Human Reproduction vol.12 no.6 pp.1203–1213, 1997

Intracytoplasmic injection of spermatids retrieved from testicular tissue: influence of testicular pathology, type of selected spermatids and oocyte activation

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Spermatid microinjection into oocytes has proven to be a successful assisted reproduction procedure in the animal model and in the human species, since in the latter a few full-term pregnancies were actually obtained. Patients entering our spermatid injection study included those with a total absence of spermatozoa in the testicular tissue notwithstanding previous positive biopsies (n = 29): an obstructive problem (n = 3), secretory azoospermia (n = 26), and those with total arrest at the spermatogenesis level in previous explorative biopsies (n = 15). In the latter group, absence of spermatids was recorded in four cases. Mature, elongated, elongating and round spermatids (ROS) were injected in respectively 3, 2, 3, and 32 attempts. A total of 260 metaphase II oocytes were injected with ROS, 36 oocytes with spermatids at other stages of maturity. The rates of oocytes showing two pronuclei (2PN) and two polar bodies reached 22% and 64% respectively after injection of round or elongated-mature spermatids. The fertilization rate after ROS injection was influenced by the percentage of spermatozoa observed in a previous biopsy. Patients with a positive preliminary biopsy had significantly more 2PN (33%) when compared to those with a severe spermatogenic dysfunction and in whom no spermatozoa were found (only 11%) ($P < 0.05$). Incubation of oocytes in calcium ionophore after ROS injection had a positive effect on the rate of 2PN formation (36 versus 16%). Ninety per cent of all the normally fertilized oocytes cleaved. The percentage of grade A and B embryos depended on the type of injected cells: 12% after ROS and 30% with the other types of haploid cells. A total of 39 transfers resulted in five pregnancies: three full term with healthy babies delivered (one after ROS injection, and two after injection of an elongating and a mature spermatid), one 4 months ongoing (after elongating spermatid injection) and one miscarriage at 4 weeks (after elongated cell injection). Compared to our conventional intracytoplasmic sperm injection–testicular sperm extraction (ICSI–TESE) programme, the implantation rate after ROS injection was very low (5.5 versus 10.5%).

Key words: ICSI/non-obstructive azoospermia/oocyte activation/spermatid injection/spermatogenic arrest

Introduction

The combination of intracytoplasmic sperm injection (ICSI) with testicular sperm extraction (TESE) has been successfully applied to alleviate male factor infertility due to obstructive azoospermia (OA) (absence of the vas, acquired epididymal obstructions following either failed reconstructive microsurgery or post-inflammatory occlusions) (Schoysman *et al.*, 1993a,b; Devroey *et al.*, 1994; Vanderzwalmen *et al.*, 1995b) and even non-obstructive azoospermia (NOA) linked to deficient spermatogenesis (Devroey *et al.*, 1995; Kahraman *et al.*, 1996; Silber *et al.*, 1995a; Yemini *et al.*, 1995).

One of the most perplexing problems in TESE procedures is the controversy regarding the predictive value of testicular size and peripheral serum follicle stimulating hormone for the outcome of the therapeutic testicular biopsy. Considering psychological and financial implications, Tournaye *et al.* (1997) suggested recently that a preliminary testicular biopsy may be preferable in patients suffering from secretory azoospermia. When in May 1993 we started the TESE–ICSI programme (Schoysman *et al.*, 1993a,b) our policy was still to perform a preliminary explorative testicular biopsy to ascertain that complete spermatogenesis would be present in at least some seminiferous tubules (positive biopsy) in order to guarantee sperm retrieval for the planned TESE–ICSI attempt. In the majority of cases, this histological examination agreed well with what we obtained at the time of the TESE–ICSI attempt since enough spermatozoa were available. However, in some patients no spermatozoa could be found in different testicular samples at the time of the TESE–ICSI attempt, notwithstanding previous positive biopsies in obstructive and non-obstructive cases.

Facing such situations, Edwards *et al.* (1994) had already suggested that spermatids could be used as substitutes for spermatozoa to achieve fertilization of human oocytes. In the literature several experiments described the obtainment of mammalian embryos with the use of spermatid cells instead of spermatozoa. In the hamster and mouse model, round spermatids (ROS) or round spermatid nuclei (ROSNI) were capable of leading to syngamy when incorporated into mature oocytes either after ICSI or electrofusion (Ogura and Yanagimachi, 1993; Ogura *et al.*, 1993). Delivery of live, healthy young and fertile offspring was reported in the mouse after electrofusion of the oocyte with ROS (Ogura *et al.*, 1994; Ogura and Yanagimachi, 1995) and in the rabbit (Sofikitis

et al., 1994, 1996a) after direct injection of isolated ROSNI. These data demonstrated that the nuclei of spermatids can provide the paternally imprinted chromosomes needed for full embryonic development.

We considered in 1994 the possibility of using human testicular spermatids for the fertilization of oocytes by ICSI. In the human, normal fertilization and cleavage up to the 4-cell stage (Vanderzwalmen *et al.*, 1995a) and a pregnancy (Fishel *et al.*, 1995) were reported after ICSI of an elongated spermatid retrieved from testicular tissue. Tesarik *et al.* (1995, 1996) reported the first two pregnancies followed by delivery of healthy babies after injection of ROS, collected from the ejaculates of patients suffering from NOA alternating with very severe oligozoospermia. Four other pregnancies were reported by Hannay (1995) after injection of ROSNI but spontaneous miscarriages occurred in all of them.

These encouraging data led us to try the use of spermatids in cases of men suffering from azoospermia due to primary testicular disorder, caused by different aetiological factors and where in a previous histological examination of the testis total absence of complete spermatogenesis was recorded (negative biopsy).

The purpose of this paper is to present our experience with ICSI of mature, elongating, elongated and round spermatids retrieved from testicular tissue. We have attempted to evaluate some of the factors affecting the outcome of fertilization, embryo quality and conception, such as the severity of the pathology, the type of injected spermatids and the effect of calcium ionophore in the oocyte activation process.

Materials and methods

Patient selection

From May 1993 until September 1996, 252 patients with various clinical and endocrine conditions ranging from purely obstructive ($n = 115$) to non-obstructive ($n = 137$) azoospermia were accepted in our TESE–ICSI programme (Table I).

At 6–20 weeks before starting the TESE–ICSI procedure, an explorative testicular biopsy was performed to evaluate the status of spermatogenesis. During the explorative procedure, the testicular tissue was divided into two pieces. One part was fixed in Bouin solution for histological examination (van de Casseye, 1994) and the second specimen was dissected with needles and crushed between glass slides before searching for spermatozoa.

Out of the 252 previous explorative biopsies, active spermatogenesis was present in 237 patients and absence of spermatozoa was noted in the testes of 15 patients. According to the percentage of tubules containing spermatozoa in the previous biopsies, the 137 NOA patients were classified into three categories; in 59 cases the percentage of tubules showing a complete spermatogenesis ranged from 20 to 50%, in 63 other cases a complete spermatogenesis was observed in <20% of tubules and in the third group no spermatozoa were found in 15 patients. In this third group the previous explorative biopsies showed an incomplete spermatogenesis with tubules containing few spermatids.

At the time of the TESE–ICSI attempts, absence of mature spermatozoa in different samples of biopsies was recorded in 30 men notwithstanding spermatozoa being present in previous biopsies. Absence of spermatozoa was recorded in three patients (3/115: 2.6%) from the obstructive group. Out of the 122 patients with previous positive biopsy composing the NOA group, no spermatozoa were present in 27 cases (22.1%). Absence of spermatozoa was noted in eight out of 59 cases (13.5%) when the previous biopsy showed <50% of spermatozoa in the tubules. In poorer quality testes (<20% of tubules with spermatozoa) absence of spermatozoa was recorded in 30.1% of patients (19/63). Informed consent was given by the 30 couples as well as the 15 patients in the NOA group with incomplete spermatogenesis; of these, 44 agreed to enter the intracytoplasmic spermatid injection programme. One couple preferred classical insemination with donor spermatozoa.

In 207 patients, spermatozoa were found in the biopsy so that they entered the normal TESE–ICSI programme. Only in three patients were fewer than five oocytes retrieved, and no evident co-existing female factors contributing to infertility, such as endometriosis, polycystic ovaries, or higher than average female age, were present in this study.

Oocyte preparation

Two to three hours after oocyte retrieval, the cumulus and corona cells were removed after incubation in 25 IU hyaluronidase/ml (type VIII; Sigma Chemical Co., St Louis, MO, USA) for up to 20–30 s. The removal of these cells was accomplished in another drop of IVF 50 (Scandinavian IVF Science, Gothenburg, Sweden) free of hyaluronidase by repeated aspiration of the complex cumulus corona in a glass pipette with an inner diameter ranging from 180 to 220 μm. The oocytes were rinsed eight times in 0.5 ml IVF 50 and incubated in IVF 50 at 37°C in an atmosphere of 5% CO_2 with air, covered with light paraffin oil until the ICSI procedure was performed on oocytes that had extruded their first polar body.

Testicular biopsy and spermatid preparation

When spermatozoa were expected, an open testicular biopsy (Vanderzwalmen *et al.*, 1994) was performed either 1 day before or on the day of egg retrieval. For the 15 patients entering the spermatid injection programme directly because of total absence of spermatozoa in the preliminary biopsy, the biopsy was performed on the day of oocyte retrieval. In brief, a small incision was made in the scrotal skin. A second incision was carried through the peritoneal tunica vaginalis and a piece of extruding testicular tissue was cut and transferred into a Falcon tube containing 1 ml of Earle's HEPES-buffered medium + 0.5% human serum albumin (HSA; Irvine, Paisley, UK). The testicular tissue was washed several times with Earle's HEPES-buffered medium + 0.5% HSA and placed in a Petri dish. The cellular contents were obtained by gentle crushing between two needles and sterile glass slides in a Petri dish containing 4 ml of medium (Vanderzwalmen *et al.*, 1995b). If no spermatozoa were immediately observed after checking at least 20 fields at ×200 magnification, other testicular samples (up to four) were collected for further analysis. The suspensions were then transferred into 15 ml Falcon tubes and agitated for 60 s on a vortex, in order to separate the different cell types obtained.

Centrifugation was performed at 600 *g* through a discontinuous Percoll gradient in three layers (50, 70, 90%) (Vanderzwalmen *et al.*, 1991) for 20 min. The 50 and 70% Percoll fractions were washed with Earle's medium supplemented with 0.5% HSA for 5 min at 1000 *g*. Spermatids were mostly found in the 50 and 70% Percoll fractions. After one washing with Earle's medium and centrifugation, the pellet was resuspended in 200 μl IVF 50. A total of 50 μl was placed in 200 μl of IVF 50 before starting isolation of round, elongating, elongated and mature spermatids.

The spermatids were first aspirated together with the red blood cells and debris, using a 10 μm pipette, and rinsed in a drop of IVF 50 medium before transferring them in a 5 μl droplet of IVF 50

Table I. Spermatid injection: distribution of patients

	Previous biopsy	Biopsy at time of oocyte retrieval	
Obstructive azoospermia (n = 115)			
Spermatozoa present	115	112	→ ICSI–TESE programme
Spermatozoa absent	0	3	→ Spermatid injection programme
Non-obstructive azoospermia (n = 137)			
Spermatozoa present	122	95	→ ICSI–TESE programme
in 20–50% of tubules	59	51	
in <20% of tubules	63	44	
Spermatozoa absent	15	42 (8[a], 19[b], 15[c])	→ Spermatid injection programme

[a]Absence of spermatozoa in the group of patients where 20–50% of tubules contained spermatozoa in a previous biopsy.
[b]Absence of spermatozoa in the group of patients with <20% of tubules containing spermatozoa in a previous biopsy.
[c]Spermatozoa entirely absent also in a previous biopsy.

adjacent to the polyvinylpyrrolidone (mol. wt 360 000, 10% wt/vol) and the injection droplets. The Petri dishes were kept at 37°C before the ICSI procedure.

Spermatid classification

In the process of spermatogenesis, haploid ROS differentiate into highly polar cells specialized for motility and fertilization. This differentiation, as shown in Figure 1, involves extensive morphological modifications. Under an inverted Olympus IX70 microscope equipped with Hoffman modulation contrast system at magnifications of ×200 and ×400, four different kinds of haploid cells were observed: ROS include the Golgi-cap phase (Figure 2) and the acrosome phase (Figure 1A), the elongating spermatids (Figures 1A, 3 and 4), the elongated spermatids composing the beginning of a maturation phase (Figures 1C and 5) and finally the mature spermatids just before their delivery (Figure 1D).

In general, ROS were distinguished from other cells (lymphocytes, monocytes, spermatocytes, spermatogonia, polymorphonuclear leukocytes) by their round shape with smooth outline and size (7.8 μm diameter). The diameter was similar to that of erythrocytes (average of 7.2 μm) and also to some small lymphocytes. Golgi phase ROS were very difficult to identify with the Hoffman modulation contrast system. The only distinguishing feature is a central rounded thickening of nucleus. The crescent-shaped dense material bordering the plasma membrane is a feature of the cap phase spermatid. The presence of a developing acrosome is a good criterion and can sometimes been seen as a small protrusion on one side of the cell.

According to the size of the tail, the spermatids (ELS) were classified as elongating (Figures 1B and 4) or elongated (Figures 1C and 5). Cells with an oval shape, with a visible head but not well-defined tail were classified in the ELS group. The mature spermatids, aspirated from the germinal epithelium (Figure 1D), appear similar to the ultimate sperm morphology. We included in this study injection of mature spermatids (n = 3) when the search for cells took >2 h. However, cases with higher concentration of spermatids, trapped between the Sertoli cells, with occasional moving tails, were not included in this study even if such cells were histologically classified as spermatids.

Intracytoplasmic spermatid injection

The injection technique of spermatids into the ooplasm was similar to the conventional ICSI method with mature spermatozoa (Van Steirteghem *et al.*, 1993; Vanderzwalmen *et al.*, 1996).

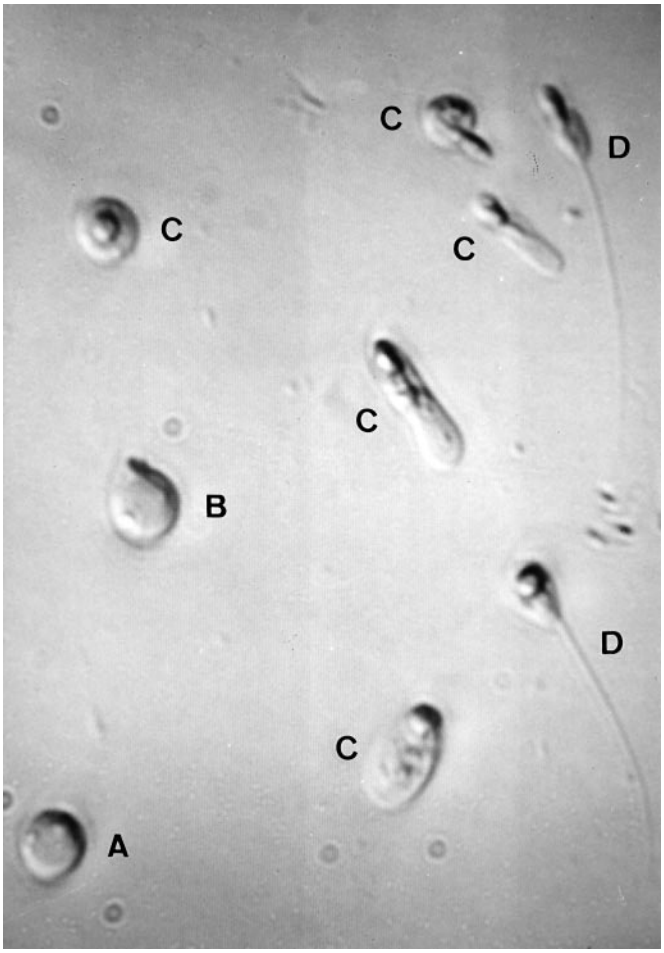

Figure 1. Hoffman modulation contrast photomicrographs (×1200) of haploid cells from the spermatogenesis stage, retrieved from testicular tissue, exhibiting spermatozoa in >50% of tubules. A, round spermatid with acrosome protrusion; B, elongating spermatid; C, elongated spermatids at different stages of development; D, mature spermatids removed from germinal epithelium cells. The use of such a mature spermatid resulted in a full-term pregnancy.

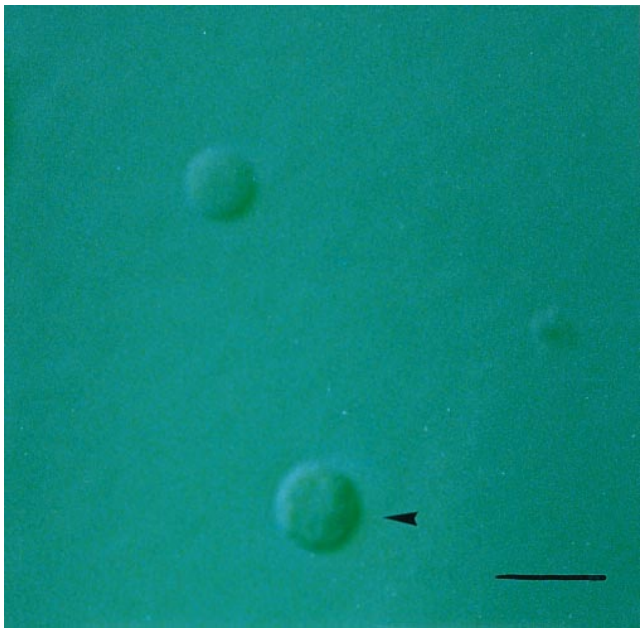

Figure 2. Round spermatid (arrow; ROS) from testicular tissue exhibiting spermatozoa in 10% of tubules (original magnification × 1600) in a preliminary biopsy. The use of such a ROS resulted in a full-term pregnancy. Scale bar = 10 μm.

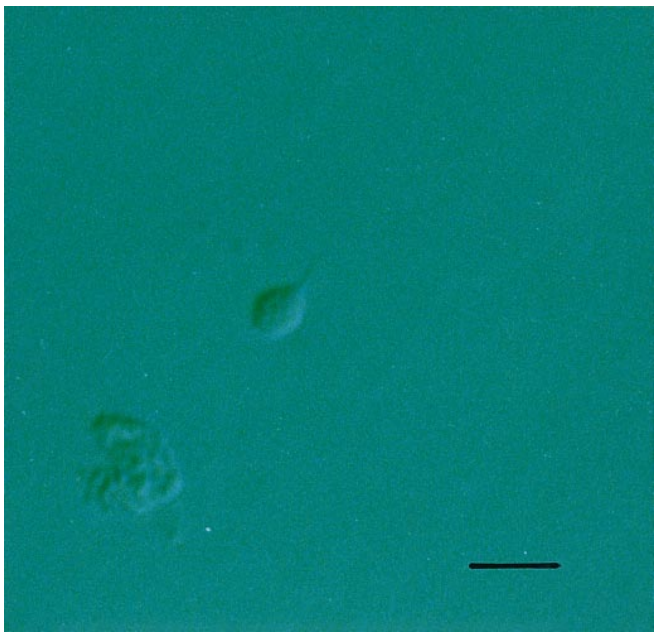

Figure 4. Elongating spermatid retrieved from testicular tissue exhibiting spermatozoa in 20% of tubules in a preliminary biopsy (original magnification ×1200). Scale bar = 10 μm. The use of such a spermatid resulted in a full-term pregnancy.

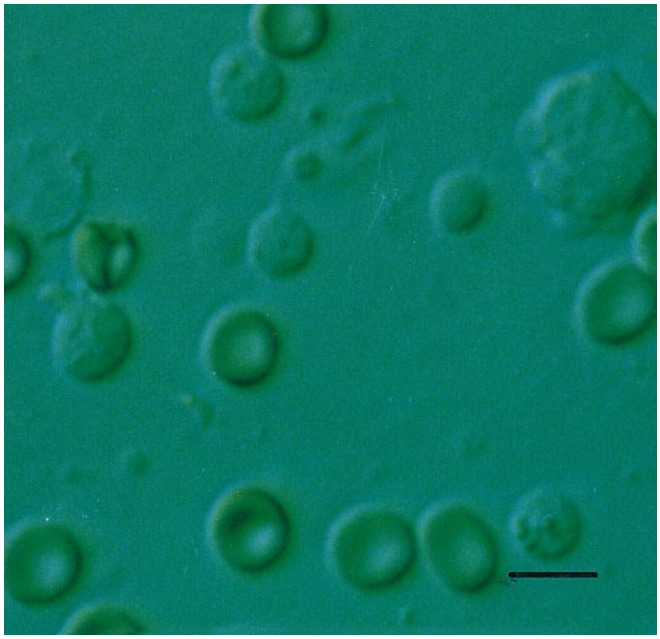

Figure 3. Round spermatid with tail starting to develop and absence of visible acrosome, retrieved from testis exhibiting spermatozoa in 10% of tubules in a preliminary biopsy (original magnification ×1200). Scale bar = 10 μm.

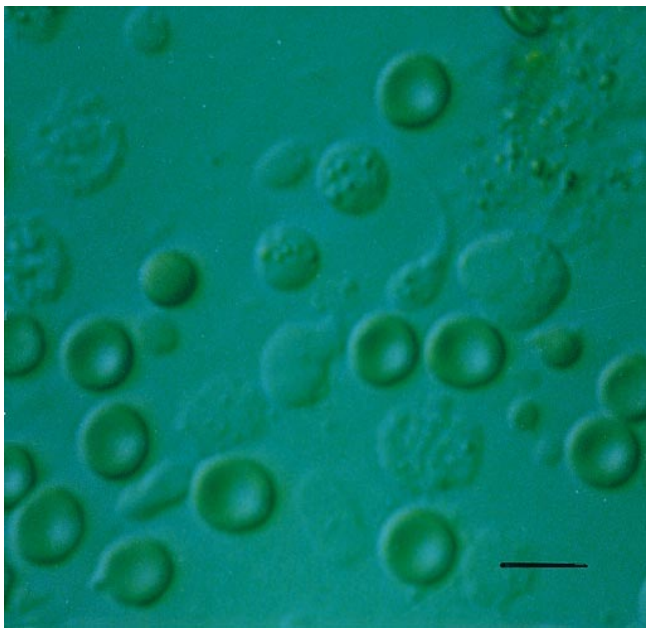

Figure 5. Elongated spermatid retrieved from testicular tissue exhibiting spermatozoa in 30% of tubules in a preliminary biopsy (original magnification ×1200). Scale bar = 10 μm.

The diameter of the ROS ranged between 7 and 8 μm. They were aspirated into an injection pipette with an inner diameter of ~6–7 μm. During this step, the ROS is compressed and deformed and takes an oval shape inside the lumen of the pipette. After aspirating the oocyte on the holding pipette (opening 15 μm), and the polar body at 12 or 6 o'clock, the injection pipette was pushed through the zona pellucida and the oolemma at the equatorial level. Before injecting spermatids, vigorous ooplasmic aspiration was performed until we visualized a rapid outflow of cytoplasm into the injection pipette. After re-injection of this cytoplasm and the spermatid, the injection pipette was withdrawn and the injected oocyte released from the holding pipette. The injected oocytes were then washed four times in fresh IVF 50 and divided amongst 4-well Nunc dishes and incubated at 37°C in an atmosphere of 5% CO_2.

At 15–20 min after ROS injections (average time for the ROS injections), some oocytes were further incubated for 15 min in 10 μM calcium ionophore, A23187, at 37°C and then washed several times in medium before culture.

Table II. Fertilization, cleavage and pregnancy rates after intracytoplasmic injection of spermatozoa and spermatids

	Injection of	
	Spermatozoa	Spermatids
No. of patients	207	40[a]
No. of injected oocytes	1826	296
2PN per injected oocytes	1114 (61)	80 (27)
1PN per injected oocytes	94 (5)	45 (15)
Degenerated oocytes	87 (5)	26 (9)
Cycles with fertilization	206	39
Cleavage per fertilized oocytes	1088 (98)	72 (90)
Grade A and B embryos	593 (55)	14 (19)
Pregancies per cycle	55 (27)	5 (13)
Ongoing pregnancies per cycle	40 (19)	4 (10)[b]
Implantation rate	10.5%	5.5%

PN = pronuclei.
Values in parentheses are percentages.
[a]Forty-four patients entered the spermatid injection programme, in four of them no spermatid was found.
[b]Three deliveries and one 4 month ongoing pregnancy.

Assessment of fertilization and embryo development

Fertilization was determined 18–22 h after injection by the presence of two pronuclei (2PN) and two polar bodies. The percentage of 1PN and degenerated oocytes was also recorded. The zygotes were cultured up to the 4-cell stage in IVF 50 medium. After 24 h of in-vitro culture, embryo quality was assessed, and the embryos were classified according to the number and form of the blastomeres and the percentage of fragments. Regular size blastomeres without fragmentation were classified as grade A, and in grade B embryos 10–20% of fragmentation was present. All the normally fertilized oocytes resulting in embryos were transferred 44 h after oocyte retrieval.

Pregnancy was detected by serum human chorionic gonadotrophin concentrations on day 15 and day 20 after transfer. An ultrasound detection was performed at the sixth week after transfer. The presence of a yolk sac, fetus and heart activity were assessed and if present a clinical pregnancy was registered.

Statistical methods

The comparison between groups of ICSI experiments was performed with Pearson's χ^2-test using Yates' correction when necessary.

Results

Table II presents the overall outcome of fertilization, embryo cleavage and pregnancies after intracytoplamic sperm or spermatid injections.

ICSI was performed in 207 attempts for patients suffering from OA or NOA. Forty-four patients entered the spermatid injection programme due to absence of spermatozoa in the testicular biopsy at the time of the egg retrieval. In 40 biopsies, spermatids were retrieved and injections performed. Four cycles were cancelled due to absence of spermatids in the testicular tissues from patients with extremely severe secretory problems.

The fertilization rate per injected oocyte observed after 18–22 h was 61% after sperm injection and 27% with spermatid injection. The rate of 1PN (5 versus 15%) and degenerated oocytes (5 versus 9%) was lower after sperm injection as compared with the spermatid injection. The percentages of cleaved embryos after injection of spermatozoa reached 98% (1088/1114) and 90% (72/80) after injection of spermatids. The percentage of grade A and B embryos was 3-fold higher in the conventional ICSI–TESE programme (55 versus 19%). Pregnancies were established in 55 women (27%) after ICSI attempts with mature spermatozoa. After spermatid injection, pregnancies were established in five women (13%), out of which three proceeded to live births of healthy babies and one is still ongoing.

In the OA group, absence of spermatozoa was recorded in only 2.6% (3/115) as compared with the NOA group where 42 (37 for spermatid injection, four with absence of spermatids, one preferring donor insemination) out of 137 testicular biopsies showed absence of spermatozoa (30.6%) (Table I).

In the NOA patients with a previous positive biopsy, the frequency of absence of spermatozoa in the testicular tissue, and henceforth the probability of spermatid injection, was related to the percentage of tubules showing spermatozoa. Out of 59 patients showing a full spermatogenesis in 20–50% of the tubules, eight (13.6%) entered the spermatid injection programme. In patients where the percentage of tubules with a complete spermatogenesis decreased to $<20\%$ at the previous biopsy, a higher percentage (30.1%: 19/63) of patients entered the spermatid programme on the day of the oocyte collection (Table I).

Table III presents the results after injection of spermatids in relation to the type of azoospermia (OA versus NOA) and the severity of the testicular pathology. No difference in the overall fertilization rate was observed between the OA and NOA groups (28 versus 27%).

In the NOA group, the rate of 2PN formation after spermatid injection decreased when we injected spermatids recovered from a testis presenting $<20\%$ of tubules with complete spermatogenesis. Out of 15 patients in whom maturation arrest, and germ cell aplasia were diagnosed, in only 11 patients could spermatids be found and a dramatic drop in the fertilization rate (11%) was observed.

The type of injected spermatids selected for the injection was correlated to the fertilization rates (Table IV). Fertilization rates per injected oocytes were significantly higher (100, 60 and 53%) when using elongated, elongating and mature spermatids as compared with the percentages of 2PN obtained after injection of ROS (22%). The cleavage rates, however, were not influenced by the type of injected spermatid (100 versus 86%). There was a tendency for the percentage of good quality embryos (grade A and B) to be higher after injection of ELS and mature spermatids (30%) as compared with injection of ROS (12%) but this was not statistically significant. Three live births of healthy babies were obtained altogether after injection of ROS, elongating and mature spermatids and one 4 month pregnancy is ongoing after elongating spermatid injection. The fifth pregnancy miscarried at 4 weeks after elongated cell injection.

Incubation of the oocytes, with calcium ionophore A23187 for 15 min at 37°C directly after microinjection, increased the fertilization rates (Table V). Out of 72 injected oocytes with ROS, 26 (36%) demonstrated normal fertilization as compared to 16% without treatment ($P < 0.05$).

Table III. Fertilization, cleavage and pregnancy rates after injection of spermatids from azoospermic patients with obstructive and non-obstructive azoospermia

	No. of cases	No. of oocytes	2PN	1PN	No. of oocytes degnerated	No. of embryos	Pregnancies
Obstructive azoospermia	3 (2.6)	25	7 (28)	2 (8)	0	7 (100)	0
Non-obstructive azoospermia							
% tubules showing spermatozoa in a previous biopsy							
20–50%	7 (27)	51	19 (37)	8 (16)	4	17 (89)	2[a]
<20%	19 (73)	141	45 (32)	23 (16)	16	40 (88)	3[b]
0%	11[c]	79	9 (11)	11 (14)	5	8 (88)	0
Total	37 (31)	271	73 (27)	43 (16)	26	65 (89)	5

PN = pronuclei.
Values in parentheses are percentages.
[a]One live birth, one biochemical pregnancy.
[b]Two live births, one ongoing pregnancy.
[c]Four out of fifteen without spermatids.

Table IV. Fertilization, cleavage and pregnancy rates according to the different types of injected spermatids: mature spermatid, elongated spermatid, elongating spermatid, and round spermatid (ROS)

Type of spermatid	Figure nos.	No. of cases	No. of oocytes	2PN	1PN	No. of oocytes degenerating	No. of embryos	Grade A and B embryos	Pregnancies
Mature spermatid	1 (D)	3	15	8 (53)	1	0	8 (100)	2 (25)	1 (live birth)
Elongated	1 (C), 5	2	3	3 (100)	0	0	3 (100)	1 (33)	1 (chemical)
Elongating	1 (B), 3, 4	3	18	12 (60)	0	3	12 (100)	4 (33)	2 (ongoing + live birth)
Total		8 (20)	36	23 (64)[a]	1	3	23 (100)	7 (30)[c]	4
ROS	1 (A), 2	32 (80)	260	57 (22)[b]	44	23	49 (86)	7 (12)[d]	1 (live birth)

PN = pronuclei.
Values in parentheses are percentages.
[a–b]χ^2: $P < 0.001$; [c–d]not significant.

Table V. Fertilization rates after injection of round spermatids into oocytes with or without calcium ionophore treatment

Calcium ionophore	No. of cases	No. of oocytes	2PN	1PN	Cleavage rate	Pregnancy
+	9	72	26 (36)[a]	16 (22)	22 (85)	1 (live birth)
–	23	188	31 (16)[b]	26 (14)	27 (87)	0

PN = pronuclei.
[a–b]χ^2: $P < 0.05$.

Discussion

The use of testicular spermatozoa was first advocated by Schoysman *et al.* (1993a,b) in cases of excretory azoospermia. Pregnancies actually occurred after ICSI of testicular spermatozoa from men with obstructive and non-obstructive azoospermia (Schoysman *et al.*, 1993a,b; Devroey *et al.*, 1994, 1995; Silber *et al.*, 1995a; Yemini *et al.*, 1995; Kahraman *et al.*, 1996; Vanderzwalmen *et al.*, 1996).

In cases of defective spermatogenesis including Sertoli cell-only syndrome, maturation arrest, cryptorchid tubular atrophy, post-mumps orchitis or Klinefelter syndrome, the chance of finding spermatozoa in a single biopsy is very limited. According to Tournaye *et al.* (1996a,b) and Silber *et al.* (1995b, 1996) areas of some persisting spermatogenesis occasionally can be detected in the above situations eventually after multiple testicular sampling.

In our TESE programme, we experienced some cases where too few or no spermatozoa at all could be found, even in different samples of testicular tissue, despite the presence of tubules with complete spermatogenesis in a previous biopsy. In these situations, we considered using spermatids, which are the end result of a completed second meiotic division and contain a haploid set of chromosomes. Mammalian embryos formed by microinjections of spermatids proved the potential of spermatids to form male PN, to participate in syngamy (Ogura and Yanagimachi 1993), to develop beyond the 2-cell stage and to produce live offspring (Ogura *et al.*, 1994; Sofikitis *et al.*, 1994, 1996a; Kimura and Yanagimachi, 1995b; Ogura and Yanagimachi, 1995).

In 1994, we reported successful fertilization of a human oocyte with an elongated spermatid, obtained by open testicular biopsy in a patient with testicular tubular atrophy, where too few spermatozoa were recovered to inject all obtained oocytes (Vanderzwalmen *et al.*, 1995a). Fishel *et al.* (1995) later

reported a pregnancy after intracytoplasmic injection of an elongated spermatid instead of morphologically abnormal spermatozoa. Tesarik *et al.* (1995, 1996) reported the birth of two healthy babies after injection of ROS found in the ejaculates of patients with NOA who had produced few spermatozoa in their ejaculates a few weeks prior to the procedure. We have so far achieved five pregnancies. Three live births of healthy babies were obtained after injection of ROS, elongating and mature spermatids, and one ongoing pregnancy is 4 months ongoing after elongating spermatid injection. The fifth case miscarriaged at 4 weeks after elongated cell injection.

Evidently, chromosomes of spermatids are potentially capable of pairing with chromosomes of oocytes and participating in syngamy and full embryonic development. This suggests that structural modification and biochemical maturation of spermatozoa is not required for embryonic development.

Compared with our TESE–ICSI results, the fertilization and conception rates after spermatid injection are dramatically lower, owing to one or more of the following: (i) the severity of the testicular pathology and the gaps in our actual knowledge of such pathology; (ii) the type of injected cells and identification of ROS; (iii) deficiency in the activation process of the oocytes; and (iv) delay in appearance of the 2PN stage.

Severity of pathology

Complete absence of spermatozoa in the testes at the time of the TESE–ICSI attempts is more common in the NOA (27 of 122) group as compared with the OA (three of 115) group, reflecting, in the NOA group, deterioration of spermatogenesis with absolute inability to produce mature spermatozoa or, at best, production of some spermatozoa in few seminiferous tubules. However, the localization of residual spermatogenic foci in the testis is difficult and unpredictable.

Acceptable fertilization rates after spermatid injection were obtained when spermatids came from a testis showing some spermatozoa in a previous positive biopsy. In these cases, higher chances of success were obtained in the OA and in the NOA groups when >20% of tubules demonstrated sperm formation.

It has been suggested that ROS may be the treatment of choice for patients with maturation arrest at the spermatid stage (Edwards *et al.*, 1994; Sofikitis *et al.*, 1994) and even that it could be a possible mode of treatment for men with spermatogenic arrest at any stage of spermeogenesis. Our contrasting observations demonstrate that for patients with complete spermatogenesis arrest, fertilization rates are extremely poor as compared to the groups with previous positive biopsies. In the latter group, persistence of active and complete spermatogenesis in even a few tubules allows the production of mature spermatozoa that are probably lost by phagocytosis before reaching the vas deferens (Schoysman and van de Casseye, 1995). It is not yet clear why the different histopathological conditions have such a major effect on the outcome of microinjection.

The low fertilization rates observed in patients with very severe defects of spermatogenesis may reflect the inability of ROS originating from such severely defective testes to achieve fertilization. It is possible that these ROS may not be wholly identical to those obtained from the testes with focal complete spermatogenesis. We suggest that the disappearance of complete spermatogenesis in all the testicular tubules, affecting negatively the quality of spermatid maturation, may have a genetic cause. For example, usually arrest of spermatogenesis can occur at the spermatid stage in cases of Y-chromosome deletions and Y-autosome translocations (Chandley, 1995). In such very severe situations, it is possible that ROS are in the final stage of gamete development, hampering the competence of the germ cell to reach the zygote stage. In these cases, we must not forget that the risk of genetic transmission of such deletions is not negligible after ROS injection treatment.

In non-genetic causes (environmental effect, physical trauma, orchitis, varicocele, cryptorchidism, etc.), a linear reduction in numbers of the spermatogenic cells at all stages of development is observed. The patient produces occasional mature spermatozoa in residual spermatogenic foci. The spermatids that are present in the biopsy are probably normal and have a higher capacity to fertilize. Severe testicular damage may result not only in quantitative alterations in spermatogenesis but also may have consequences on the quality (i.e. reproductive capacity) of the generated ROS.

However, in a recent study Sofikitis *et al.* (1996b) artificially induced testicular damage and found a detrimental effect of varicocele on the fertilizing and developmental potential of ROS in the rabbit.

To our knowledge, as in our experience, pregnancies and births of healthy babies have been observed in patients where some spermatozoa had been previously identified, either in the ejaculate or the testis (Tesarik *et al.*, 1995). In cases of ROS retrieved from patients with a history of spermatogenetic block at the ROS stage, the pregnancies obtained resulted in abortion, increasing the suspicions of a genetic factor (Hannay, 1995).

It is currently impossible to distinguish between live and dead cells without staining. We also do not know how to differentiate genomically normal from abnormal spermatids without destroying the cell (Ogura and Yanagimachi, 1995). Edwards *et al.* (1994) suggested that isolation and culture of pre-spermatid cells could be a solution for the above-defined group of patients if the cause of spermatogenic arrest proved to be non-genetic. Rassoulzadegan *et al.* (1993) reported in-vitro transmeiotic differentiation of male germ cells. Goto *et al.* (1996) recently showed that spermatids obtained after in-vitro division of secondary spermatocytes and injected into the cytoplasm of bovine oocytes can promote development to the blastocyst stage. Another possibility exists in the use of nuclei of secondary spermatocytes in mice, as recognized by Kimura and Yanagimachi (1995a).

Type of injected cells and identification of ROS

Fertilization is affected by the type of male germ cells (round, elongating, elongated spermatids) found in biopsies and injected into oocytes. We have reported results where most injections were performed with round, instead of elongating, elongated or mature spermatids (87% with ROS, 13% with ELS). Figure 1 shows the evolution of a haploid cell during spermatogenesis in a testis exhibiting >50% of tubules with

complete spermatogenesis. But in our clinical application, when we were dealing with severe secretory azoospermia, we rarely encountered cells with the same morphology as those shown in Figure 1.

The presence of these different cell types depended on the severity of pathology. In spite of the small group of patients, we observed more frequently ELS in the group of failed surgery (one case of two) and in the NOA group where spermatozoa were observed in 20–50% of the tubules (two cases of four), than in groups with severely limited spermatogenesis (one of 12). Of nine cases of full maturation arrest, so-called Sertoli cell-only syndrome, ROS were found in seven testicular biopsies. Isolation and handling of ROS are critical for these cases since they often present as cells mostly arrested at the cap phase.

In our study, the majority of the cells that we found after some hours of examination of the testicular tissue were only ROS, whereas Silber *et al.* (1996) did not find ROS in the absence of ELS. They postulated that failure of meiosis could explain a maturation arrest and not a problem in the development of the haploid cell from ROS to ELS.

Although Tesarik *et al.* (1996) reported successful pregnancies using ROS from the ejaculate, a great majority of the spermatids were elongated. A month earlier, the husbands had produced samples showing a few spermatozoa in the pellet after whole ejaculate centrifugation, suggesting persistent active spermatogenesis. When this occurs, morphologically different stages of spermatids can be recovered from either testicular biopsies or from ejaculates.

ELS should be the more suitable for injection for the following reasons. Firstly, our results show higher fertilization, good quality embryos and pregnancy rates after injection of elongating and elongated spermatids. The pregnancy rate after ROS injection is extremely poor as compared with ELS injection. If the number of embryos of good quality selected for transfer were low, this could be due to the low number of good quality embryos available for transfer. Janny and Ménézo (1994) identified a strong paternal effect on embryo development and blastocyst formation. Since we transferred all the embryos, no supernumerary embryos were available for further in-vitro culture up to the morula–blastocyst stage. Therefore we cannot yet confirm the hypothesis of complete embryo blockage. However, it is possible that defects at the DNA level prevent ROS from completing embryogenesis.

Simerly *et al.* (1995) reported the paternal inheritance of the human centrosome. It is therefore possible, according to Asch *et al*. (1995), that abnormal centrosome and consequently abnormal spindle formation can explain an arrest during mitotic cell cycle progression and anomalies arising in embryos after the use of ROS. For such reasons, the normality of the centrosome by ROS needs to be ascertained (Fishel *et al.*, 1996).

The existence of a cytoplasmic layer surrounding the ROS nucleus may impede its transformation into the male PN. Different authors (Ogura and Yanagimachi, 1993; Kimura and Yanagimachi, 1995b; Sofikitis *et al.*, 1996a; Yamanaka *et al.*, 1997) have suggested that the fertilization rate after cytoplasmic injection of intact ROS in rodents is low, as compared with the injection of ROSNI. Moreover, injection of ROS demands a larger diameter pipette than that used for nuclei injection with a consequently higher degeneration rate of oocytes.

Secondly, since the ELS are in the final stages of spermatogenesis, the histone–protamine transition and nuclear DNA processing have already begun and more gene transcription steps are completed, keeping in mind that DNA methylation of mouse male germ cells continues during the transit of the spermatozoa in the epididymis (Ariel *et al.*, 1994). In mice, remethylation is part of the process of sperm maturation which occurs in the epididymis.

Thirdly, the ability to activate oocytes may be lower with ROS than after injection of ELS. The oocyte-activating factor (OAF) oscillin (Parrington *et al.*, 1996) seems to be expressed during the transformation of ROS to ELS and finally to spermatozoa.

Fourthly, morphologically ELS are more easy to recognize than ROS cells. Even under the Hoffman modulation contrast microscope, a clear identification of ROS remains difficult. The criteria that we use for distinguishing ROS from other round cell types are quite similar to those of Tesarik and Mendoza (1996a). Through the Hoffman differential contrast microscope, ROS were isolated according to their shape (round) and size (~8 μm). Rotating and observing the ROS, we tried to ascertain the presence of a developing acrosomal structure as a bright spot adjacent to the spermatid nucleus.

The observation of a round nucleus, regular zone of cytoplasm surrounding it and a developing acrosome structure was more frequently visualized in patients with residual spermatogenic foci in the testes (Figure 1). But in cases of severe testicular dysfunction (very few tubules with spermatozoa, or maturation arrest at the ROS level), the identification of ROS is very hard, the qualitative criteria being more difficult to recognize. Indeed, in such cases, the nucleus is rarely visible and a slight dark dot or a slight swelling are the only useful parameters. As reported by Yamanaka *et al.* (1997) such qualitative criteria are highly susceptible to intra- and extra-observer variations. Therefore, in order to ascertain that our criteria used for the identification of ROS by standard microscopic techniques are useful, there is a need for confirmation by other methods.

Recently, Yamanaka *et al.* (1997) applied quantitative criteria for the characterization of the ROS that they selected on morphometric parameters by computer-assisted image analysis. In order to ascertain that the cells were ROS, they processed one sample for transmission electron microscopy and another for confocal scanning laser microscopy. Since such evaluation techniques are not available in the majority of IVF laboratories, a more simple method for confirming ROS selection by qualitative criteria was recently proposed by Mendoza and Tesarik (1996). They evaluated the occurrence of ROS in the ejaculate of men suffering from NOA by three different staining methods: Papanicolaou, fluorescein-labelled *Pisum sativum* agglutinin binding and antiacrosin antiserum immunolabelling.

As mentioned by Yamanaka *et al.* (1997), it is clear that the methodology for ROS isolation is a major factor affecting the outcome of spermatid injection. The evaluation of ROS

by confocal scanning laser microscopy is a good method because the cells remain suitable for injections. However, this technique is expensive and cannot be introduced in all laboratories before the spermatid injection technique has definitely proven its value.

During spermatogenesis, no cell division is involved; the process is a metamorphosis, converting conventional cells to highly organized motile structures. Earlier studies, using ultrastructural and cytochemical methods, clarified the cytological changes characterizing spermatogenesis. Some authors adopted a classification according to a series of developmental steps (six or eight) involving different cellular organelles (acrosome swelling, nuclear changes, development of the tail, cytoplasm reorganization). Unfortunately, '*in situ*' under the Hoffman modulation contrast microscope, it is more difficult to make a strict distinction between the different stages involving the beginning of spermatogenesis, as described by Clermont (1963) and Holstein (1976).

Those using spermatids mainly for clinical use need simple criteria, based on size and morphological aspects, for selecting the cells. In pathological cases, different cell shapes are even more difficult to define. It is also difficult to distinguish 'mature spermatids' that are trapped between the Sertoli cells at the level of the lumen from normal-looking spermatozoa. In fact the mature spermatid with a moving tail, just before its delivery from the germinal epithelium, may resemble a fully developed spermatozoon. We observed such mature spermatids in crude preparations after separating the testicular tissue with needles. After a vortexing step, the majority of them are detached and can be used with success in a conventional ICSI–TESE programme.

Need of oocyte activation?

After ICSI with mature spermatozoa, oocyte activation is an essential step and spermatozoa activated the majority of the oocytes by releasing a cytosolic oocyte activating factor (OAF), oscillin (Parrington *et al.*, 1996).

At the time of our spermatid injections in October 1995 we assumed that, unlike spermatozoa, ROS may lack an active cytosolic factor and that therefore oocytes injected with ROS remained inactivated. Data presented by Kimura and Yanagimachi (1995b) suggested that in mouse ROS the OAF is present and could sensitize Ca^{2+} oscillations but not induce them, so that electrical stimulation of the oocytes was necessary for zygote development. Sofikitis *et al.* (1996a) also reported in the rabbit the beneficial effects on fertilization and embryonic development of electrical oocyte stimulation preceding ROSNI injection.

Therefore, since our fertilization rate following ROS injection was initially low, experiments were conducted to determine whether oocytes should be incubated in the presence of an artificial OAF such as ionophore. After using this pharmacological compound, higher fertilization rates were observed and birth of one healthy baby ensued. Hoshi *et al.* (1995) also activated microinjected human oocytes with A23187 and achieved an ongoing pregnancy and live birth of a healthy baby. Meanwhile, however, Souza *et al.* (1996) showed that in human ROS the activity of the OAF was sufficient to induce the Ca^{2+} oscillation mechanism in the oocyte. They showed that the activity of this factor develops between the secondary spermatocyte and the ROS stage. Yanamaka *et al.* (1997) have also provided strong evidence that human OAF has already appeared or been primed by the first stage of spermatogenesis. They observed that 95% of the injected oocytes showed activation after the injection of ROSNI without any electrical or mechanical ooplasmic prestimulation.

We suggest that the cytoplasm of the ROS from severe pathological cases is not mature enough as compared with its nucleus, and that perhaps a deficiency in OAF results in a lack of Ca^{2+} oscillation response.

It has been demonstrated that, in the absence of oocyte activation, the nucleus of the spermatid is exposed to metaphase-promoting factor and cytostatic factor; these may drive the ROSNI in G1 phase prematurely to metaphase, since the spermatid DNA devoid of protamine is not easily protected against such factors (Fishel *et al.*, 1996; Tesarik, 1996). Therefore, it is logical that artificial activation of the oocyte several hours before the injection should be the best solution.

However, at the time we started to induce artificial oocyte activation, we activated the oocytes 15–20 min after the ROS injection, for two reasons. Firstly, artificial induction of oocyte activation with calcium ionophore triggers Ca^{2+} oscillations in oocytes that have been previously injected with spermatozoa (Tesarik and Testart, 1994) and consequently fertilization rates were increased after ICSI (Tesarik and Sousa, 1995). Secondly, Tesarik and Sousa (1995) reported that vigorous aspiration of the cytoplasm before sperm expulsion produces a massive Ca^{2+} flux into the oocyte which acts as a booster for oocyte activation. The delivery of an oocyte-activation signal leads to the inhibition of the metaphase-promoting and cytostatic factors. Such conditions probably help to avoid premature chromosome condensation in the spermatid nucleus. It seems also that the time of activation of the oocyte is not of great importance because the ROS and oocyte being out of phase does not affect the outcome of ROS injection (Fishel *et al.*, 1995, 1996; Tesarik *et al.*, 1995, 1996; Kimura and Yanagimachi, 1995a).

Delay in appearance of 2PN stage

In our centre, fertilization was generally evaluated 20 h after microinjection. According to Tesarik and Mendoza (1996a), the 2PN stage can be easily missed if evaluation is performed later than 10–12 h after injection. Sofikitis *et al.* (1994) using rabbit, and Ogura and Yanagimachi (1993) using hamster, noted that PN formation after ROSNI is faster as compared to classical ICSI. The earlier male PN formation may be due to the decondensed state of the spermatids. Their nuclei are composed primarily of a protein, histone, which contains few disulphide bonds. Perreault *et al.* (1987) showed that the timing of sperm nuclear decondensation and male PN formation is directly related to the sperm nuclear disulphide bond contents. Consequently, the percentage of oocytes exhibiting 1PN 20 h after ROSNI is relatively higher than in those injected with mature spermatozoa (5 versus 24%). The size of the PN was in some cases larger than after classical ICSI. Tesarik and Mendoza (1996a) showed that a single syngamy

nucleus, larger than a PN, was observed in most of the zygotes 16–18 h after spermatid injection. This lower fertilization rate observed by us could be due to confusion of such diploid nucleus with a haploid female PN. According to Tesarik and Mendoza (1996b), the number of nucleolar precursors, which is 2-fold higher in the diploid PN, could account for the difference between the two types of nuclei. An additional explanation may be that some of the oocytes injected with ROS have been activated but not fertilized.

Our results, as well as other published data (Fishel *et al.*, 1995; Tesarik *et al.*, 1995, 1996; Yamanaka *et al.*, 1997) with the use of spermatids for microinjection of eggs, indicate that such cells can provide the paternally imprinted genes needed for embryonic development. Gamete imprinting of mouse (Ogura *et al.*, 1993,1994; Kimura and Yanagimachi, 1995a,b) and human (Fishel *et al.*, 1995, 1996; Tesarik and Mendoza, 1996a,b) spermatogenic cells is already completed either in the testis before the second meiotic division, or within the cytoplasm of the mature oocyte after spermatid injection**.** The excellent health of all of the offspring and their fertility potential support our suggestion that the nucleus of the ROS is genetically ready to participate in a normal fertilization process.

It remains mandatory that we should make careful investigations concerning the gene expression and genomic imprinting when using pre-sperm cells in clinical application. After erasure of the previous imprint probably in the primordial germ cells or replicating spermatogonia, it seems that re-establishment of the imprint must begin at some point prior to the pachytene stage of meiosis and consolidation of the imprint may well be ongoing in the spermeogenesis step (Tycko, 1997). A better knowledge of the different imprinted genes, of their consolidation and the mechanism of reactivation of normally silent alleles could reduce epigenetic risk factors which might turn out to be involved. It has been advocated by Jegou (1992) that experimental work on mice could answer these questions, but this requires animal models that can mimic human infertility with precision and will anyway not offer a complete safety for further work in the human.

At the present state of knowledge, the spermatid option seems more favourable for azoospermic men who with proven capacity to produce albeit only a few spermatozoa previously. But in severe pathological cases, the usefulness of ROS in ICSI remains to be proved on a large scale. The risk of genetic transmission of Y-chromosome deletions and of genomic imprinting anomalies should not be overlooked and care should be taken to avoid dramatic consequences of such pathologies.

In order to obtain the full range of clinical benefits from this procedure, more intensive work is needed to improve selection and handling of cells and to ascertain genomic imprinting and gene expression necessary for embryonic and post-embryonic development. Therefore, when using immature cells for conception, screening of patients is indicated. Clearly this goes well beyond a simple karyotype and demands further work on the genes of the Y chromosome. This will obviously be a target for further development in this area of the field of male infertility. Further research is needed to evaluate the required culture conditions to induce progression of the ROS into a more elongated stage.

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Received on October 25 1996; accepted on April 9, 1997