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A cytogenetic study of in-vitro matured murine oocytes after ICSI by human sperm

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A cytogenetic study of in-vitro matured murine oocytes after intracytoplasmic sperm injection by human spermatozoa

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A running title: Chromosome and IVM

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Abstract

BACKGROUND: The purpose of this study was to investigate the chromosomal normality and developmental potential of in-vitro matured murine oocytes following ICSI by human spermatozoa.

METHODS: Heterologous ICSI fertilization between mouse oocytes and human spermatozoa were employed in order to overcome the reduced fertilization rates after conventional IVF due to zona hardening during the in-vitro maturation, and to assess separately maternal and paternal chromosome complements. Cytogenetic analysis were performed in four types of oocytes : 1) in-vitro matured MII oocytes; 2) in-vivo matured MII oocytes; 3) in-vitro matured oocytes after ICSI; 4) in-vivo matured oocytes after ICSI.

RESULTS: Activation rates after ICSI of in-vitro matured oocytes was lower than that of in-vivo matured oocytes (69.9% vs. 97.2%, $p< 0.01$), and premature chromosomal condensation was only observed in in-vitro matured oocytes. However, there were no significant differences in developmental rates after successful activation between in-vivo matured and in-vitro matured ICSI oocytes (69.7% vs. 76.6%). The incidences of aneuploidy and structural aberrations were similar between the ICSI embryos and non-ICSI (MII) oocytes. Furthermore, the frequency of chromosomal aberrations was not associated with in-vitro or in-vivo maturation. Similar analysis in paternal chromosomes indicated that there was no significant difference in the incidence of chromosomal aberrations between the

embryo derived from in-vitro and in-vivo matured oocytes.

CONCLUSIONS: These results suggest that in-vitro matured oocytes following ICSI do not lead to an increase in the frequency of aneuploidy and structural aberrations when human sperm are injected into mouse oocytes.

Introduction

It is well known that ovarian stimulation induces asynchrony of follicular growth and oocyte maturation, and it is occasionally observed that the oocytes retrieved for assisted reproductive technology (ART) are in various stages of meiotic maturation (Mandelbaum et al., 1996). A decrease in the total number of oocytes at the metaphase II (MII) stage in ART leads to reductions in the number of embryos suitable for transfer and the pregnancy rates. Recently, some attempts have been made to perform second day intracytoplasmic sperm injection (ICSI) after in-vitro maturation of metaphase I (MI) or germinal vesicle (GV) oocytes in order to increase the number of embryos for transfer (Nagy et al., 1996; Edirisinghe et al., 1997). Moreover, in vitro fertilization (IVF) after in vitro maturation of immature oocytes retrieved from patients with polycystic ovary or developing ovarian hyperstimulation could be a useful alternative to conventional IVF (Barnes et al., 1995; Jaroudi et al., 1997).

It has been reported that the fertilization rates of in-vitro matured oocytes after insemination are very low since zona hardening occurs during the in-vitro maturation of mouse, rat and human oocytes (Beckers et al., 1999; Choi et al., 1987; Zhang et al., 1991). Therefore, ICSI could be an important technique to achieve successful fertilization of in-vitro matured oocytes.

There are conflicting reports regarding the chromosomal normality of in-vitro matured oocytes. It was demonstrated (Cooper et al., 1998) that the chromosomal normality was not significantly different between in-vitro and in-vivo matured murine oocytes in stimulated cycles, but an another report (Badenas et al., 1989) indicated that insemination prior to completion of oocyte maturation resulted in chromosomal aberrations. The amount of data concerning the normality of in-vitro matured oocytes following ICSI is very limited. It was reported (De Vos et al., 1999) that the activation rates after ICSI in in-vitro matured human oocytes were lower than those in in-vivo matured oocytes, but developmental competence after successful fertilization was similar in the two groups. However, cytogenetic normality of in-vitro matured oocytes after ICSI was not evaluated in those studies. Although it has been reported that there was no differences in numerical chromosomal abnormalities between embryos generated by ICSI and conventional IVF in human, relatively high incidences of aneuploidy and chromosomal mosaics has been suggested (Edirisinghe et al., 1997; Wall et al., 1996; Munne et al., 1998).

The purpose of this study was to investigate the chromosomal normality, activation and developmental potential of in-vitro matured mouse oocytes following ICSI by human spermatozoa. Heterologous ICSI fertilization were employed in order to overcome the reduced fertilization rates after conventional IVF due to zona hardening during the in-vitro maturation, and to assess separately maternal (mouse oocyte-derived) and paternal (human sperm-derived) chromosomes at the first cleavage metaphase. We also investigated the chromosomal normality of in-vitro matured and in-vivo matured MII oocytes. Therefore, in-vitro and in-vivo matured MII oocytes, and in-vitro and in-vivo matured oocytes after ICSI were analyzed.

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Materials and Methods

Preparation of Gametes

All of the oocytes were obtained from 3-5 week old B6D2F1 mice. Mice were primed with 5 IU pregnant mare serum gonadotropin (PMSG, serotropin; Teikokuzouki, Tokyo, Japan) and injected with 5 IU of hCG (HCG mochida; Mochida Pharmaceutical, Tokyo, Japan) 48 hr later. In-vivo matured oocytes were collected from oviducts 16 h after hCG administration. They were freed from cumulus cells by treatment with 0.1% hyaluronidase in Hepes-buffered CZB medium and were kept in CZB medium until ICSI.

Free-grown GV intact oocytes were obtained from B6D2F1 mouse ovaries after priming with 5 IU of PMSG. Forty-eight hours later, the ovaries were removed, placed in HEPES-buffered human tubal fluid (modified HTF; Irvine Scientific, Santa Ana, CA, USA) medium supplemented with 4 mg/ml BSA. The oocytes were isolated by manual puncture of the large antral follicles using 27-gauge sterile needles. Collected cumulus-oocyte complexes (COCs) were washed with modified HTF medium and transferred to a 4-well culture dish (Greiner, Germany) containing 0.2 ml of HTF medium supplemented with 4 mg/ml BSA within 20 min of collection. The COCs were cultured for 18 hr at 37℃ under 5% CO_2 in air. After 18 h of culture, the cumulus cells were removed mechanically with a Pasteur pipette or two hypodermic needles in Hepes-buffered CZB medium containing 0.1%

hyaluronidase within a few minutes.

The cumulus-free oocytes were morphologically assessed under an inverted microscope and spermatozoa were injected into only MII oocytes.

Frozen-thawed human spermatozoa

Semen samples were collected from volunteer donors. Sperm concentration, motility, and vitality were assessed according to World Health Organization (WHO) guidelines (1993), and "normal" samples were selected. Written consent was obtained from the donors and the local ethics committee approved this experiment. Spermatozoa were cryopreserved by the dropwise addition of TEST (TES and Tris) yolk buffers (Irvine Scientific, Santa Ana, CA, USA) to a 1:1 ratio of semen: TEST yolk buffers. Diluted samples were frozen rapidly in liquid N_2 vapor for 20 min before being submerged into liquid N_2. For thawing, the cryovials were placed into a waterbath at 37℃ for a few minutes. The thawed samples were washed by centrifugation with BWW medium at 2000g for 5 min.

Microinjection of spermatozoa and oocytes culture

In this study, heterologous fertilization between mouse oocytes and human spermatozoa

were performed in order to assess separately maternal and paternal chromosome complements.

Injection of human spermatozoa into mouse MII oocytes was performed using a piezo-electric pipette-driving unit (Kimura et al., 1995). In brief, an oocyte was held on a holding pipette and its zona pellucida was drilled by applying a few piezo pulses. A single human spermatozoon was immobilized by the application of piezo pulses to the upper one-third of the tail, and then sucked up into the injection pipette. After breaking the oolemma with one or two piezo pulses, the spermatozoon was injected. All procedures were performed in Hepes-CZB medium at room temperature and injection of 30 to 40 oocytes each time was completed within 120 min.

Injected oocytes were transferred to CZB medium and cultured for 6-8 hr at 37℃ under 5% CO_2 in air. After incubation, they were transferred into another droplet of CZB medium containing 0.006 μ g /ml vinblastine (Sigma, St Louis, MO, USA) for 10 h and slides were prepared for chromosomal analysis.

Cytological and chromosomal preparations

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Oocytes with apparently normal morphology were prepared for chromosomal analysis by a gradual-fixation/air-drying method (Mikamo et al., 1983). The oocytes were treated with

1% (w/v) pronase (Kaken Pharmaceuticals, Tokyo, Japan) for 5 min to remove zonae pellucidae and then treated with a hypotonic solution (1:1 mixture of 1% sodium citrate and 30% fetal bovine serum) for 10 min at room temperature. Oocytes were fixed with fixative I (methanol: acetic acid; H_2O=5:1:4) for a few minutes, mounted on a glass slide, and covered with fixative II (methanol: acetic acid =3:1). Thereafter, the slide was dipped into fixative II for 30 min. Finally, it was fixed with fixative III (methanol: acetic acid: H_2O=3:3:1) for 1 min, and gently dried with a warm moist airflow. Fixed preparations were stained with 2% Giemsa stain for 7-8 min. After conventional chromosome analysis, the chromosomes underwent C-banding to detect acentric and discentric chromosomes (Summer, 1972).

Four types of oocytes were analyzed: 1) in-vivo matured MII oocytes (control/MII); 2) in-vitro matured MII oocytes (IVM/MII); 3) oocytes that had undergone ICSI after in-vivo maturation (control/ICSI); 4) oocytes that had undergone ICSI after in-vitro maturation (IVM/ICSI)

Statistical analysis

Data were compared between the experimental groups using the chi-square test and Fisher's exact test as appropriate. The differences were considered significant at a level of $p < 0.01$.

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Results

Activation rate and developmental rate after ICSI

In the control/ICSI group and IVM/ICSI group, 191 and 361 MII oocytes, respectively, were used for ICSI. In the control/ICSI group, 145 oocytes were available for cytological assessment (Table I). The activation rate and the number of oocytes reaching mitotic metaphase were 97.2% (141/145) and 76.6% (108/141), respectively, in control/ICSI group. In contrast, 269 oocytes in IVM/ICSI group were available for cytological assessment and activation rate of IVM/ICSI group was significantly lower than that of control/ICSI group (69.9% vs 97.2%, $p < 0.01$). However, the number of oocytes reaching mitotic metaphase after successful activation was not significantly different between the two groups (76.6% in control/ICSI group and 69.7% in IVM/ICSI group, respectively).

Twenty-five oocytes in the control/ICSI group and 109 oocytes in IVM/ICSI group arrested their development before forming male pronucleus (Table II). Of these oocytes, 4 (16.0%) in the control/ICSI group and 81(74.3%) in IVM/ICSI group were not activated and remained at metaphase II stage. All the 4 non-activated oocytes in control/ICSI group and only 30 out of 81 non-activated oocytes (37.0%) in IVM/ICSI group had no sperm component. Premature chromosomal condensation (PCC) and decondensed sperm heads were frequently observed in non-activated IVM/ICSI oocytes. By contrast, neither PCC nor decondensed

sperm heads were observed in non-activated control/ICSI oocytes. In oocytes arrested after activation, we never found intact sperm heads. Fifteen out of 21 (71.4%) of activated control/ICSI oocytes and 12 out of 28 (42.9%) of activated IVM/ICSI oocytes contained the swollen sperm head (Figure 1A). A similar incidence of oocytes with no sperm component in activated oocytes was observed between the control/ICSI and IVM/ICSI groups.

Chromosome analysis

Results of the chromosome analysis are summarized in Table III and IV. Male (human sperm origin) and female (mouse oocyte origin) pronuclear chromosomes in 1-cell zygotes are easily distinguishable by numbers (n=23 and 20, respectively) and morphology (Figure 1B). The zygotes with clumped, overlapping chromosomes and highly dispersed metaphase plates were excluded in data analysis. Finally 103 and 112 ova were suitable for cytogenetic analysis of maternal chromosomes in control/ICSI and IVM/ICSI groups, respectively. One hundred twenty oocytes in control/MII and 110 oocytes in IVM/MII were similarly analyzed (Table III). There were no significant differences in the aneuploidy or the structural aberration rates between the four groups. A relatively high incidence of polyploidy was observed in the control/ICSI and IVM/ICSI groups, but there was no significant difference between the two ICSI groups (12.6% in control/ICSI group, 8.0% in

IVM/ICSI group, respectively). All polyploidy oocytes that were observed in both ICSI groups showed two sets of maternal chromosomes and one set of paternal chromosomes.

Similar analyses in paternal chromosomes were performed in 108 control/ ICSI oocytes and 131 IVM/ICSI oocytes, respectively. There was no significant difference in aneuploidy, polyploidy or structural aberration rates between the two groups (Table IV).

Discussion

This study demonstrated that the activation rate after ICSI of in-vitro matured oocytes was significantly lower than that of in-vivo matured oocytes. The failure of oocyte activation could be caused by either unsuccessful release of the activation signal by the spermatozoa (sperm associated oocyte activating factor; SAOAF) or lack of a response of the oocytes to the activation signal. In human oocytes, it has been reported that, after ICSI, 50-80% of unfertilized oocytes remained at the MII stage despite the presence of decondensed spermatozoa within their cytoplasm (Flaherty et al., 1995; Kovacic et al., 2000). However, the M II oocytes containing a swollen sperm head that was arrested at various stage of decondensation was only observed in IVM/ICSI group in this study. The sperm nuclear decondensing activity of the oocyte has been connected with the level of glutathione, which is acquired during maturation (Perrault et al., 1992). Insufficient uptake of glutathione or its depletion occurred during IVM and postmaturation aging impairs the process of sperm chromatin decondensation (Sutovsky and Schatten., 1997, Goud et al., 1999). Furthermore, only normal spermatozoa were used in this study. Therefore, this aetiology may be mainly related to oocyte immaturity or postmaturation aging during IVM rather than ICSI procedure or sperm defects. However, atypical decondensation of human sperm nuclei with the retention of the perinuclear theca has been demonstrated when human sperm injected into

hamster oocytes (Terada et al., 2000). The retention of perinuclear theca during decondensation of sperm nuclei after ICSI may lead to oocyte activation failure after ICSI since the presence of an egg activating factor in perinuclear region has been suggested (Kimura et al., 1998).

The observation of PCC is associated with prolonged activity of maturation promoting factor (MPF) composed of heterodimer of $p34^{cdc2}$ and cyclin B. (Kubiak et al., 1993). The relationship between PCC and oocyte cytoplasmic immaturity has been described (Calafell et al., 1991). The fact that PCC was only observed in IVM/ICSI oocytes (both non-activated and activated oocytes) further supports the concept that in-vitro matured oocytes do not complete cytoplasmic maturation. Taken together, the significant differences found in the activation rates between the control/ICSI and IVM/ICSI groups in our study might be explained by the cytoplasmic immaturity of in-vitro matured oocytes.

In the present study, a similar incidence of developmental arrest before pronuclear formation after successful activation was seen in the two ICSI groups. We frequently found a swollen sperm head (SSH) in these arrested oocytes. Control/ICSI oocytes showed a relatively high incidence of SSH compared with IVM/ICSI oocytes, but this difference might be merely contributed to the observed occurrence of PCC in IVM/ICSI group. It has been reported that ooplasmic factors regulate sperm head decondensation, and that the inability of activated oocytes to fully process the injected spermatozoa may be indicative of oocyte

immaturity or oocyte defects. (Perreault, 1992, Flaherty et al., 1995). Our findings that SSH was observed in both in-vivo and in-vitro matured ICSI oocytes suggest that specific oocyte defects after ICSI procedure, rather than oocyte immaturity, may be associated with the SSH in activated oocytes. Several factors may be attributed to developmental arrest in an ICSI program. One possible reason for developmental arrest may be damage to the MII spindle or oocyte cytoskeleton during ICSI procedure while another possibility could be asynchrony in oocyte activation and sperm chromatin decondensation. It has been suggested that the aging effect prior to fertilization has been associated with poor embryonic quality (Chen et al., 2000) and postmature oocytes are in a dynamic state that is poised for entry into the interphase. Oocytes in such a partially activated state would be prone to activation, therefore, even minor stimuli such as the injection procedure could result in parthenogenetic activation (Goud et al., 1998, 1999; Alvarez et al., 1997). The sperm nuclear decondensing activity of the oocyte is closely related to the period of oocyte activation, and may be exhausted after particular time interval after the onset of oocyte activation, resulting in the arrest of sperm chromatin decondensation. Spermatozoa with intact sperm plasma membrane and acrosome injected during ICSI may also be involved in asynchrony between oocyte activation and sperm nuclear decondensation, since a high rate of PN formation after microinjection of human acrosomeless sperm has been reported (Lanzendorf et al., 1988).

A few reports are available on the cytogenetic normality of in-vitro matured oocytes. A

recent report on cytogenetic analysis has indicated that the aneuploidy rate of in-vitro matured metaphase II oocytes was 15% in mouse (Frydman et al., 1997), a value much higher than our result, 0.9%, in IVM/MII oocytes. However, in their report they do not compare in-vitro matured oocytes with in-vivo matured oocytes. Our results indicate that there is no significant difference in the incidence of aneuploidy and structural abnormality between the in-vivo matured and in-vitro matured oocytes, and this would suggest no influence of in-vitro maturation on the chromosomal aberrations of oocytes.

In this study, the polyploidy was observed in about 10% of ICSI oocytes. Tripronucleate zygotes have been reported after the injection of only one spermatozoon into human oocytes, and it has been suggested (Palermo et al., 1993, Flaherty et al., 1995) that formation of tri-pronucleus was attributable to the failure of second polar body (PB II) extrusion. It was also reported (Grossmann et al., 1997) that none of the tripronucleate zygotes following ICSI showed two Y signals using FISH. Our study demonstrated that all tripronucleate zygotes showed diploid in maternal (mouse) chromosome. Therefore, this study provides cytogenetic evidence that tripronucleate zygotes derived from ICSI would result from the retention of the PB II. The mechanisms underlying failure of extrusion of the second PB II after ICSI remain to be elucidated. Several factors, including damage to the metaphase plate, oocyte cytoskeleton, increased female age, oocyte immaturity and sperm characteristics have been suggested as causes for retention of the PB II. However, oocyte immaturity and sperm

characteristics are not likely to be involved in the occurrence of polyploidy since similar incidences of polyploidy between in-vivo and in-vitro matured oocytes was observed, and normal spermatozoa were employed in this study. Recently, it has been reported that the first polar body does not always reside close to the MII spindle in mouse, rhesus monkey and human oocytes (Kono et al., 1991; Hewitson et al., 1999; Hardarson et al., 2000). Although great care was taken to avoid passing the injection pipette through the spindle region during the ICSI procedure, this precaution does not completely prevent the damage to the MII spindle. Another explanation for the failed of the PB II extrusion may be related to the postmaturation aging of the oocytes occurred during IVM or culture periods for ICSI procedure. It has been suggested that high sensitivity of post mature oocytes to parthenogenetic activation is the main interfering factor responsible for suppression of PB II extrusion when human sperm is inseminated to the hamster egg (Alvarez et al., 1997).

Several authors reported that no differences in numerical chromosomal abnormalities were observed between embryos generated by ICSI and conventional IVF in humans (Edirisinghe et al., 1997; Wall et al., 1996; Munne et al., 1998). Although we have not investigated the chromosomal abnormality of conventional IVF in this study, our results may suggest that the ICSI procedure itself has no adverse effects on the chromosomal normality of oocytes because there were no differences in the incidence of chromosomal aberrations, except polyploidy rate, between ICSI oocytes and metaphase II oocytes or in-vitro matured

and in-vivo matured oocytes.

In addition, our findings demonstrated that there were no significant differences in aneuploidy, polyploidy or structural aberration rates in paternal chromosomes between the two ICSI groups. This incidence of sperm chromosomal aberration is similar to the reported data of the spermatozoa from normal men using the sperm penetration assay with zona-free golden hamster eggs and using microinjection into mouse oocytes (Martin et al., 1983; Rybouchkin et al., 1996). Taken together, it seems to be likely that in-vitro maturation and the ICSI technique do not induce sperm chromosomal abnormalities.

In conclusion, our results demonstrate that in-vitro maturation and the ICSI procedure do not cause an increase in the frequency of aneuploidy and structural aberrations when human sperm injected into mouse oocytes, although activation rates after ICSI were low in in-vitro matured oocytes. Therefore, in-vitro matured oocytes following ICSI may become a useful new technology for the treatment of human infertility. However, our heterologous system may not completely reflect the homologous fertilization (human oocytes and human spermatozoa), especially, the centrosomal inheritance is different between mice and human. During fertilization, the centrosome is introduced by the sperm in human, whereas, mouse fertilization is accomplished by maternally inherited. Further studies are needed to confirm the safety and the efficacy of in-vitro matured oocytes following ICSI.

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