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

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BACKGROUND: The purpose of this study was to investigate the chromosomal complement and developmental potential of in-vitro matured murine oocytes following ICSI by human sperm. METHODS: Heterologous ICSI fertilization between mouse oocytes and human sperm was employed in order to overcome the reduced fertilization rates observed after conventional IVF due to zona hardening during in-vitro maturation, and to assess separately maternal and paternal chromosome complements. Cytogenetic analyses were performed in four types of oocytes: (i) in-vitro matured metaphase II (MII) oocytes; (ii) in-vivo matured MII oocytes; (iii) in-vitro matured oocytes after ICSI; (iv) 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 versus 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 and in-vitro matured ICSI oocytes (69.7 versus 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 analyses of paternal chromosomes indicated that there were no significant differences in the incidence of chromosomal aberrations between the embryos 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.

Key words: chromosomal analysis/human sperm/ICSI/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 reproduction techniques 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 assisted reproduction leads to reductions in the number of embryos suitable for transfer and the pregnancy rates. Some attempts have been made to perform second day ICSI after in-vitro maturation (IVM) 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, IVF after IVM of immature oocytes retrieved from patients with polycystic ovaries, or from those at risk of developing ovarian hyperstimulation syndrome, 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 IVM of mouse, rat and human oocytes (Choi et al., 1987; Zhang et al., 1991; Beckers et al., 1999). 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 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 were no differences in numerical chromosomal abnormalities between human embryos generated by ICSI and conventional IVF, relatively high incidences of
aneuploidy and chromosomal mosaicism have been suggested (Wall et al., 1996; Edirisinghe et al., 1997; Munne et al., 1998). The purpose of this study was to investigate the chromosomal complement, activation and developmental potential of in-vitro matured mouse oocytes following ICSI by human sperm. Heterologous ICSI fertilization was employed in order to overcome the reduced fertilization rates observed after conventional IVF due to zona hardening during the IVM, 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 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 analysed.

Materials and methods

Preparation of gametes

All of the oocytes were obtained from 3- to 5-week-old B6D2F1 mice. Mice were primed with 5 IU pregnant mare serum gonadotrophin (PMSG, Serotropin; Teikokuzouki, Tokyo, Japan) and injected with 5 IU of HCG (HCG Mochida; Mochida Pharmaceutical, Tokyo) 48 h 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 Chatot, Zimuk and Bavister (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. The ovaries were removed 48 h later, placed in HEPES-buffered human tubal fluid (modified HTF; Irvine Scientific, Santa Ana, CA, USA) medium supplemented with 4 mg/ml bovine serum albumin (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, Kremsmuenster, Austria) containing 0.2 ml of HTF medium supplemented with 4 mg/ml BSA within 20 min of collection. The COCs were cultured for 18 h at 37°C under 5% CO2 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 sperm were only injected into MII oocytes.

Frozen–thawed human sperm

Semen samples were collected from volunteer donors. Sperm concentration, motility and viability were assessed according to World Health Organization guidelines (World Health Organization, 1992), and ‘normal’ samples were selected. Written consent was obtained from the donors and the local ethics committee approved this experiment. Sperm were cryopreserved by the dropwise addition of TEST (TES and Tris) yolk buffers (Irvine Scientific) to a 1:1 ratio of semen: TEST yolk buffers. Diluted samples were frozen rapidly in liquid N2 vapour for 20 min before being submerged into liquid N2. The cryovials were placed into a waterbath at 37°C for a few minutes to thaw and the thawed samples were washed by centrifugation with BWW medium at 2000 g for 5 min.

Microinjection of sperm and oocytes culture

In this study, heterologous fertilization between mouse oocytes and human sperm was performed in order to separately assess maternal and paternal chromosome complements.

Injection of human sperm into mouse MII oocytes was performed using a piezo-electric pipette-driving unit (Kimura and Yanagimachi, 1995). In brief, an oocyte was placed on a holding pipette and its zona pellucida was drilled by applying a few piezo pulses. A single human spermatoozoon 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 spermatoozoon was injected. All procedures were performed in HEPES–CZB medium at room temperature and injection of 30–40 oocytes each time was completed within 120 min.

Injected oocytes were transferred to CZB medium and cultured for 6–8 h at 37°C under 5% CO2 in air. After incubation, they were transferred into another droplet of CZB medium containing 0.006 µg/ml vinblastine (Sigma, St Louis, MO, USA) for 10 h and slides were prepared for chromosomal analysis.

Cytological and chromosomal preparations

Oocytes with apparently normal morphology were prepared for chromosomal analysis by a gradual-fixation/air-drying method (Mikamo and Kamiguchi, 1983). The oocytes were treated with 1% (w/v) pronase (Kaken Pharmaceuticals, Tokyo) for 5 min to remove zona 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:H2O in a 5:1:4 ratio) for a few minutes, mounted on a glass slide and covered with fixative II (methanol:acetic acid in a 3:1 ratio). Thereafter, the slide was dipped into fixative II for 30 min. Finally, it was fixed with fixative III (methanol:acetic acid:H2O in a 3:3:1 ratio) 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 dicentric chromosomes (Summer, 1972).

Four types of oocytes were analysed: (i) in-vitro matured MII oocytes (control/MII); (ii) in-vitro matured MII oocytes (IVM/MII); (iii) oocytes that had undergone ICSI after in-vitro maturation (control/ICSI); (iv) oocytes that had undergone ICSI after in-vitro maturation (IVM/ICSI)

Statistical analysis

Data were compared between the experimental groups using the χ2 test and Fisher’s exact test as appropriate. The differences were considered significant at a level of P < 0.01.

Results

Activation rate and developmental rate after ICSI

In the control/ICSI and IVM/ICSI groups, 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 the control/ICSI group. In contrast, 269 oocytes in the IVM/ICSI group were available for cytological assessment and the activation rate of the IVM/ICSI group was significantly lower than that of the control/ICSI group (69.9 versus 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 the control/ICSI group and 69.7% in the IVM/ICSI group).

Twenty-five oocytes in the control/ICSI group and 109
Table I. Activation and developmental rates of in-vivo or in-vitro matured oocytes after heterologous ICSI

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Injected (%)</th>
<th>Analysed (%)</th>
<th>Non-activated (%)</th>
<th>Activated (%)</th>
<th>Activated oocytes in different developmental stages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/ICSI</td>
<td>191</td>
<td>145 (75.9)</td>
<td>4 (2.8)</td>
<td>141 (97.2)</td>
<td>Arrested before PN 21 (14.9) Arrested at 2PN 12 (8.5) Reached mitotic formation 108 (76.6)</td>
</tr>
<tr>
<td>IVM/ICSI</td>
<td>361</td>
<td>269 (74.5)</td>
<td>81 (30.1)a</td>
<td>188 (69.9)a</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.01 for difference between the control/ICSI and IVM/ICSI groups.

PN = pronucleus; IVM = in-vitro maturation.

Table II. Cytological findings in the oocytes which failed to form a male pronucleus

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total</th>
<th>Non-activated oocytes with (%)</th>
<th>Activated oocytes with (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Condensed sperm</td>
</tr>
<tr>
<td>Control/ICSI</td>
<td>25</td>
<td>4 (16)</td>
<td>0</td>
</tr>
<tr>
<td>IVM/ICSI</td>
<td>109</td>
<td>81 (74)</td>
<td>4 (4.9)</td>
</tr>
</tbody>
</table>

SSH = swollen sperm head; PCC = premature chromosome condensation.

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 sperm (sperm associated oocyte activating factor; SAOAF) or lack an oocyte’s response 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 sperm within their cytoplasm (Flaherty et al., 1995; Kovacic and Vlaisavljevic, 2000). However, the MII oocytes containing a swollen sperm head that was arrested at various stages of decondensation were only observed in the 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 (Perreault, 1992). Insufficient uptake of glutathione or its depletion, occurring during IVM and post-maturation ageing, impairs the process of sperm chromatin decondensation (Sutovsky and Schatten, 1997; Goud et al., 1999). Furthermore, only normal sperm were used in this study. Therefore, this aetiology may be mainly related to oocyte immaturity or post-maturation ageing during IVM, rather than the 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 were injected into hamster oocytes (Terada et al., 2000). The retention of the perinuclear theca during decondensation of sperm nuclei after ICSI may lead to oocyte activation failure after ICSI, since the presence of an oocyte activating factor in the 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 a heterodimer of p34<sup>cdc2</sup> 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 merely be attributed to the observed occurrence of PCC in the 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 sperm may be indicative of oocyte immaturity or oocyte defects (Perreault, 1992; Flaherty et al., 1995). Our observation of SSH in both in-vivo and in-vitro matured ICSI oocytes suggests that specific oocyte defects after the 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 programme. One possible reason for developmental arrest may be damage to the MII spindle or oocyte cytoskeleton during the ICSI procedure, while another possibility could be asynchrony in oocyte activation and sperm chromatin decondensation. It has been suggested that the ageing effect prior to fertilization has been associated with poor embryonic quality (Chen et al., 2000) and post-mature 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 (Alvarez et al., 1997; Goud et al., 1998, 1999). The sperm nuclear decondensing activity of the oocyte is closely related to the period of oocyte activation, and may be exhausted after a particular time interval after the onset of oocyte

Table III. Chromosome analysis of MII oocytes and oocyte-derived genomes in 1-cell zygotes after heterologous ICSI

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of oocytes or zygotes karyotyped</th>
<th>No. (%) of ova or oocytes with chromosomal aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aneuploidy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyper</td>
</tr>
<tr>
<td>Control/MII</td>
<td>120</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IVM/MII</td>
<td>110</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Control/ICSI</td>
<td>103</td>
<td>2 (1.9)</td>
</tr>
<tr>
<td>IVM/ICSI</td>
<td>112</td>
<td>1 (0.9)</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.01 compared with the control/MII and IVM/MII groups.
activation, resulting in the arrest of sperm chromatin decondensation. Sperm with intact plasma and acrosome membranes injected during ICSI may also be involved in asynchrony between oocyte activation and sperm nuclear decondensation, since a high rate of pronuclei 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 MII 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 and in-vitro matured oocytes, and this would suggest no influence of IVM on the chromosomal aberrations of oocytes.

In this study, polyploidy was observed in ~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 a tripronucleus was attributable to the failure of the second polar body (PBII) extrusion. It was also reported (Grossmann et al., 1997) that none of the tripronucleate zygotes following ICSI showed two Y signals when analysed using fluorescent in-situ hybridization. Our study demonstrated that all tripronucleate zygotes were diploid in maternal (mouse) chromosome. Therefore, this study provides cytogenetic evidence that tripronucleate zygotes derived from ICSI would result from the retention of the PBII. The mechanisms underlying failure of extrusion of the PBII 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 PBII. 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 were observed, and normal sperm 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 damage to the MII spindle. Another explanation for the failure of the PBII extrusion may be related to the post-maturation ageing of the oocytes occurring during IVM or culture periods for the 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 PBII extrusion when human sperm are inseminated into hamster oocytes (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 (Wall et al., 1996; Edirisinghe et al., 1997; Munnè 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 MII oocytes, or in-vitro 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 sperm from normal men using the sperm penetration assay with zona-free golden hamster oocytes and using microinjection into mouse oocytes (Martin et al., 1983; Rybouchkin et al., 1996). Taken together, it seems to be likely that IVM and the ICSI technique do not induce sperm chromosomal abnormalities.

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

### Table IV. Chromosome analysis of human sperm-derived genomes in 1-cell zygotes after heterologous ICSI

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of metaphasic zygotes</th>
<th>No. of zygotes karyotyped</th>
<th>Aneuploidy</th>
<th>Polyploidy</th>
<th>Structural aberration</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hyper</td>
<td>Hypo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control/ICSI</td>
<td>108</td>
<td>87</td>
<td>1 (1.1)</td>
<td>5 (5.7)</td>
<td>0</td>
<td>9 (10.3)</td>
</tr>
<tr>
<td>IVM/ICSI</td>
<td>131</td>
<td>83</td>
<td>1 (1.2)</td>
<td>1 (1.2)</td>
<td>0</td>
<td>3 (3.6)</td>
</tr>
</tbody>
</table>

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References


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Chromosomes and IVM