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A detailed cytogenetic analysis of large numbers of fresh and frozen–thawed human sperm after ICSI into mouse oocytes

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BACKGROUND: Since information about chromosome aberrations in micro-manipulated sperm is still inadequate, cytogenetic analysis was performed on large numbers of fresh and frozen–thawed (FT) human sperm after injection into mouse oocytes. The effects of the ICSI procedure on oocytes are also discussed based on analysis of the mouse chromosome complements. METHODS: After the injection of fresh and FT human sperm into mouse oocytes, chromosomes of the hybrid oocytes were analysed at first cleavage metaphase. RESULTS: Incidences of the hybrid oocytes at the first cleavage metaphase were significantly different between fresh (71.5%) and FT sperm groups (80.1%) ($P < 0.05$). The chromosome analysis of 477 fresh and 141 FT sperm showed no difference in the incidences of aneuploidy (1.6/0.7%), structural aberrations (8.8/7.8%) or diploidy (0.0/0.0%) between these categories. The cytogenetic result did not differ from our previous result using IVF between human sperm and hamster oocytes. In an additional cytogenetic study on 615 mouse chromosome complements, the incidence of diploidy (5.4%) was significantly higher than those (0.3–2.8%) in the previous mouse cytogenetic studies, and the hybrid oocytes with no mouse chromosomes (2.0%) existed. CONCLUSIONS: This result suggests that the ICSI procedure induces no sperm chromosome aberrations but increases numerical aberrations in oocyte chromosome complements.

Key words: chromosome aberrations/human sperm/ICSI/mouse oocytes

Introduction

The cytogenetic analysis of human sperm has been conventionally performed using IVF with zona-free hamster oocytes. However, as the ICSI technique, which bypasses sperm–oocyte membrane fusion, has been widely used in treatment for patients with male factor infertility, the necessity for a human sperm chromosome assay system using ICSI has arisen. The first trial for this technique was attempted in human sperm injected into Syrian hamster oocytes (Martin *et al*., 1988). However, the success rate of human sperm karyotyping was extremely low (3.4%), and it was pointed out that the frequent chromosome aberrations (39%) observed were possibly caused by experimental conditions. It is because of these phenomena that Syrian hamster oocytes are now used less frequently for ICSI. On the other hand, an efficient and stable ICSI method was developed in mouse by Kimura and Yanagimachi (1995) and, subsequently, the application of mouse oocytes to the human sperm chromosome assay was attempted by a number of investigators (Rybouchkin *et al*., 1995; 1996; 1997; Lee *et al*., 1996). However, information about chromosome aberrations in micro-manipulated human sperm is still inadequate because sperm sample sizes in the previous studies were so small.

Recently, the success rate of human sperm karyotyping was improved by combining the ICSI method of Kimura and Yanagimachi with the gradual fixation–air-drying method, which is an efficient chromosome preparation technique for early mammalian embryos (Mikamo and Kamiguchi, 1983). In this assay system, furthermore, no ageing-related increase of chromosome aberrations occurs in either sperm or oocyte, providing the injection is finished within 3 h after oocyte collection (Watanabe and Kamiguchi, 2001a). It therefore satisfies the conditions for efficient cytogenetic analysis of human sperm using ICSI. In this study, the aim was to determine the risk of chromosome abnormalities in micro-manipulated human sperm. To rectify the defect of sample size in the previous ICSI studies, therefore, a large quantity of fresh and frozen–-thawed human sperm were injected into mouse oocytes, and cytogenetically analysed in detail. Then, the result in the present ICSI study was compared with the result in our previous IVF study. At the same time, since mouse chromosome complements were also analysable in the 1-cell hybrid oocytes, the effects of micro-manipulation on female chromosome complements, for which information was not available, was additionally estimated.

Materials and methods

Collection of oocytes

B6D2F1 female mice, 6–11 weeks old, underwent ovulation induction by i.p. injection of 7 IU pregnant mare's serum gonadotrophin (Teikoku-zoki, Tokyo) followed by i.p. injection of 7 IU hCG (Mochida Pharmac., Kyoto) 48 h later. Oocytes were collected from oviducts 16 h after the hCG injection and were then freed from cumulus cells by 5 min treatment of 0.1% hyaluronidase dissolved in HEPES–CZB medium (Kimura and Yanagimachi, 1995). The oocytes were stored in CZB medium (Chatot *et al*., 1989; 1990) at 37°C under 5% CO_2 in air for up to 3 h prior to sperm injection.

Collection of human sperm

Human semen samples used for this study were obtained from a fertile donor showing normozoospermia according to World Health Organization (1999) criteria. Fresh semen was liquefied for 30 min at 37°C in air. The sample was washed twice by centrifugation (700 *g* for 5 min) along with 6 ml HEPES–Biggers–Whitten–Whittingham medium containing 0.3% bovine serum albumin (BSA) (Watanabe and Kamiguchi, 2001a). After the sperm were suspended in 5% polyvinylpyrrolidone (PVP) dissolved in Dulbecco's phosphate-buffered saline (Dulbecco and Vogt, 1954), the sperm suspension was placed in a manipulation chamber for sperm selection.

Some of the semen samples were used after being frozen and thawed (Kobayashi *et al*., 1991). The frozen samples were prepared in the same manner as the fresh samples after thawing at 37°C.

Preparation of manipulation chamber

For the injection of human sperm into mouse oocytes, a manipulation chamber was prepared by placing four kinds of droplets in a line on the cover of a 10 cm plastic dish (Falcon Plastics, USA) and covering them with mineral oil. The first droplet was 5 µl of 10% PVP for washing the injection pipette. The second droplet was 10 µl of sperm suspension. The third droplet was 5 µl of 10% PVP for the immobilization of sperm. The fourth droplet was 20 µl of HEPES–CZB medium for the oocytes. The injection chamber was prepared immediately before use and placed on the cooling microplate (Kitazato supply, Tokyo) of the inverted microscope with Hoffman's modulation contrast optics. The temperature of the microplate was maintained at 17–18°C during micro-manipulation (Kimura and Yanagimachi, 1995).

Injection of human sperm into mouse oocytes

Human sperm were injected into mouse oocytes using a piezo-driven micromanipulator according to Kimura and Yanagimachi (1995). Only motile human sperm with normal-shaped heads (3–5 µm in length and 3 µm in width; Menkveld *et al*., 1990) were selected for ICSI, to exclude chromosome aberrations implicated in morphological abnormality or immotility. Motile sperm were transferred from the sperm droplet into the 10% PVP droplet, and piezo-pulses were applied a few times at the midpiece of the sperm to immobilize them. The sperm thus immobilized were injected into metaphase II mouse oocytes with the first polar body in the HEPES–CZB droplet. Since the meiotic spindle of mouse oocytes was indicated by a hump in the cortex, it was possible to inject the sperm so as to prevent damaging this spindle (Kimura and Yanagimachi, 1995). The hybrid oocytes

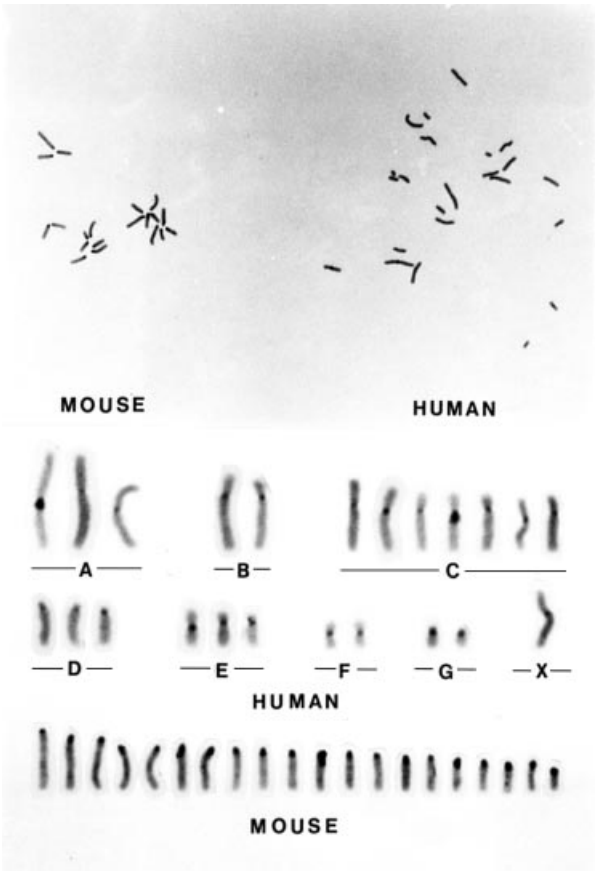

Figure 1. A chromosome spread of a hybrid 1-cell embryo between a human sperm and a mouse oocyte and its karyotype (C-band staining).

Table I. Activation and development of mouse oocytes penetrated with fresh or frozen–thawed (FT) human sperm

Sperm	No. of oocytes injected	No. of surviving oocytes (%)	No. of oocytes successfully prepared	No. of oocytes activated (%)	No. of activated oocytes with sperm transformed into (%)[a]		
					SH or PCC	PN	Mitotic chromosomes
Fresh	965	739 (76.6)	713	705 (98.9)	148 (21.0)	53 (7.5)	504 (71.5)
FT	242	190 (78.5)	187	186 (99.5)	31 (16.7)	6 (3.2)	149 (80.1)[b]

[a]% = per no. of oocytes activated×100.
[b]χ^2-Test, $P < 0.05$.
SH = swollen sperm head; PCC = premature chromosome condensation; PN = pronucleus.

were transferred into the culture dishes which had been prepared by placing CZB medium droplets (0.2 ml) on the 35 mm dish and covering them with mineral oil. All oocytes were used for ICSI within 3 h after collection.

Preparation of chromosome slides

The hybrid oocytes were incubated in the culture dish for 6 h and were then transferred into the CZB medium droplets (0.2 ml) containing 0.006 μg/ml vinblastin to block karyogamy and mitotic spindle formation. When the hybrid oocytes reached the first cleavage metaphase, 16–24 h after the micro-injection, they were prepared for chromosome slides. After zona pellucida had been removed by 5 min treatment of 0.5% actinase E (Kaken Pharmac., Japan), the hybrid oocytes were treated with hypotonic solution (0.5% sodium citrate containing 15% BSA) for 10 min at room temperature. For the preparation of chromosome slides, the gradual fixation–air-drying method was used (Mikamo and Kamiguchi, 1983). The analysis of the chromosome slides was carried out twice after successive staining with 2% Giemsa and C-banding (Figure 1). Those hybrid oocytes that contained one, two or three visible pronuclei 24 h after ICSI were also prepared in the same way.

Statistical evaluation

The χ^2-test was used and differences were considered significant at $P < 0.05$.

Results

The success rate of ICSI is summarized in Table I. In the fresh sperm group, 965 mouse oocytes were penetrated with fresh human sperm. The oocyte survival rate was 76.6% (739/965). Of the 739 oocytes that survived, 713 were successfully prepared for chromosome slides, but the rest burst in the hypotonic solution. Oocyte activation, which was assessed by the completion of the second meiosis resulting in the presence of mouse pronucleus (PN) or mitotic chromosomes, was recognized in 98.9% (705/713) of the oocytes prepared. The rest (1.1%) were not activated, showing meiotic mouse chromosomes along with swollen human sperm head (SH) or prematurely condensed human chromosomes (PCC). The oocytes activated were classified into three categories depending on the sperm transformation. The majority (71.5%, 504/705) of them developed into the first cleavage metaphase where both human sperm and mouse oocyte chromosome complements were contained (Figure 1). In the oocytes (21.0%, 148/705) classified in the second largest group, sperm remained SH or PCC, although mouse nuclei formed PN or mitotic chromosomes (Figure 2). The smallest group comprised the oocytes containing di- or tri-pronuclei (7.5%, 53/705). In the frozen–thawed sperm group, 242 mouse oocytes were penetrated with the frozen–thawed human sperm, and 78.5% (190/242) of them survived ICSI. The difference in the oocyte activation rate between the fresh and the frozen–thawed sperm group was not significant. However, the incidence of the ICSI oocytes at the first cleavage metaphase was significantly higher (80.1 versus 71.5%, $P < 0.05$) in the frozen–thawed sperm group than the fresh sperm group, suggesting that frozen sperm have the ability to form PN and later condense into mitotic chromosomes.

The results of cytogenetic analysis in the fresh and frozen–thawed human sperm injected into mouse oocytes are summarized in Table II. The incidences of the fresh human sperm with aneuploidy, diploidy and structural chromosome

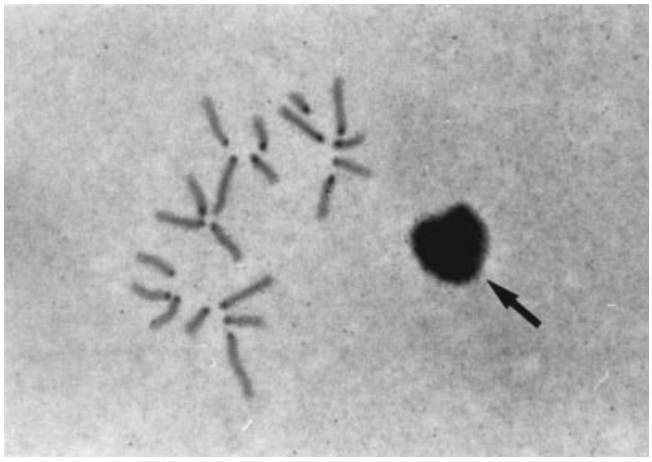

Figure 2. A chromosome spread of a hybrid 1-cell embryo containing swollen human sperm head and mouse mitotic chromosomes. The mouse oocyte was activated and then mouse chromosomes were duplicated and condensed into mitotic chromosomes, but human sperm did not develop beyond swelling of head (arrow).

Table II. Chromosome aberrations in the fresh or frozen–thawed (FT) human sperm injected into mouse oocytes

Sperm	No. of sperm analysed	No. of aberrant sperm (%)				No. of structural chromosome aberrations (per sperm)				
		Aneuploidy		Diploidy	Structural aberrations					
		Hyper	Hypo			chrb	chre	chtb	chte	Total[a]
ICSI (Fresh)	477	5 (1.0)	3 (0.6)	0 (0.0)	42 (8.8)	31 (0.065)	4 (0.008)	9 (0.019)	5 (0.010)	49 (0.103)
ICSI (FT)	141	0 (0.0)	1 (0.7)	0 (0.0)	11 (7.8)	7 (0.050)	0 (0.000)	3 (0.021)	4 (0.028)	14 (0.099)
ICSI Total	618	5 (0.8)	4 (0.6)	0 (0.0)	53 (8.6)	38 (0.061)	4 (0.006)	12 (0.019)	9 (0.015)	63 (0.102)
IVF[b] (FT)	383	5 (1.3)	5 (1.3)	NA	39 (10.2)	32 (0.084)	1 (0.003)	8 (0.021)	4 (0.010)	45 (0.117)

[a]The higher total no. of structural aberrations compared with the no. of the aberrant sperm shows that some cells had multiple aberrations.
[b]Watanabe and Kamiguchi (2001).
chrb = chromosome breakage; chre = chromosome exchange; chtb = chromatid breakage; chte = chromatid exchange; NA = not analysed.

aberrations (Figure 3) were 1.6, 0.0 and 8.8% respectively. In the frozen–thawed human sperm, no significant increase of these three types of aberrations (0.7, 0.0 and 7.8%) was found as compared with the fresh human sperm.

For the donor used in this study, a cytogenetic analysis of the frozen–thawed sperm using IVF with zona-free hamster oocytes had already been performed (Watanabe and Kamiguchi, 2001b). The present cytogenetic data using ICSI (the 'total' category in Table II) were then compared with the previous cytogenetic data using IVF (see the 'IVF' category in Table II) to estimate the effect of micro-manipulation on sperm chromosomes. The incidences of aneuploidy (2.6 versus 1.4%) and structural aberrations (10.2 versus 8.6%) in human sperm were not significantly different between ICSI and IVF, although there was no comparable data about diploidy in IVF.

Along with human sperm chromosome complements, 615 mouse oocyte chromosome complements were analysed to evaluate the effects of micro-manipulation on female chromosomes in this study (Table III). The incidence of mouse chromosome complements with aneuploidy was 4.0%. There was no discernible evidence of mouse chromosomes in 2.0% of the oocytes. The incidences of diploidy and structural chromosome aberrations were 5.4 and 1.3% respectively.

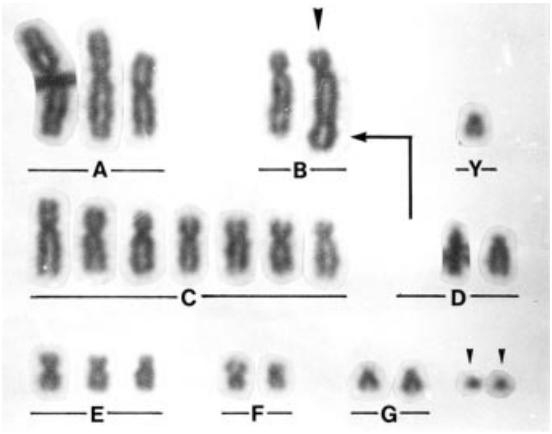

Figure 3. Karyotype of a human sperm with a chromosome exchange type of structural chromosome aberration (Giemsa staining). The reconstructed chromosome (large arrowhead) resulting from translocation between B and D group chromosomes and the resultant chromosome fragments (small arrowheads) were observed.

Discussion

Investigation of human sperm chromosomes had been performed mainly by using IVF with zona-free Syrian hamster oocytes before the introduction of ICSI with mouse oocytes (Martin *et al*., 1991; Kamiguchi *et al*., 1994). However, nobody has attempted a comparative study between these methods which would thereby make it possible to estimate the sperm DNA damage that may have been induced by micro-manipulation. In this study, chromosome analysis using ICSI was performed on motile human sperm with morphologically normal heads from a fertile donor, whose sperm had been previously cytogenetically examined using IVF. It was found that the incidence of chromosomally aberrant human sperm did not significantly differ between ICSI and IVF (Table II). Since

Table III. Chromosome aberrations in mouse chromosome complements after injection of the fresh or frozen–thawed human sperm

No. of mouse chromosome complements analysed	No. of aberrant mouse chromosome complements (%)					No. of structural chromosome aberrations (per oocyte)			
	Aneuploidy								
	Hyper	Hypo	Zero[a]	Diploidy	Structural aberrations	chrb	chre	chtb	chte
615	6 (1.0)	6 (1.0)	12 (2.0)	33 (5.4)	8 (1.3)	5 (0.008)	0 (0.000)	1 (0.002)	2 (0.003)

[a]The zygotes in which no mouse oocyte chromosomes was found. See Table II for explanation of abbreviations.

Table IV. Comparison of the present and previous studies examining chromosome aberrations in motile human sperm with normal heads injected into mouse oocytes

Study	Donor	No. of sperm analysed	Chromosome aberrations (%)			
			Aneuploidy	Diploidy	Structural aberrations	Total
Rybouchkin *et al.* (1996a)	Fertile	33	0.0	0.0	9.1	9.1
Rybouchkin *et al.* (1996b)	Fertile	41	2.4	0.0	4.9	7.3
Lee *et al*. (1996)	Fertile	159	1.3	0.0	6.9	8.2
Rybouchkin *et al.* (1997)	Infertile	41	4.9	0.0	0.0	4.9
Present study	Fertile	618	1.4	0.0	8.6	10.0

the repair capacity of sperm DNA lesion differs between mouse oocytes used for ICSI and Syrian hamster oocytes used for IVF (Matsuda *et al*., 1995; Tateno *et al*., 1996), this comparison is not exactly ideal for determining the risk of structural chromosome aberrations. However, the present result is sufficient to conclude that the influence of micro-manipulation is extremely low, even if DNA damage is induced in human sperm by micro-manipulation itself.

It has not been possible to estimate exactly the genetic constitution of human sperm injected into mouse oocytes, because the incidences and types of chromosomally abnormal sperm varied widely among the previous ICSI studies in the motile human sperm with morphologically normal heads (Table IV). This wide variation could be due to spontaneous variation in sperm from donor to donor, as found in a previous IVF study using hamster oocytes (Kamiguchi *et al*., 1994). However, infertility might also be one of the factors affecting the incidence of aberrant sperm, since the incidence of aneuploidy was comparably higher in an infertile donor (4.9%) who was used by Rybouchkin *et al*. (1997) than fertile donors (0.0–2.4%) used by the other authors (Rybouchkin *et al*., 1996a;b; Lee *et al*., 1996). Moreover, it is also possible that the small sperm number in the previous ICSI studies is the cause of this wide variation, although the sperm number was adequate in each case for the authors to elucidate the correlation between chromosome abnormalities and sperm phenotypes (morphology or motility). Rybouchkin *et al*. (1997) actually detected no structural chromosome aberration in sperm (Table IV), although such a case was never found in the cytogenetic data of 51 fertile donors gathered by Kamiguchi *et al*. (1994) using IVF between hamster oocytes. To settle the problem of small sample and obtain further information about the risk of chromosome aberrations in micro-manipulated human sperm, a detailed cytological and cytogenetic analysis was performed on a large number (a total of 618 cells) of human sperm injected into mouse oocytes in the present study. Before comparing this result with those in the previous ICSI studies (Table IV), it is important to determine whether our result using sperm from the same individual reflects typical normal variation, or results from spermatogenesis unique to the subject. Our previous IVF study on the sperm from this donor (Watanabe and Kamiguchi, 2001b), which is shown in Table II, is a clue to help clarify this. The cytogenetic result in our IVF study is directly comparable with those from 51 donors in the IVF study of Kamiguchi *et al*. (1994), since both studies were performed using the same method and the same equipment. According to Kamiguchi *et al*., sperm samples from 51 normal donors contained aneuploidy ranging from 0 to 4% (1.4 ± 1.0% average) and structural chromosome aberrations ranging from 3.6 to 24.6% (14.1 ± 4.1% average). In our IVF study, the incidences of those types of aberrations were 2.6 and 10.2% respectively and these values were within the range of normal variations of aneuploidy and structural chromosome aberrations shown by Kamiguchi *et al*. (1994). Therefore, it is concluded that there is no variation caused by individual characteristics in the spermatogenesis of the particular donor.

When the result of the present ICSI study was compared with those of previous ICSI studies (Table IV), the proportions of the three types of aberrations were very similar to those in two other studies (Lee *et al*., 1996; Rybouchkin *et al*., 1996a). In particular, the incidences of structural chromosome aberrations were more than five times as high as the incidences of aneuploidy in each of these three studies. Moreover, the study of Lee *et al*., using >100 sperm samples, did not have the problem of small sample size and can be considered to be more reliable. The agreement between the present and the previous two studies seems to indicate that these studies detected more exactly the genetic constitution of human sperm injected into mouse oocytes. In contrast, the rest (Rybouchkin *et al*., 1996b; 1997) showed different patterns in the ratio of structural chromosome aberrations to aneuploidy (1:2 or 2:1), while there were no such patterns found in the 51 normal donors examined by Kamiguchi *et al*. (1994). Therefore, the influence of the small sperm number on the incidences of the chromosomally aberrant sperm cannot be excluded in these two studies. On the other hand, despite the difference in the ratio of structural chromosome aberrations to aneuploidy, it is noteworthy that the total incidences of chromosome aberrations were very similar in the present and the previous ICSI studies. This seems to indicate that the total incidences of chromosome aberrations are comparatively precise even in studies on less than 50 sperm samples. Taking these results of the ICSI studies into consideration, one can conclude that, roughly, the estimated risk of chromosome aberrations in micro-manipulated motile human sperm with normal heads is almost 10%, a value composed of aneuploidy, diploidy and structural chromosome aberrations in the ratio 1:0:5. As described above, our cytogenetic data on large numbers of sperm contributed for understanding the risk of chromosome aberrations in micro-manipulated motile human sperm with normal heads. On the other hand, it is regrettable that only a single donor was examined in this study. Due to a change in the Japanese law, however, it is no longer possible to carry out inter-species IVF or ICSI, and hence it is not possible to extend these studies to other individuals.

In this study, no diploidy was found in 618 motile human sperm with normal heads which were obtained from a fertile donor. The same was also true of the previous ICSI studies (Table IV). Paradoxically, this fact strongly suggests the correlation between diploidy and sperm head abnormality. It is easy to think of large-headed sperm as the morphological abnormality related to diploidy. However, Lee *et al*. (1996) reported that there were no diploid cells in 11 large-headed human sperm injected into mouse oocytes. Furthermore, Calogero *et al*. (2001) did not refer to the correlation between diploidy and large sperm head in the semen samples from the patients with oligoasthenoteratozoospermia, which show a significantly higher incidence of diploid sperm in a fluorescence in-situ hybridization method, and the average rate of diploidy among the patients was extremely low (0.09%). These findings indicate that large sperm heads do not necessarily contain a diploid nucleus, although this does not mean that diploid sperm have large heads. On the other hand, the possibility has been reported that a diploid sperm without any morphological abnormality was contained in ejaculate from patients with oligoteratozoospermia (Rosenbush *et al*., 1998).

Table V. Comparison of present ICSI and previous in-vivo and in-vitro studies examining chromosome aberrations in mouse female chromosome complements

Study	Fertilization	No. of mouse oocytes analysed	Chromosome aberration (%)		
			Aneuploidy	Diploidy	Structural aberrations
Fraser and Maudlin (1979)	In-vivo	935	8 (0.9)	?	?
	In-vitro	1925	28 (1.5)	?	?
Martin-Deleon and Boice (1983)	In-vitro	321	7 (2.2)	1 (0.3)	0 (0.0)
Santalo *et al*. (1986)	In-vivo	850	17 (2.0)[a]	24 (2.8)	7 (0.8)
	In-vitro	882	11 (1.2)[a]	22 (2.5)	2 (0.2)
Present study	ICSI	615	24 (4.0)[b] hyper- and hypo 12 (2.0) no chromosomes 12 (2.0)	33 (5.4)[c]	8 (1.3)

[a]The incidence of hyperploidy.
[b]χ^2-Test (Fraser and Maudlin, 1979), $P < 0.001$.
[c]χ^2-Test, $P < 0.01$.

Therefore, diploidy may not be implicated in this particular morphological abnormality.

In the present study, the first attempt at cytogenetic analysis of mouse chromosome complements was made after ICSI into mouse oocytes. These data were compared with the data in previous mouse in-vivo and in-vitro studies (Table V) to estimate the risk of chromosome abnormalities in female chromosome complements after micro-manipulation. The incidences of structural chromosome aberrations in female chromosome complements did not differ between our ICSI study and the previous studies. This result may be explained by the fact that the meiotic spindle of mouse oocytes locates right under the cortex where a hump is formed (Kimura and Yanagimachi, 1995). Since the hump was easily confirmed under a light microscope, human sperm could be injected into the mouse oocyte without any direct damage to mouse meiotic chromosomes. The aneuploidy rate in this study (4.0%) was significantly higher than the rates reported by Fraser and Maudlin (1979) (0.9 or 1.5%, $P < 0.001$) and higher (but not significantly so) than the rate reported by Martin-Deleon and Boice (1983) (2.2%). Santalo *et al*. (1986) did not count hypoploidy cells. However, when the rate in in-vitro study of them (1.2%) is doubled on the assumption that hyper- and hypoploidy are induced at 1:1 ratio, the resultant rate (2.4%) is lower (but not significantly so) than our rate (4.0%). Remarkably, ICSI oocytes with no mouse chromosomes accounted for a half of our aneuploidy rate (Tables III and V). When the rate of this type of aberration (2.0%) was subtracted from the overall rate of aneuploidy (4.0%), the remainder (2.0%), which consisted of hyper- and hypoploidy, did not differ from the aneuploidy rates in the previous studies. This type of aberration, to which none of the authors has referred in the previous reports shown in Table V, is probably induced by micro-manipulation. The most probable mechanism causing the loss of female chromosome complements is extrusion of the whole meiotic chromosomes into the second polar body. The diploidy rate was also significantly higher in ICSI than in-vivo and in-vitro fertilization (5.4 versus 0.3–2.5%, $P < 0.01$), suggesting that micro-manipulation induces diploidy in female chromosome complements. Since the MII mouse oocytes with the first polar body were selected for injection in this study, the diploid mouse chromosome complements must have been attributed to failure to expel the second polar body. Macas *et al*. (1996) has reported that most human multipronuclear ICSI oocytes contained the diploid female chromosome complements which resulted from failure to extrude the second polar body. Although the number of pronuclei was not counted before chromosome preparation in this study, six of 33 diploid mouse oocytes contained two individual mouse metaphases which probably originated from two individual pronuclei. Moreover, 19 tripronuclear hybrid oocytes were found in 59 hybrid oocytes arrested at the pronuclear stage after injection of the fresh or frozen–thawed human sperm (Table I). It is therefore considered that multipronuclei were formed in some ICSI mouse oocytes owing to failure to extrude the second polar body. Failure of the second meiosis described above indicates that micro-manipulation has a harmful effect on the oocyte cytoskeleton system. Macas *et al*. (1996) proposed the possibility that external calcium ions injected along with sperm disorganized microtubules constructing the meiotic spindle. This may be the cause of frequent hyper- and hypoploidy in human tripronuclear ICSI oocytes observed by Macas *et al*. (1996), since an increase of hyper- and hypoploidy has been reported in hamster primary oocytes exposed to colchicine, suggesting that depolymerization of microtubules is implicated in the induction of hyper- and hypoploidy (Sugawara and Mikamo, 1980). Moreover, Harderson *et al*. (2000) examined meiotic spindles of human oocytes visualized with microtubule immunostaining, and found that the position of the meiotic spindle cannot be predicted by the location of the first polar body. However, human sperm are traditionally injected into human oocytes on the assumption that meiotic spindles are located under the cortex close to the first polar body, although the spindles are actually invisible under the light microscope. Hence, there is the possibility in human oocytes that the meiotic spindle is exposed to calcium ions originating from the injection pipette, the tip of which is inserted close to the spindle. In contrast to

the result in human ICSI oocytes, no significant increase of hyper- and hypoploidy was observed in mouse chromosome complements, although the complete loss of mouse chromosome complements was frequently induced in this study. Similarly, the incidence of diploidy was extremely low in hamster primary oocytes exposed to colchicine. Moreover, the possibility that the meiotic spindle was exposed to calcium ions seems to be comparably low in mouse oocytes, the spindles of which could easily be identified under a microscope. These facts suggest that the induction of diploidy may be attributed to a different mechanism from the one inducing hyper- and hypoploidy. The possible explanation may be that the stretching of the oocyte plasma membrane by micro-injection disturbs the microfilament network underneath oocyte plasma membrane, which plays a role in the formation of the contractile ring where the site of cleavage is determined (White and Borisy, 1983; Rappaport, 1986; Tolle *et al*., 1987). To resolve this problem, further experiments on the alteration of the cytoskeleton network during the extrusion of the second polar body are needed.

The present comparative analysis between the fresh and the frozen–thawed human sperm injected into mouse oocytes revealed that cryopreservation frequently allowed sperm nuclei to develop into first mitotic chromosomes. This result indicates that the cryopreservation of sperm may be available as one of the treatments for those infertile males with defects in their sperm plasma membrane. The most probable reason for such an effect is the freezing-induced plasma membrane damage, which was observed directly in ram (Holt *et al*., 1992) and human sperm after freezing and thawing (Nogueira *et al*., 1999). Kasai *et al*. (1999) found that human sperm treated with Triton X-100, a detergent that disrupts the lipid bilayer of plasma membranes, became frequently more swollen than sperm with intact plasma membranes after micro-injection into mouse oocytes. It is therefore little wonder that mouse oocytes inseminated with human sperm in which freezing-induced plasma membrane damage had occurred, develop well into the first cleavage metaphase. In this study, the incidences of chromosome aberrations were also compared between fresh and frozen–thawed human sperm; a similar study was attempted using IVF between human sperm and zona-free hamster oocytes (Martin *et al*., 1991). Both results agree that cryopreservation did not influence the frequency or type of chromosome aberrations in human sperm. It is therefore confirmed that frozen–thawed human sperm are available to ICSI as well as IVF. However, this fact does not refer to the morphologically aberrant sperm or immotile sperm because the present study was conducted on the motile human sperm with normal heads. Similarly, it is considered that the chromosomes of these kinds of abnormal sperm were not evaluated by the sperm chromosome assay using IVF methods, since it is doubtful whether these aberrant sperm are potent enough to penetrate oocytes. Up to now, there has been no study on the effect of cryopreservation on abnormal human sperm.

In this study, detailed cytogenetic data were collected from a large number of human sperm using ICSI. On the basis of the results, further detailed investigations are being conducted to assess the risk of chromosome abnormalities in the morphologically abnormal human sperm and immotile human sperm, for which information remains inadequate.

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