Correction

DEVELOPMENTAL BIOLOGY


The authors note that on page 14633, left column, second paragraph, lines 2–5, “The Institutional Review Boards of the Saint Mother Obstetrics and Gynecology Clinic and of the Ministry of Health, Labor, and Welfare of Japan approved this study on December 20, 2009 and July 3, 2011, respectively” should instead appear as “The Institutional Review Board of the Saint Mother Obstetrics and Gynecology Clinic approved this study on December 20, 2009 and approved the submission for the clinical trial registration on July 3, 2011.”

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Fourteen babies born after round spermatid injection into human oocytes

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During the human in vitro fertilization procedure in the assisted reproductive technology, intracytoplasmic sperm injection is routinely used to inject a spermatozoon or less a mature elongating spermatid into the oocyte. In some infertile men, round spermatids (haploid male germ cells that have completed meiosis) are the most mature cells visible during testicular biopsy. The microsurgical injection of a round spermatid into an oocyte as a substitute is commonly referred to as round spermatid injection (ROSI). Currently, human ROSI is considered a very inefficient procedure and of no clinical value. Herein, we report the birth and development of 14 children born to 12 women following ROSI of 734 oocytes previously activated by an electric current. The round spermatids came from men who had been diagnosed as not having spermatozoa or elongated spermatids by andrologists at other hospitals after a first Micro-TESE. A key to our success was our ability to identify round spermatids accurately before oocyte injection. As of today, all children born after ROSI in our clinic are without any unusual physical, mental, or epigenetic problems. Thus, for men whose germ cells are unable to develop beyond the round spermatid stage, ROSI can, as a last resort, enable them to have their own genetic offspring.

Azoospermia, a condition in which there are no spermatozoa in the ejaculate, occurs in 1% of men in the general population (1). For men with obstructive azoospermia, microsurgical aspiration of epididymal spermatozoa has been performed, and the pregnancy rate following injection of oocytes with epididymal spermatozoa is comparable to that following injection of ejaculated spermatozoa (2). However, up to 94% (17 of 18) of azoospermic men are diagnosed as having nonobstructive azoospermia (3). For such men, microscopic testicular sperm extraction (micro-TESE) has been widely performed to obtain testicular spermatozoa, which could participate in syngamy. Subsequently, they formed research; K.K. contributed new reagents/analytic tools; A.T., H.K., M.I., and R.Y. conducted research; and K.K. contributed new reagents/analytic tools; A.T. and R.Y. wrote the paper.

Identifying the round spermatids: The authors claim that they were able to identify these cells based on their structural and physical characteristics (verified by karyotyping and FISH). Round spermatid injection was effectively used in our clinic and resulted in the birth of 14 healthy babies. Although the current success rate of round spermatid injection is not very high compared with intracytoplasmic sperm injection, this procedure can be the last resort for men who cannot produce spermatozoa but wish to use their own genetic material to produce offspring.

The authors declare no conflict of interest.

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Author contributions: A.T. designed research; A.T., M.N.N., Y.T., I.T., S.W., and S.T. performed research; K.K. contributed new reagents/analytic tools; A.T., H.K., M.I., and R.Y. analyzed data; and A.T. and R.Y. wrote the paper.

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As shown in Fig. 2, round spermatids were the smallest spermatogenic cells (6–8 μm in diameter). Unlike other spermatogenic cells, they did not have distinct nucleoli and the rim of cytoplasm surrounding the nucleus was thinner than that in the spermatogonium. Protruded active pseudopodia were often seen in spermatogonia (26), but not in round spermatids. An acrosome vesicle or cap, definitive evidence for the cell being a spermatid, was seen in less than 10% of the presumptive spermatids we examined. Another important characteristic of the round spermatid was evident in the fact that its cytoplasm could be readily separated from the nucleus when the cell was drawn back and forth within the injection pipette. Thus, although some spermatogonia did resemble round spermatids, pipetting did not separate their cytoplasm from the nucleus as readily as in spermatids. Lymphocytes, about the size of round spermatids, had a “tough” plasma membrane that, unlike that of round spermatids, could not be broken even by vigorous pipetting.

**ROSI and Oocyte Activation.** Fig. 4 shows an oocyte injected with a round spermatid whose plasma membrane ruptured when the cell was sucked into the injection pipette (Fig. 4B). Although ROSI alone could induce oocyte activation, this outcome was not consistent. Therefore, because repetitive intracellular Ca$^{2+}$ oscillations are an essential component of oocyte activation and a prerequisite of normal embryo development (27), we compared the pattern of Ca$^{2+}$ oscillations in oocytes after vigorous ooplasmic aspiration (16), electric stimulation, ROSI, and ROSI plus electric stimulation. As shown in Fig. S2, ROSI combined with electric stimulation (Fig. S2D) was most effective in inducing large, repetitive Ca$^{2+}$ oscillations. Because our previous study had shown that oocytes stimulated with electric current before ROSI developed into cleaving embryos more often than those without such treatment (55% vs. 32%) (Table S1), we subjected all oocytes to electro-stimulation ∼10 min before ROSI. An example of ROSI embryo developing in vitro after this stimulation is shown in Fig. S3.
Fig. 3. (A) Twelve presumptive round spermatids randomly selected and adherent to a glass slide. (B) The same cells after FISH, each showing a single spot of either chromosome X (green) or Y (orange). (Magnification: A and B, 400x.)

Live Birth of Healthy Babies Following ROSI. Table 1 summarizes the results of embryo transfer after ROSI. To compare the efficiency of ROSI with that of intracytoplasmic sperm injection (ICSI), part of the data collected in our clinic after TESE and ICSI (28, 29). Currently, ROSI is less efficient than ICSI in obtaining live offspring. Note that in both ROSI and ICSI, embryos that had been kept frozen before transfer to mothers had better chance to develop to term than those that had not been frozen (P < 0.05).

Table 2 summarize patients’ information, developmental stages of embryos at the time of transfer, karyotypes, and gender of fetuses, and the growth data of babies born after ROSI. As shown in Table 2, all 14 babies were karyotypically normal. The mean (±SD) gestational age and body weight at birth were 38.5 wk (±1.19, n = 12 deliveries) and 2,832.8 g (±497.0, n = 14 babies), respectively. Pathological examination of all miscarriages following ROSI revealed no partial or complete hydatidiform moles. As of today, all children born after ROSI are growing well without any physical or mental problems and with no evidence of Prader–Willi syndrome, Angelman syndrome, or Wiskott–Aldrich syndrome or any other unusual physical and mental aberrations.

Discussion

The Use of ROSI in Cases of Spermatogenetic Arrest at the Round Spermatid Stage. Silber et al. (21) maintain that whenever round spermatids exist in the human testes, there are also mature spermatozoa present. In other words, there is no spermatogenesis arrest at the spermatid stage. Clearly this is not the case for animals. For example, the testes of mutant male mice homologous for the Crem gene are devoid of spermatozoa, the most advanced spermatogenic cells in these males being round spermatids. Although these males are, of course, infertile (12, 30), ROSI can help to overcome this problem (13). According to He et al. (31), human males with Crem gene variants are also azoospermic, with the most advanced spermatogenic cells in such men being round spermatids. Moreover, Weinbauer et al. (32) reported the importance of Crem protein for the initiation of human spermiogenesis. In mice, Crem is an autosomal recessive gene, and therefore when spermatids of Crem-null males were injected into oocytes of wild-type (Crem<sup>−/−</sup>) females, all male offspring were fertile (13). This was true for mutant mice lacking such genes as bs (blind-sterile) (13), ring finger protein 17 (RNF17), CUG triplet repeat, RNA binding protein 1 (CUGbp1), and jumonji domain-containing 1a (Jmjd1a) (33–36). According to Ayhan et al. (37), in men with mutations of two autosomal recessive genes, TAF4B and ZMYND15, their spermatogenesis is arrested at the round spermatid stage. It would be interesting to know if their specific type of infertility can be overcome by ROSI.

Table 2 includes histology-based Johnsen's scores (38) of the 12 men who participated in this study. Note that the husband of wife 1 had a Johnsen's score of 1 after initial histological examination of testis biopsies. This meant that neither spermatogenic cells nor Sertoli cells were found in biopsied samples (Fig. S4A). However, later examination of other portions of the samples revealed spermatid-like cells within some of the tubules (Fig. S4B). Intensive searches for spermatozoa and spermatogenic cells in the testis eventually made it possible for us to find spermatids suitable for ROSI. Johnsen's scores (38), which are solely dependent upon histological examination of seminiferous tubules collected from very limited regions of the testis, should be considered as merely representing the predominant pattern of spermatogenesis (39).

Possible Reasons for Previous Failures of Human ROSI. In the mouse, identification of round spermatids is not difficult because they exhibit a distinct centrally located chromatin granule observed readily under the interference-contrast microscope used for microsurgical operation of oocytes (40). Round spermatids are the smallest spermatogenic cells in the testis and can be readily distinguished from small blood cell leukocytes. In contrast to the mouse, human spermatids do not show a distinct chromatin granule, at least under an inverted microscope with Hoffman modulation contrast optics. It is very likely that many previous investigators, who attempted ROSI, actually chose the wrong cells in that one cannot expect normal development of oocytes injected with spermatozon or somatic cells. Nuclei of spermatozoites (tetraploid or diploid) cannot participate in embryo development.

Fig. 4. The procedure of ROSI. (A) Immediately before picking up one spermatid. (B) A spermatid aspirated into the injection pipette, the plasma membrane being broken. The white arrowhead indicates the spermatid nucleus. (C–F) Oocytes before, during and after injection of spermatid. Arrowhead indicates the spermatid nucleus. (Magnification: A–F, 400x.)
without first completing meiosis within oocytes, which is very difficult, if not impossible (40–42). An acrosome vesicle or acrosomal cap detected on the nucleus of small round cells indicates that they are round spermatids. However, the absence of such structures does not mean that the cell in question is not an early-stage round spermatid. Even though FISH and karyotyping are a reliable means through which to identify them, we cannot use such invasive techniques for ROSI, which requires live cells. The day may come when we can identify round live spermatids correctly by using an antibody specific to their surface. Computer-assisted identification of live spermatids and other types of spermatogenic cells may become available in the near future.

Another reason for the failure of many attempts using ROSI could be because of incomplete activation of the recipient oocytes, despite the fact that human round spermatids may contain at least some oocyte-activating ability (43). According to Ogonuki et al. (44), fresh round spermatids injected into mouse oocytes were unable to activate oocytes, whereas spermatids frozen and thawed before injection could do so, suggesting that some endogenous oocyte-activating factor is mobilized by the freeze–thawing routine used in this study. Although round spermatid injection alone could induce small Ca

2

 oscillations (Fig. S2C), ROSI with prior electrical stimulation was most effective in inducing repetitive, large Ca

2

 oscillations (Fig. S2D). We need further systematic studies to identify the best method to activate human ROSI oocytes. Other oocyte-activating agents to be considered include: Ca

2

 ionophore (20, 45, 46), phosphatidyl inositol (47, 48), phospholipase Cζ (49–51), 4- (diethylamino) pyridine (52), and post-acrosomal protein PAWP (53–55).

Interestingly, pregnancy and delivery rates following ROSI were higher when embryos were first kept frozen for some time as opposed to their transfer soon after ROSI (Table 1). Because this was true for ICSI (Table 1), it was perhaps because of better preparation of the uterine endometrium when recipients were allowed to resume their normal ovulation cycle before transfer.

Safety of ROSI. The safety of any of the assisted fertilization technologies is a prime concern. Some investigators suspect that the inefficacy of ROSI might be because of incomplete male-specific DNA methylation imprinting in round spermatids, but at least in the mouse, DNA methylation is completed in round spermatids (56) and even before meiosis begins (57). One possible factor might be the inefficient demethylation and remethylation of DNA after introduction of spermatid nuclei into mature oocytes (58).

### Table 1. Clinical outcome of ROSI partners of 76 women in this table had round spermatids as their most advanced spermatogenic cells

<table>
<thead>
<tr>
<th>Assisted fertilization</th>
<th>Cryopreservation of embryos</th>
<th>Total no. of women</th>
<th>Total no. of treatment cycles</th>
<th>Percentage of oocytes fertilized (fert/total) (%)</th>
<th>Percentage Oocytes cleaved (cleaved/total) (%)</th>
<th>Total no. of transferred embryos</th>
<th>No. and percentage of recipients (%)</th>
<th>Total no. of live offsprings</th>
<th>Pregnancy</th>
<th>Miscarriage</th>
<th>Live offspring delivered</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROSI (present study)</td>
<td>No</td>
<td>58</td>
<td>162</td>
<td>55.6 (330/594)</td>
<td>44.9 (267/594)</td>
<td>152</td>
<td>121</td>
<td>20 (16.5)</td>
<td>13 (65.0)</td>
<td>7 (58.5)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>28</td>
<td>42</td>
<td>76.4 (107/140)</td>
<td>71.4 (100/140)</td>
<td>56</td>
<td>42</td>
<td>10 (23.8)</td>
<td>5 (50.0)</td>
<td>5 (11.9)</td>
<td>5</td>
</tr>
<tr>
<td>TESE and ICSI</td>
<td>No</td>
<td>260</td>
<td>540</td>
<td>44.9 (833/1855)</td>
<td>43.5 (807/1855)</td>
<td>562</td>
<td>468</td>
<td>134 (28.6)</td>
<td>27 (20.1)</td>
<td>107 (22.9)</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>140</td>
<td>295</td>
<td>68.2 (825/1210)</td>
<td>65.3 (790/1210)</td>
<td>377</td>
<td>290</td>
<td>174 (60.0)</td>
<td>14 (8.0)</td>
<td>160 (55.2)</td>
<td>176</td>
</tr>
</tbody>
</table>

Partners of 76 women in this table had round spermatids as their most advanced spermatogenic cells. To compare the efficiencies of ROSI and ICSI, part of the data collected in our clinic after TESE and ICSI are included in this table. § vs. §§ and ¶ vs. ¶, P < 0.05; Fisher’s protected least significant difference test. *No and percentage of recipients with positive germinal sacs detected by ultrasound scanning between 4 and 5 wk of gestation. Ten of these 28 patients failed to get pregnancy previously when they received unfrozen ROSI embryos.

### Table 2. Clinical data of 14 offspring developed from ROSI oocytes

<table>
<thead>
<tr>
<th>Parents</th>
<th>Data from ROSI to embryo transfer</th>
<th>Data of birth</th>
<th>Medical check up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Wife Johnson's score* in TSE</td>
<td>Initial Oocytes fertilized (fert/total) (%)</td>
<td>Developmental stage x No. of embryos transferred</td>
</tr>
<tr>
<td>1</td>
<td>37 36 1</td>
<td>33.3 (2/6)</td>
<td>8 cell ×2</td>
</tr>
<tr>
<td>2</td>
<td>28 28 1</td>
<td>50.0 (9/18)</td>
<td>8 cell ×2</td>
</tr>
<tr>
<td>3</td>
<td>34 37 3</td>
<td>50.0 (1/2)</td>
<td>Blastocyst ×1</td>
</tr>
<tr>
<td>4</td>
<td>36 32 6</td>
<td>50.0 (5/10)</td>
<td>10 cell ×1+</td>
</tr>
<tr>
<td>5</td>
<td>40 39 4</td>
<td>65.0 (13/20)</td>
<td>11 cell ×1+</td>
</tr>
<tr>
<td>6</td>
<td>28 28 7</td>
<td>50.0 (7/14)</td>
<td>5 cell ×2</td>
</tr>
<tr>
<td>7</td>
<td>34 39 3</td>
<td>75.0 (3/4)</td>
<td>12 cell ×2</td>
</tr>
<tr>
<td>8</td>
<td>36 35 7   ³</td>
<td>50.0 (2/4)</td>
<td>Morula ×1+</td>
</tr>
<tr>
<td>9</td>
<td>26 23 6</td>
<td>42.1 (8/19)</td>
<td>8 cell ×2</td>
</tr>
<tr>
<td>10</td>
<td>38 37 7</td>
<td>23.1 (3/13)</td>
<td>10 cell ×2</td>
</tr>
<tr>
<td>11</td>
<td>41 40 7</td>
<td>58.8 (15/18)</td>
<td>8 cell ×2</td>
</tr>
<tr>
<td>12</td>
<td>43 34 7</td>
<td>56.3 (9/16)</td>
<td>Blastocyst ×1</td>
</tr>
</tbody>
</table>

BW, body weight (g); F, female; M, male; N, normal; ND, No data; PCD, physical and cognitive development.

*Johnson (38).

¹Monozygotic twins.

²Dizygotic twins, **3 mo, *9 mo, /7 mo.

³Examination of biopsied sample and re-examination of histology slide revealed the presence of few highly deformed elongated spermatids, but we did not use these cells for this study because they were not tolerant to freeze–thawing the procedure we used routinely for ROSI.
Materials and Methods

Ethical Aspects. This study was conducted with the informed consent of all participating patients. The Institutional Review Boards of the Saint Mother Obstetrics and Gynecology Clinic and of the Ministry of Health, Labor, and Welfare of Japan approved this study on December 20, 2009 and July 3, 2011, respectively. This study was registered and adhered to International Committee of Medical Journal Editors criteria. The University Hospital Information Network Clinical Trials Registry is UMIN000006117.

Subjects. We conducted Micro-TESE on 730 nonobstructive azoospermic men who had already undergone a previous Micro-TESE conducted by andrologists from other institutions and had been found not to have normal spermatogenic cells beyond the round spermatid stage. We found round spermatids in 76 (10.4%) of the 730 men. These 76 men, aged 22-46 y (33.4 ± 5.2), participated in a total of 163 transfer cycles in our clinic between September 2011 and March 2014.

Collection and Cryopreservation of Spermatogenic Cells from Azoospermic Men. Testicular tubular biopsies (20-50 mg each) were obtained from not only the surface, but also from deep inside the testis until we found spermatzoa or elongated spermatids. If no such cells were found despite our continuing efforts, but we saw many normal-looking spermatids, all collected tubules were dissociated enzymatically and cryopreserved. Details are provided in SI Materials and Methods.

Thawing of Frozen Testicular Cell Samples and Identification of Round Spermatids Immediately Before ROSI. Thawing was carried out at 30 °C. Using a differential interference microscope, morphological and physical features of individual cells were examined. Their small size (6-8 μm in diameter), thin rim of cytoplasm around the nucleus, and ready separation of cytoplasm from the nucleus were important identifying features of round spermatids. The accuracy of the visual identification was checked by karyotyping and FISH, which proved to be near 100%. Details are provided in SI Materials and Methods.

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Statistical Analysis. Data were analyzed using the Microsoft Excel Add-in software (MacToukai-kaiseki v2.0; Esumi).


