates of the Ca ionophore and Ca+R groups were 22.2% (2/9) and 43.8% (7/16), respectively (Table 2). The time-series of these cases demonstrated that the activation rate of the Ca+R group tended to become higher than that of the Ca ionophore group over time (Figures 1 and 2).

Table 1. The rate of oocyte activation

	No. of oocytes treated	No. of activated oocytes with 2PN2PB (%)
control	112	4 (3.5)
Ca ionophore	26	7 (26.9) *
Ca ionophore with roscovitine	53	17 (32.1) *

*$P < 0.05$ vs control

Table 2. The rate of oocyte activation (TESE)

	No. of oocytes treated	No. of activated oocytes with 2PN2PB (%)
Ca ionophore	9	2 (22.2)
Ca ionophore with roscovitine	16	7 (43.8)

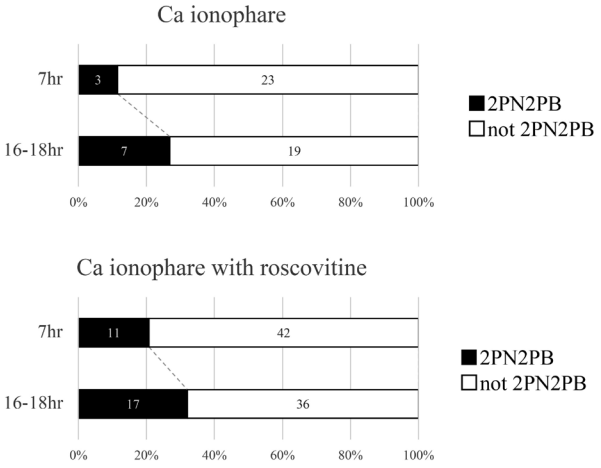

Fig 1. Effects of treatment with a Ca ionophore alone or a Ca ionophore with roscovitine on the oocyte activation rate at 7 and 16–18 hr after ICSI in the non-TESE-treated cases

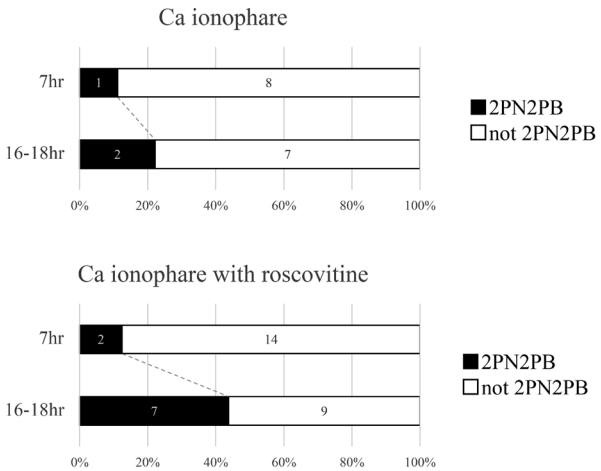

Fig 2. Effects of treatment with a Ca ionophore or a Ca ionophore with roscovitine on the oocyte activation rate at 7 and 16–18 hr after ICSI in the TESE-treated cases

DISCUSSION

The present study showed that artificial oocyte activation with a combination of roscovitine and a Ca ionophore was able to activate oocytes as well as treatment with the Ca ionophore alone, even in cases in which TESE was employed, in which Ca oscillation may be unlikely to occur, and the oocyte activation rate tended to be higher in the Ca+R group.

It has been reported that fertilization failure occurs in 2-3% of ICSI cycles and that most cases in which ICSI fails to fertilize oocytes are due to the failure of oocyte activation (13-15). In addition, it is known that sperm are present in approximately 83% of oocytes that are determined to be unfertilized after ICSI (16), and if these unfertilized oocytes are properly activated they may show bipolar anterior nuclei, like normally fertilized oocytes, after activation and continue to develop normally thereafter. In fact, there have been many reports of artificial oocyte activation using chemical agents, such as Ca ionophores or electric pulses, after ICSI in cases of globozoospermia or repeated fertilization failure after ICSI, which resulted in pregnancy (1-6). The use of Ca ionophores or other chemical agents or electric pulses after ICSI has been reported in many cases of globozoospermia (17).

We have previously developed a method for artificially activating ovulated oocytes in mice, in which we used a combination of a Ca ionophore and puromycin to obtain a high rate of half-length eggs (7). In addition, we have reported a method for artificially activating unfertilized human oocytes using a combination of a Ca ionophore and puromycin (8). It has been confirmed that this method activates unfertilized human oocytes after ICSI at 20 hours after the injection of sperm and that the rate of pronucleus appearance was approximately 90%, the division rate was approximately 65%, and the percentage of embryos with a good morphology was approximately 45% (8, 14). Furthermore, another clinical study showed that oocytes activated with this method were responsible for live births (5). However, the mechanism by which puromycin causes oocyte activation is still unclear, and there is concern that the use of puromycin, a protein synthesis inhibitor, early in development may induce malformations or genetic abnormalities.

On the other hand, since roscovitine is a selective inhibitor of MPF, a complex of cyclin-dependent kinase 1 (Cdk1) and cyclin B, its pharmacological mechanism is evident. Although roscovitine exerts its effects by competing for the ATP-binding domain of Cdk, it does not affect other related kinases, such as extracellular signal-regulated kinases 1 and ERK2, and mitogen-activated protein kinase, which appears to be involved in oocyte activation along with MPF (18, 19). Therefore, it is considered that the use of roscovitine is safer than the use of puromycin for inducing fertilization and subsequent development. Although roscovitine inhibits the activity of Cdk2-cyclin A and Cdk2-cyclin E, which are involved in the cell cycle (18, 19), these actions of roscovitine are known to be reversible ; i.e., they cease just after the removal of roscovitine (18, 20, 21). Furthermore, although roscovitine inhibits mRNA synthesis by suppressing Cdk (7, 22) early oocyte development utilizes maternally derived mRNAs that are present in the oocyte, and hence, the effects of roscovitine may be small. However, because adverse effects of roscovitine on oocytes and embryos cannot be completely ruled out, the dividing ability, developmental potential, etc., of oocytes subjected to roscovitine treatment should be further evaluated prior to the drug's clinical application. Especially, chromosomal analysis of activated oocyte may be essential to determine the safety of this method.

In the present study, although there was no significant difference in the oocyte activation rate between the Ca ionophore alone and Ca+R methods in the cases treated with TESE-ICSI, the activation rate tended to be higher in the cases treated with a combination of roscovitine and a Ca ionophore. This tendency was observed at 16-18 hours after ICSI, but not at 7 hours after ICSI. In our preliminary experiment, it was confirmed that the suppressive action of combined treatment with a Ca ionophore and roscovitine on MPF activity was sustained longer than was seen after treatment with the Ca ionophore alone (unpublished data). Thus, it is speculated that the lack of reduction in MPF activity may have caused the low fertilization rate seen in the TESE-ICSI-treated cases and that longer-lasting effects of roscovitine on MPF may be effective in terms of fertilization. Further examinations would be needed to confirm this hypothesis.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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