Fertilization failure and oocyte activation

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Abstract : Recent reports have suggested that when unfertilized oocytes with a spermatozoon after intracytoplasmic sperm injection (ICSI) are properly activated, the activated oocytes develop normaly similar to fertilized oocytes. However, human oocytes do not easily respond to universal activators of mammalian oocytes like ethanol or calcium ionophore A23187, which induce a calcium transient in ooplasm. Puromycin activates human oocytes at a rate of 90%, but more than two thirds of the parthenogenones possess 2 haploid pronuclei or 1 diploid pronucleus without extrusion of the second polar body. Therefore, the activation method which produces one pronucleus with extrusion of the second polar body in oocytes without a spermatozoon is necessary for producing embryos with normal karyotypes. Recently, we found the oocyte activation method which produced parthenogenones displaying one pronucleus with extrusion of the second polar body. Using our method (a combination of calcium ionophore A23187 and puromycin), the activation rate was approximately 90% and the proportion of parthenogenones displaying one pronucleus with extrusion of the second polar body was approximately 80% in human aged and mouse young oocytes. When human unfertilized oocytes following ICSI were activated by this method, two pronuclei were formed with extrusion of the second polar body in 30% of the oocytes. Four cleaved parthenogenones (or embryos) showed normal karyotypes. However, the cytotoxic, teratogenetic and mutagenetic activity of Ca ionophore and puromycin should be approved prior to the clinical adaptation of the method. J. Med. Invest. 47 : 1-8, 2000

Key words **:** *fertilization, oocyte activation, parthenogenone, MPF, MAPK*

INTRODUCTION

In-vitro fertilization (IVF) is a technique which offers a possibility for fertilization to infertile couples and has been used world-wide for aiding infertile couples with tubal blockage. However, the technique is not effective for severe oligozoospermic patients because few spermatozoa of the patients can penetrate the zonae pellucidae. For more than a decade, the fertilization failure of spermatozoa from oligozoospermic patients was one of the most important problems to be solved for improving the pregnancy rate of oligozoospermic patients. The appearance of intra-cytoplasmic sperm injection (ICSI) dramatically improved the fertilization and pregnancy rates of oligozoospermic patients and ICSI showed that penetration of spermatozoa through the zonae pellucidae and fusion between a sperm cell membrane and oolenma are not essential for early development of embryos (1). However, fertilization failure still occurs in ICSI (1-3). Approximately 80% of unfertilized oocytes following ICSI contain a spermatozoon (4). This suggests that the process from meiosis to mitosis was impaired in the unfertilized oocytes and the fertilization failure is defined as failure of the M-G1 transition in metaphase II (MII) oocytes. The failure of the M-G1 transition may be repaired by artificial oocyte activation. In this review, we discuss the possible treatment for failure of the M-G1 transition in MII oocyte.

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FAILURE OF OOCYTE ACTIVATION IN ICSI

In mammals, except dogs and foxes, oocytes which are ovulated from ovaries are arrested at M II and they contain a high level of maturation (or M-phase) promoting factor (MPF : the universal regulator of the G 2-M transition in eukaryotic cells, which consists of cdc2 kinase and cyclin B). Cytostatic factor (CSF), which stabilizes MPF activity is also kept high in MII oocytes (5). When one spermatozoon contacts the oolenma or penetrates into the ooplasm, calcium oscillation occurs (6-8). The mechanism that induces calcium oscillation in ooplasm remains unknown. However the success of ICSI strongly suggests that a factor which induces calcium oscillation in ooplasm (so called oscillin) is present in spermatozoa and is directly introduced into the ooplasm, not via a sperm receptor (4). Calmodulin-dependent kinase II (CaM KII) is activated by a Ca^{2+} transient and the activation of the kinase probably turns on the cyclin degradation machinery, resulting in cyclin B degradation and sister chromatid separation (9) (Fig.1). The process of M-G1 transition in MII oocytes is called" oocyte activation ". In in vitro fertilization (IVF) 5~32% of unfertilized oocytes have a spermatozoon in the ooplasm (10-15). This suggests that at least two-thirds of spermatozoa, which penetrate oocytes possesses an ability to activate MII oocytes.

ICSI is the most efficient and widely accepted treatment for aiding severe oligozoospermic patients, a group of azoospermic patients and patients who experienced previous fertilization failure using conventional IVF and embryo transfer (ET) treatment. The overall fertilization rate by ICSI is between 50% and 70% (1-3). In contrast to IVF, approximately 80% of unfertilized oocytes which have no signs of activation following ICSI have a spermatozoon in their ooplasm, and in the majority of these oocytes, the sperm head was partially or completely decondensed (4). Many studies have suggested that failure of oocyte activation is the principal cause of fertilization failure in ICSI (15-17). Even spermatozoa with abnormal morphology and elongated spermatids have an ability to activate oocytes similar to normal mature spermatozoa. Considering these findings, cell membranes of spermatozoa which fuse to oolenma during normal fertilization still remain in the ooplasm and may interfere with the migration of oscillin into ooplasm at the proper time, resulting in failure of oocyte activation. Recently, Yanagida *et al*, reported that in 0.9% of ICSI cases with motile spermatozoa, the motile spermatozoa failed to fertilize the oocytes despite an apparently successful ICSI procedure (18). Futhermore, in patients with round-headed sperm the fertilization rate following ICSI was extremely low compared with patients

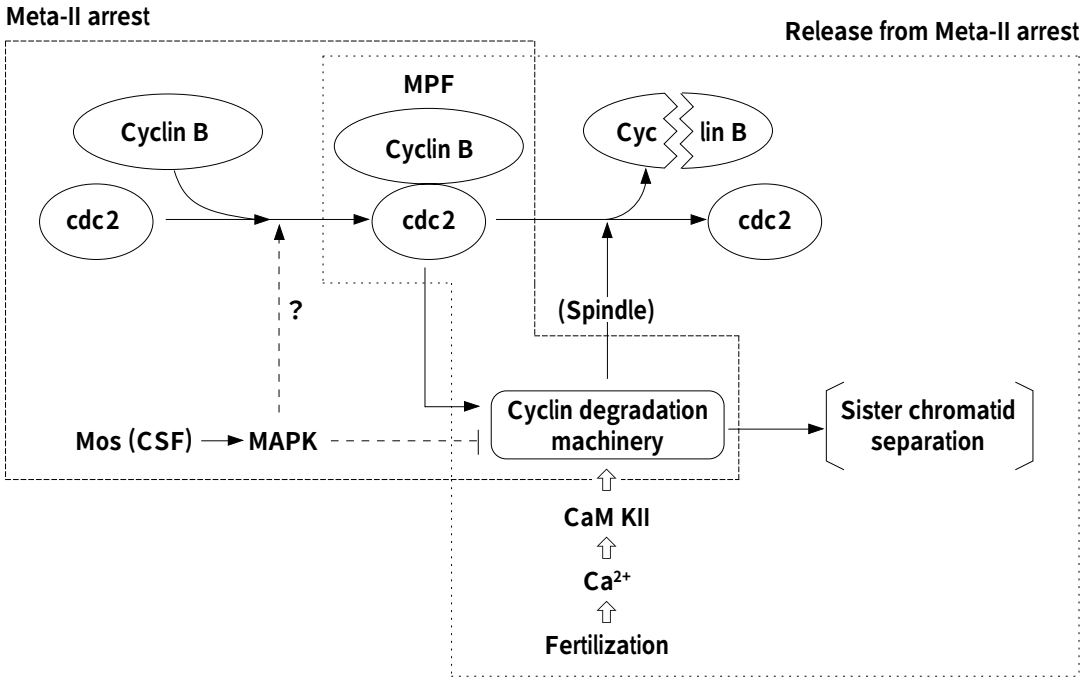

Fig.1. Model for the mechanisms of metaphase II arrest and release in vertebrate eggs. Fertilization triggers the activation of calmodulin-dependent kinase II via a Ca^{2+} transient, which probably activates the cyclin degradation machinery, resulting in cyclin B degradation and sister chromatid separation (Sagata, 1996)

with other types of male infertility due to the absence of oocyte activation capacity (19, 20). If unfertilized oocytes after ICSI are properly activated, they may form two pronuclei with extrusion of the second polar body, and cleave and develop normally. Indeed, oocytes injected with a spermatozoon unable to fertilize MII oocytes were immediately activated with electric pulse or calcium ionophore A23187. Consequently, successful pregnancies were achieved and two healthy babies were born (18, 20). The activation methods they used produced two pronuclei with extrusion of the second polar body in oocytes with a spermatozoon. This suggests that the activation method which produces one pronucleus with extrusion of the second polar body in oocytes without a spermatozoon is necessary for producing embryos with normal karyotypes.

OOCYTE ACTIVATION WITH CALCIUM IONOPHORE A23187 AND PUROMYCIN

There are many physical and chemical stimuli for inducing parthenogenetic or oocyte activation in mammals, such as ethanol (21, 22), calcium ionophore A23187 (Ca ionophore) (23, 24), inhibitors of protein synthesis (25, 26) and electric pulse (24, 27-29). Mouse aged oocytes are easily activated at a consistently high rate by Ca ionophore or ethanol which induce a calcium transient in the ooplasm (30). In contrast to mouse oocytes, Ca ionophore does not effectively activate human aged oocytes (31, 32). In general, human aged oocytes are less sensitive to widely used stimuli like Ca ionophore and ethanol than mouse aged oocytes. However, puromycin, an inhibitor of protein synthesis, produces parthenogenones in about 90% of human oocytes (31, 33, 34). However, 77% of the human parthenogenones activated by puromycin possessed 2 haploid pronuclei or 1 diploid pronucleus without extrusion of the second polar body (34). Moses and Masui showed that 6-dimethylaminopurine (6-DMAP)-an analogue of puromysin-suppressed extrusion of the second polar body in mouse oocytes (35). As mentioned above, an increase in intracellular Ca concentration and MPF inactivation are the main molecular events of M-G1 transiton in MII oocytes and to mimic these events, human aged unfertilized oocytes following IVF were activated using various combinations of Ca ionophore and puromycin or 6-DMAP. When oocytes activated with 5 μM calcium ionophore A23187 (Sigma, St. Louis, Mo, USA) for 5 minutes were incubated in HTF medium containing 10μg/ml puromycin (Sigma, St. Louis, Mo, USA) for 5 hours, the activation rate was approximately 90%, which is similar to the rates reported by De Sutter et al. (33, 34) and Balakier et al. (31). Furthermore, in approximately 80% of the activated oocytes, one pronucleus was formed with extrusion of the second polar body (Fig 2). However, when oocytes were treated with Ca ionophore and 6-DMAP, they did not extrude the second polar body. In the next experiment, the activated oocytes displaying one pronucleus with extrusion of the second polar body were cytogenetically analysed. Cytogenetical analysis showed a normal haploid set of chromosomes in 78% of the activated oocytes. These findings suggested that the activated oocytes displaying one pronucleus with extrusion of the second polar body were haploid. If unfertilized oocytes with a spermatozoon are activated with a combination of Ca ionophore and puromycin, the activated oocytes should possess two pronuclei -one female and one male pronuclei- with extrusion of the second polar body similar to normal fertilized

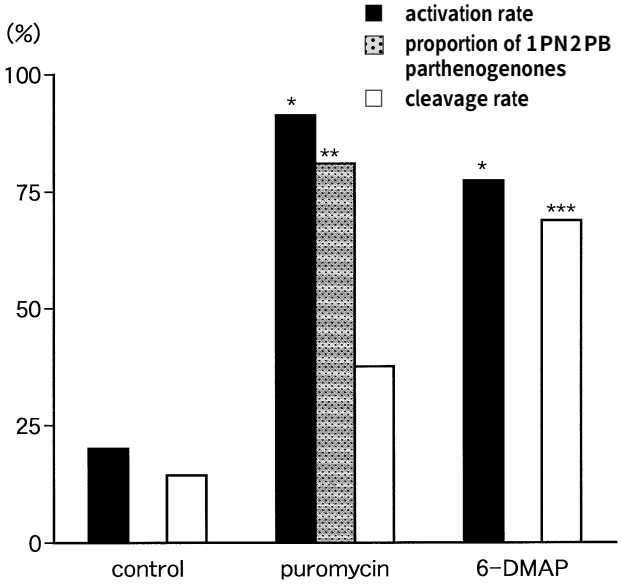

Fig.2. The activation rate, proportion of parthenogenones displaying one pronucleus with extrusion of the second polar body and cleavage rate in human aged oocytes activated by calcium ionophore and protein phosphorylation inhibitors. Human aged unfertilized oocytes activated with 5 μM calcium ionophore A23187 for 5 minutes were incubated in HTF medium containing 10 μg/ml puromycin for 5 hours. After incubation for 13 hours, the oocytes were examined using an inverted microscope with Nomarski interference phase-contrast optics." Activated oocytes "were defined as oocytes with at least one pronucleus or that had immediately cleaved.
*p<0.05 : puromycin and 6-DMAP vs control
**p<0.05 : puromycin vs 6-DMAP and control
***P<0.05 : 6-DMAP vs puromycin and control

oocytes. In our preliminary study, when unfertilized oocytes following ICSI were treated with Ca ionophore and puromycin, two pronuclei and the second polar body appeared in 30% of the oocytes 18 hours after treatment with Ca ionophore. Seventy percent of the activated oocytes developed as far as the 8-cell stage 42 hours after the Ca ionophore treatment and in cytogenetical analysis all cleaved parthenogenones (n=4) showed normal karyotypes. However, whether one of the appeared pronuclei was derived from a spermatozoa or not remains unknown. The sex ratio of the cleaved parthenogenones should be determined to clarify this, because half of the cleaved parthenogenones must have Y chromosomes if the genome derived from a spermatozoa is normally involved in the early development.

Young oocytes are not easily activated by a calcium transient. Kubiak *et al*. (22) and Vincent *et al*. (36) reported that mouse young oocytes activated with Ca ionophore or 7% ethanol did expel a second polar body, but became arrested again at so-called metaphase III (MIII), in which unichromatid chromosomes were scattered in misshapen microtuble spindles. However, 6-DMAP induced mouse young oocytes that were activated with Ca ionophore, but arrested at MIII, to enter interphase (35). In the present study, when mouse young oocytes were exposed to 5 μM Ca ionophore and incubated in a medium containing 10 μg/ml puromycin for 4 hours, the activation rate was 90%. More than 80% of the activated oocytes showed one pronucleus with extrusion of the second polar body (Table 1, Fig. 3). These findings are similar to those of human aged oocytes. Futhermore, the DNA content of the pronucleus of the activated oocytes was half the DNA content of a set of MII chromosomes and the most highly activated oocytes possessed 20 chromosomes (normal haploid set). In conclusion, a combination of Ca ionophore and puromycin produced haploid parthenogenones in young mouse oocytes. This strongly suggests that the combination is effective for inducing haploid parthenogenones from young human oocytes although we did not activate human young oocytes in the present study.

MOLECULAR EVENTS INDUCED BY CALCIUM IONOPHORE AND PUROMYCIN

It is unknown how the combination of Ca ionophore and puromycin produces parthenogenones displaying one pronucleus with extrusion of the second

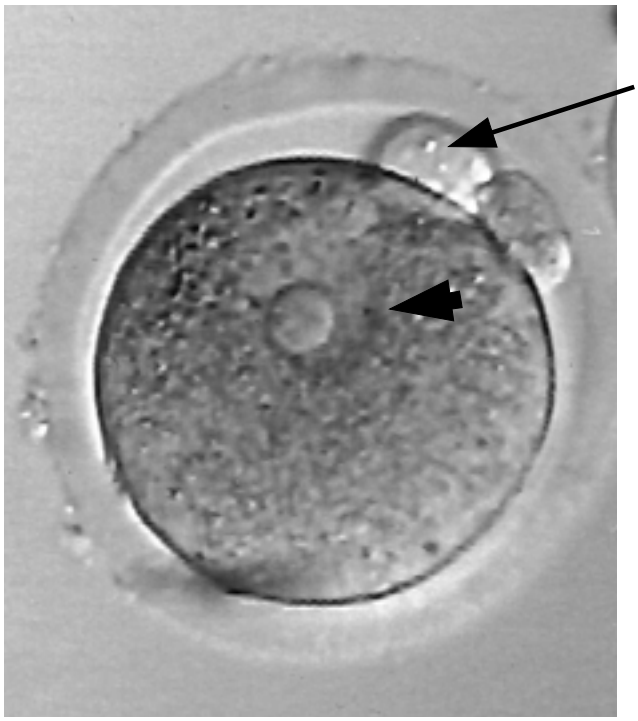

Fig. 3. Parthenogenone diaplaying one pronucleus with extrusion of the second polar body. arrow : the second polar body, arrow head : pronucleus

Table 1. Effect of calcium ionophore and/or protein phosphorylation inhibitors on oocyte activation of mouse young oocytes.

			Activated oocytes (%)	
		No. of oocytes treated	Total No. of activated oocytes (%)	No. of parthenogenones with 1PN2PB (%)
	Control	90	8 (8.9)	3 (3.3)
Ca ionophore (+)	Puromycin	116	103*(88.8)	94*(81.0)
	DMAP	93	41 (44.1)	28 (30.1)

*p<0.0001 (the puromycin group vs the control and DMAP groups)
PN : pronucleus
PB : polar body
DMAP : 6-dimethylaminopurine
Activated oocytes means oocytes which had formed at least one pronucleus.

polar body. A Ca^{2+} transient induced by the Ca ionophore must trigger the M-G1 transition. Rinaudo *et al*. reported that human oocytes exposed to Ca ionophore exhibited an isolated elevation of intracellular calcium concentration, followed by a prompt return to baseline levels (32). A Ca^{2+} transient activates calmodulin-dependent kinase II (CaM KII), which probably turns on the cyclin degradation machinery, resulting in cyclin B degradation and sister chomatid separation (9). In the present study a Ca^{2+} transient induced by Ca ionophore probably inactivated MPF activity via CaM KII. However, a Ca^{2+} wave could be a positive signal that activates myosin light chain kinase (37). This kinase is activated through the Ca^{2+}/calmoduline complex and activates the contractile ring which consists of actin and myosin in cytokinesis. The extrusion of the second polar body is an uneven cleavage of the cell. A Ca^{2+} transient induced by Ca ionophore might play an important role of extruding the second polar body.

In our experiments, 25~50% of oocytes activated by Ca ionophore alone extruded the second polar body without formation of a pronucleus (M III stage). Cotreatment with puromycin increased the rate of oocytes with a pronucleus or pronuclei to approximately 90%. Verlhac *et al*, reported that when freshly ovulated MII mouse oocytes were treated with ethanol, MPF activity dropped to one fourth at the time of polar body extrusion and this activity increased again in MIII oocytes to a level slightly lower than that observed in MII oocytes (38). MPF activity of MIII oocytes might have inceased again in the present study. Puromycin and 6-DMAP are known to inhibit mitogen activating protein kinase (MAPK) which is a component of CSF in mouse (39) and bovine oocytes (40). Sagata reported that MAPK probably activates a positive regulator of MPF (e.g. cdc 25) or inhibits negative regulators (e.g. weel or proteinphosphatase 2 A) (9). So, this suggests that puromycin decreased MPF activity via suppression of MAPK, resulting in the formation of a pronucleus.

Cotreatment with 6-DMAP completely suppressed extrusion of the second polar body in human aged oocytes, but not cotreatment with puromycin. This finding agrees with the study by Liu *et al*. (41) that the combination of Ca ionophore and 6-DMAP disrupted the oocyte spindle and effectively produced parthenogenones diaplaying one pronucleus without extrusion of the second polar body. However, treatment of puromycin without Ca ionophore produces parthenogenones displaying 2 haploid pronuclei or 1 diploid pronucleus without extrusion of the second polar body. It is unkown how the combination of Ca ionophore and puromycin can extrude the second polar body. Some molecular events provoked by the Ca ionophore might be suppressed by 6-DMAP, but not puromycin, resulting in suppression of extrusion of the second polar body.

A major difference between puromycin and 6-DMAP is that puromycin inhibits protein synthesis, while 6-DMAP does not. Rime *et al*. (42) reported that puromycin provokes a approximate 60% decrease in protein synthesis together with the formation of a pronucleus. However, the emission of the second polar body does not appear to be attributed to the inhibition of protein synthesis.

CLINICAL ADAPTATION OF THE OOCYTE ACTIVATION

Clinical adaptation of this method may be possible, but it is still far from reality at present. Prior to the clinical adaptation, the safety of rescuing unfertilized oocytes and the cytotoxic, teratogenetic and mutagenetic activity of Ca ionophore and puromycin should be approved. Many cytogenetical analyse show that approximately 25% of unfertilized oocytes following IVF have chromosomal aberrations (11, 14, 15, 43, 44, 45-47). Pickering *et al*. reported that aged unfertilized oocytes (24~48 h) displayed a higher incidence (approximately 30%) of disrupted or abnormal spindles (48). Furthermore, Dozortsev recently reported that swollen nuclei with fragmented pieces of chromosomes were observed in 51% of unfertilized oocytes after ICSI and these probably corresponded to degenerating nuclei (16). If unfertilized oocytes are evenly activated with Ca ionophore and puromycin, activated oocytes with chromosomal aberrations and/or swollen nuclei with fragmented pieces of chromosomes must be discarded. However, in the present study approximately 80% of oocytes, which had been unfertilized after IVF, showed a normal haploid set of chromosomes after activation. This may suggest that MII oocytes respondent to Ca ionophore and puromycin have 23 chromosomes with 2C and have an ability to finish meiosis. In respect to organelles other than chromosomes, Sousa and Tesarik reported that no obvious ultrastructual signs of degeneration (vacuolation, mitchondrial swelling) were apparent in any of 15 oocytes that failed to fertilize by 44 h

after ICSI although the functions of organelles in the oocytes remain unknown (49).

In ICSI cases with spermatozoa unable to fertilize oocytes, successful pregnancies were achieved using a combination of ICSI and oocyte activation (18, 19). Young oocytes are difficult to activate because the activities of MPF and MAPK are stable. However, in mice, 90% of newly ovulated oocytes were activated with Ca ionophore and puromycin and approximately 80% of the activated oocytes showed one pronucleus with extrusion of the second polar body. This suggests that the combination of Ca ionophore and puromycin is effective for activating young human oocytes. In cases of activating young oocytes, the adverse effects of aging on oocyte quality was not found to be an important consideration.

Ca ionophore A23187 does not appear to be cytotoxic to oocytes if the concentration is low (50). Indeed, in mice, oocytes activated by Ca ionophore developed as far as the post-implantation stage. If oocytes survive an isolated elevation of intracellular calcium concentration induced by Ca ionophore, the activated oocytes with a diploid set of chromosomes developed as far as the post-implantation stage. However, these parthenogenones do not have a paternal genome and some genes like the igf2 gene are not expressed because of genomic imprinting. So, the ability to develop as far as the post-implantation stages does not mean that the genes are normal. Further studies are required to approve the safety of Ca ionophore, although a healthy baby was born after treatment with Ca ionophore.

A high dosage and/or prolonged treatment with puromycin can irreversibly damage the chromosomes (pulverization, breakage), but not a low dosage and a short treatment (33). Indeed, in the present human and mouse cytogenetical analysis, 70~80% of oocytes activated with Ca ionophore and puromycin showed a normal haploid set of chromosomes with normal morphology. Bovine oocytes activated with Ca ionophore and 6-DMAP developed to the blastocyst and hatched blastocyst stages similar to those derived from IVF-ET (41). These findings suggest that inhibition of MAPK activity do not appear to affect the developmental abilty of embryos.

At present there are no methods to select fresh normal embryos. By culturing embryos to the blastocyst stage, genetically abnormal embryos may be selected out through aberrations in their development (51) and may produce the most viable embryos without abnormal organelles. However, the ability to develop as far as the post-implantation stages does not indicate the genes are developing normally. At present, the teratogenetic and mutagenetic activity of Ca ionophore and puromycin are under investigation in our center using a mouse model.

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