

Fertilization with human testicular spermatids: four successful pregnancies

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Between July 1995 and May 1996, 36 patients with non-obstructive azoospermia of secretory origin underwent intracytoplasmic injection of spermatids. A previous histological biopsy was performed on all patients: 15 had spermatogenic arrest, a further 13 had Sertoli cell-only syndrome, and the remaining eight had post-cryptorchidism tubal atrophy. The ejaculate was duly examined and a complete absence of spermatozoa and spermatids was confirmed, with only bacteria and debris being found. Testicular sperm extraction (TESE) was then performed. In 19 out of 36 cases round spermatids only were found, while elongated spermatids were found in the remaining 17. Both round and elongated spermatids were isolated and used for injection. A total of 135 oocytes at metaphase II were recovered from 19 partners and injected with round spermatids, while 123 mature oocytes from 17 partners were injected with elongated spermatids. The number of oocytes fertilized, as judged by the presence of two pronuclei, was 75 (55.5%) and 71 (57.7%) respectively. By 34 h after injection, the number of embryos which had cleaved to the 2-cell stage was 56 (74.6%) with round spermatids and 55 (77.4%) with elongated spermatids. All cleaved embryos were transferred into the uterus of the partners. Clinical pregnancies were established in two cases of round spermatid cycles (10.5%) (both are still ongoing), and three cases of elongated spermatid cycles (17.6%) (two are still ongoing; one was lost after 8 weeks of gestation). Chromosomal analysis showed that all fetuses had a normal karyotype (three male and one female) with no chromosomal abnormalities.

Key words: intracytoplasmic spermatid injection/non-obstructive azoospermia/spermatogenic arrest

Introduction

The advances made in the research into human gamete biology and in specialized techniques have caused an explosion in the use of micromanipulation for assisted fertilization in couples suffering from severe male infertility. Previously, procedures such as partial zona dissection (PZD) (Malter and Cohen, 1989) and subzonal insemination (SUZI) (Fishel *et al.*, 1990, 1992) were used to achieve fertilization and pregnancy in such couples. Despite the successful applications of these techniques

in terms of pregnancy rates, the results were very poor. The introduction of intracytoplasmic sperm injection (ICSI) revolutionized the treatment of severe forms of male infertility (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993). In fact, ICSI is widely applied in the world's major in-vitro fertilization (IVF) centres to treat couples with severe male infertility and even those with azoospermia (Silber *et al.*, 1994, 1995a).

Azoospermia is a phenomenon currently encountered in many infertile men. This condition is caused generally either by obstruction of the male genital tract or by spermatogenic dysfunction. Infertility of the former can be treated surgically, by retrieving spermatozoa from the epididymis or directly from the testis. The first successful pregnancies using epididymal spermatozoa in conjunction with standard IVF were reported by Temple-Smith *et al.* (1985) and by Silber *et al.* (1988). However, with conventional IVF, fertilization and pregnancy rates have remained low (Silber *et al.*, 1990; Baldou *et al.*, 1991; Hirsh *et al.*, 1993; Hovatta and Von Smitten, 1993). More recently the use of epididymal spermatozoa, in combination with ICSI, has given higher fertilization and pregnancy rates when compared with IVF (Silber *et al.*, 1994, 1995a; Tournaye *et al.*, 1994). When no epididymal spermatozoa were found, testicular spermatozoa were used for IVF although fertilization was still very low (Craft *et al.*, 1993; Schoysman *et al.*, 1993a,b). On the other hand, the use of testicular spermatozoa in conjunction with ICSI has proved to be a much more successful technique (Silber *et al.*, 1994, 1995a).

For many azoospermic patients with secretory dysfunction of spermatogenesis, a new era of treatment began following the most recent experiments that used spermatids for injections into oocytes (Edwards *et al.*, 1994). Animal studies have shown that the nuclei of round spermatids from hamster and mice are able to duplicate DNA and participate in syngamy when incorporated into oocytes either microsurgically (Ogura and Yanagimachi, 1993) or by electrofusion (Ogura *et al.*, 1993). Ogura *et al.* (1994) reported the first successful birth of normal young mice after electrofusion of oocytes with round spermatids. Sofikitis *et al.* (1994a,b) also reported the achievement of three pregnancies after the injection of round spermatids into rabbit oocytes. In a recent study (Sofikitis *et al.*, 1996), they demonstrated that a combination of electrical stimulation and ooplasmic mechanical manipulation during round spermatid injection is a more efficient procedure than mechanical stimulation alone for activation of rabbit oocytes, fertilization and subsequent embryo development. These results have shown the potential capacity of round spermatids to achieve fertilization and the birth of offspring, and suggested a new approach for the treatment of azoospermic cases with spermatogenic dysfunction (Edwards *et al.*, 1994).

Table I. Histological diagnosis and sperm analysis of patients

Histological diagnosis	No. patients	No. round spermatids	No. elongated spermatids
Spermatogenic arrest	15	9	6
Sertoli cell-only syndrome	13	7	6
Post-cryptorchidism tubal atrophy	8	3	5

Tesarik *et al.* (1995) reported a successful pregnancy following the injection of round spermatids, isolated from the ejaculate of patients with non-obstructive azoospermia, into human oocytes. Moreover, Vanderzwalmen *et al.* (1995) demonstrated that late-stage spermatids are also able to achieve fertilization and embryo cleavage when injected into oocytes. A successful pregnancy using late-stage spermatids for intracytoplasmic injection was also reported by Fishel *et al.* (1995). All these results have encouraged us to introduce into our ICSI programme the treatment of azoospermic patients using spermatids that have been surgically retrieved from the testis. In this paper we report on the treatment of azoospermic patients in which round and elongated spermatids were used for injection into human oocytes, and the resulting pregnancies.

Materials and methods

Patient selection

Between July 1995 and May 1996, 36 patients with non-obstructive azoospermia were entered into our spermatid intracytoplasmic injection programme. A previous extensive examination of the ejaculate revealed the complete absence of sperm cells. A histological biopsy showed the presence of only round spermatids in 19 patients and elongated spermatids in 17 patients. All patients had high concentrations of follicle stimulating hormone (FSH) with a mean value of >22 IU/l and small testicular volumes <15 ml. The histological diagnosis and sperm analysis of these patients is described in Table I.

Testicular biopsy preparation and isolation of spermatids

Testicular samples ($n = 5-10$, each one 6–9 mm³) were surgically removed from different areas of the testicle, collected in a large volume of Earle's balanced salt solution (EBSS) and rinsed twice. The testicular samples were minced into small pieces using a pair of scissors. The suspension was then collected in a Jar tube and pulsed using a vortex. This is an easy method of causing cell disaggregation without damaging the germ cells. Aliquots of the suspension were then incubated at room temperature for 5 min in drops of culture medium (Medicult) under oil in Petri dishes (6–8 drops per dish). The drops were then examined under an inverted microscope for the presence of spermatozoa or spermatids (Figure 1). Both round and elongated spermatids were found in all patients, but no spermatozoa were present. The round spermatids were easily distinguishable by their small size, the round nucleus and the presence of an acrosomic granule adjacent to the nucleus (Figure 1). The spermatids were isolated using an injection pipette (7 and 9 mm outer and inner diameters respectively) and collected in culture medium (Medicult).

ICSI procedure

The injection of spermatids into the oocyte cytoplasm was performed using the method of Palermo *et al.* (1992). Since spermatids are

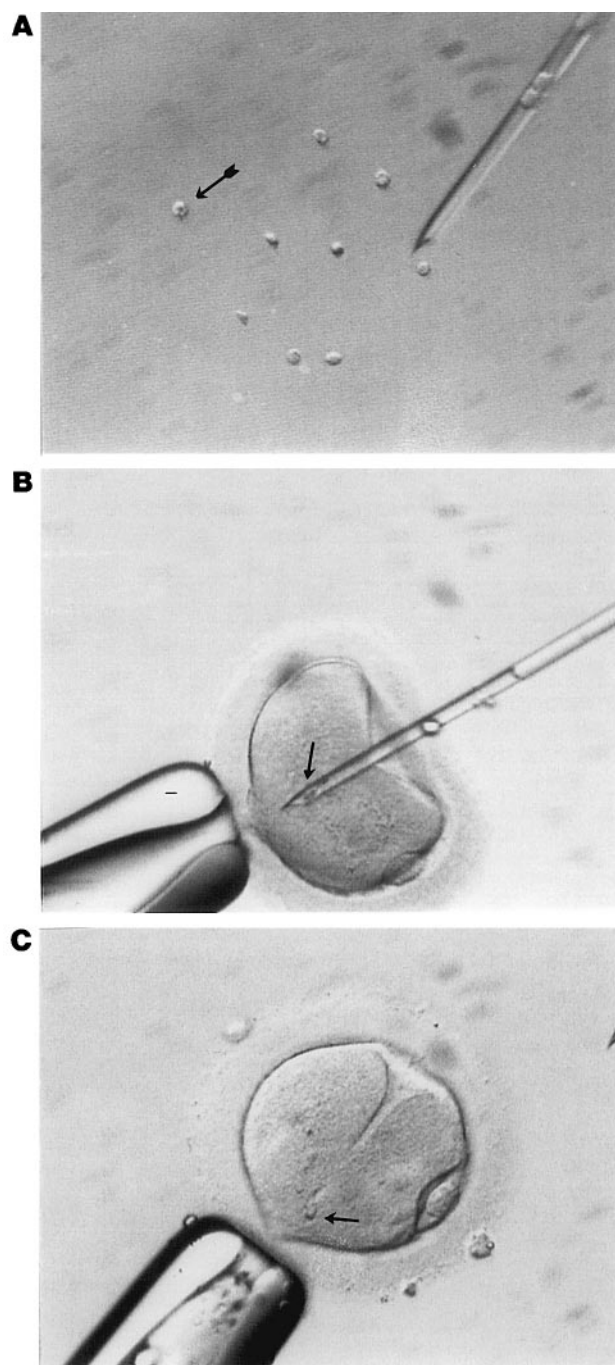


Figure 1. Injection procedure: (A) a round spermatid is aspirated into the injection needle; (B) the round spermatid is then expelled into the cytoplasm of a metaphase II oocyte; (C) following deposition into the oocyte cytoplasm, the round spermatid is slightly deformed, due to its passage through the injection needle.

slightly larger than normal spermatozoa, some modifications of the injection needle were necessary to adapt it for these cells. The injection needle used was of 6–7 mm inner and 8–9 mm outer diameter. The polar body was at 6 or 12 o'clock and the point of injection at 3 o'clock. Oocyte activation was facilitated by a vigorous cytoplasmic aspiration during the injection (Figure 1). The formation of zygotes was confirmed 12 h after injection by the presence of two pronuclei. By 18 h, a single slightly larger syngamous pronucleus was observed and by 32–34 h the zygotes developed into 2-cell stage embryos (Figure 2).

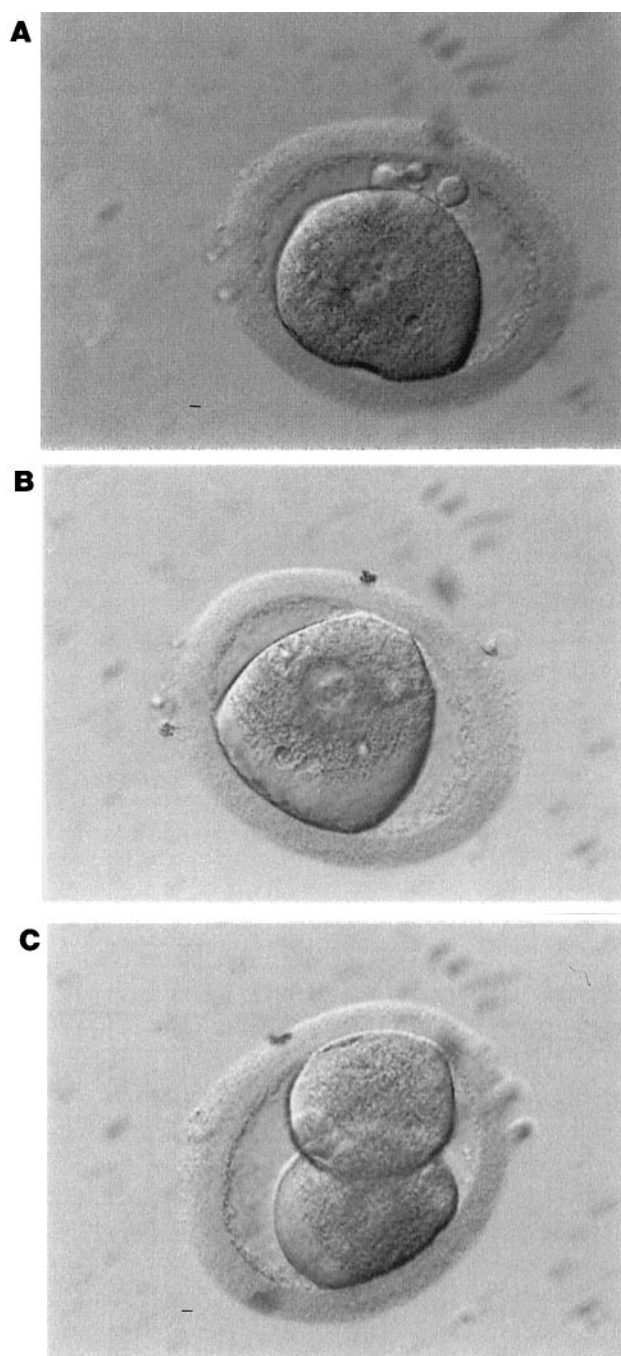


Figure 2. Fertilization and embryo development following intracytoplasmic injection of spermatids. (A) Two pronuclear stage 12 h following injection; (B) fusion of the 2 pronuclei into a large syngamous nucleus by 18 h; (C) embryo development to the 2-cell stage by 36 h after spermatid injection.

Results

A total of 36 patients with non-obstructive azoospermia underwent testicular sperm extraction (TESE). A total of 135 oocytes at metaphase II were recovered from 19 patients and injected with round spermatids, while 123 mature oocytes recovered from 17 patients were injected with elongated spermatids. The fertilization rates observed 12 h after injection were 75/135 (55.5%) in the round spermatid cycles and 71/123 (57.7%) in the elongated spermatid cycles. The percentages of cleaved embryos 34 h after injection were 56/75 (74.6%) and 55/71

Table II. Fertilization, embryo development and pregnancies after injection of round and elongated spermatids into human oocytes. Figures in parentheses are percentages

	Round spermatids	Elongated spermatids
Patients	19	17
Oocytes retrieved	187	172
Metaphase II oocytes	135	123
Injected oocytes	135	123
1PN	19 (14)	16 (13)
2PN	75 (55.5)	71 (57.7)
3PN		
Cleaved embryos ^a	56 (74.6)	55 (77.4)
Embryo quality (%) ^b		
I	36 (64.2)	37 (67.2)
II	20 (35.7)	18 (32.7)
III		
IV		
Embryos transferred	56	55
Mean no. embryos transferred per patient	2.9	3.2
Pregnancies ^{a,c}	2 (10.5)	3 (17.6)
Ongoing pregnancies	2	2
Miscarriages	1 (5.8)	

^aThe results of the two groups were not significantly different.

^bEmbryo quality was detected on the basis of: regularity and symmetry of the blastomeres, fragmentation and cytoplasmic homogeneity. Grade I = regular size blastomeres without fragmentation and clear cytoplasm; grade II = regular size blastomeres with <10% fragmentation and clear cytoplasm; grade III = irregular size blastomeres with >20% fragmentation and grainy cytoplasm; grade IV = irregular size blastomeres with >50% cytoplasmic fragmentation and dark and grainy cytoplasm.

^cDefined by the presence of a fetal heart beat.

(77.4%) respectively. The mean number of embryos transferred was 2.9 in the first group and 3.2 in the second group. Pregnancies (as defined by the presence of a heart beat) were established in two (10.5%) women from the round spermatid group and three women (17.6%) from the elongated spermatid group. In the two groups, four pregnancies are ongoing while in the elongated spermatid group one pregnancy was lost (see Table II). Chromosomal analysis showed that all fetuses had a normal karyotype with no genetic abnormalities.

Discussion

The introduction of intracytoplasmic injection of spermatids into oocytes has been successfully used to treat patients with non-obstructive azoospermia who were previously excluded from assisted reproduction techniques. Round and elongated spermatids isolated either from the ejaculate (Tesarik *et al.*, 1995, 1996; Tesarik and Mendoza, 1996), or directly from testicular tissue (Fishel *et al.*, 1995; Chen *et al.*, 1996), have been used successfully to treat azoospermic patients with primary testicular dysfunction. Even patients with Sertoli cell-only syndrome, when treated with testicular exploration by TESE, are able to produce round, elongated spermatids, and even spermatozoa, to be used for ICSI (Devroey *et al.*, 1995; Silber *et al.*, 1995b). This may be due to the fact that many cases of Sertoli cell-only syndrome have occasional foci of normal seminiferous tubules in which spermatogenesis occurs normally, or foci at which maturation arrest has occurred (Silber *et al.*, 1995b). However, the use of spermatids for assisted fertilization techniques may invoke some important

problems with regard to DNA maturity, genomic imprinting and Y chromosome abnormality that must be considered.

DNA maturity

It has been shown in rats and rams that, during spermiogenesis, the haploid nucleus of the spermatid shows a widely dispersed chromatin with a few heterochromatin clumps and small spherical nucleus. The dispersed chromatin of the round spermatid undergoes a process of DNA maturation during chromatin condensation. Two major modifications take place in the basic nucleoprotein composition of the nucleus: the first involves the replacement of several testis-specific histones, present in spermatocytes and round spermatids, by spermatid-specific basic proteins. This replacement occurs gradually during nuclear elongation and chromatin condensation. In the second spermatid proteins are replaced by one or more basic sperm proteins (protamines), particularly rich in arginine and cysteine. The association of these protamines with the DNA may contribute to the condensation and stabilization of the chromatin, but the exact role of the nucleoprotein in this process remains unclear (Meistrich *et al.*, 1978; Loir and Courtens, 1979; Loir and Lanneau, 1984). These data were confirmed by Grimes (1986) who suggested that the process of histone-protamine transition has hardly begun in the elongated spermatids. Furthermore, as shown by Hecht (1986, 1987, 1988), the protamines are synthesized toward the end of spermiogenesis and the chromatin of the male germ cells undergoes a major shift in function during mid-spermiogenesis, when condensation-related events in the spermatid nucleus terminate transcription. This pause in RNA synthesis is maintained until after fertilization, when the protamines of the male pronucleus are replaced by histones (Hecht and Williams, 1978). These findings could explain the slightly higher fertilization rate using elongated spermatids obtained by Fishel *et al.* (1995) in comparison with our results, but serious investigation is needed to determine at which stage the spermatids have optimal DNA and are suitable for injection.

Genomic imprinting and DNA methylation

The importance of genomic imprinting (reviewed in Fishel *et al.* 1996) in mammalian development was first recognized by Lyon and Glenister (1977). They stated that differential expression of the maternal and paternal chromosomes depends on whether they are inherited from the mother or father. Developmental studies of isoparental mouse embryos have provided the evidence for the differential role of maternal and paternal genomes (Surani, 1984, 1991; Solter, 1988). Gene inactivation experiments have confirmed that imprinted genes regulate embryonic and placental growth and that DNA methylation is a part of the imprinting mechanism (Barlow, 1995). In mammals, DNA methylation is restricted to cytosine, usually in a CpG dinucleotide. It becomes hemimethylated after DNA replication and acts as unique substrate for DNA-methyltransferase (DNMTase) (Razin and Cedar, 1991, 1994). The action of DNA-methyltransferase is restricted to previously methylated sequences and propagates the chromosome-specific methylation pattern to daughter cells. Furthermore, in keeping with the imprinting, genomic maturation patterns are removed

and reset during gametogenesis and in the preimplantation embryo (Brandeis *et al.*, 1993). All imprinted genes contain DNA sequences methylated in a parental-specific manner (Razin and Cedar 1994; Fishel *et al.*, 1996). Abnormality or any alteration of the imprinting process may be lethal to the embryo or cause genetic aberrations in the offspring (Cattanach and Beechey, 1990; Hall, 1990; Cattanach *et al.*, 1992).

For example, aberrant promotor methylation of tumour suppressor genes has now emerged as an epigenetic inactivation pathway contributing to tumour formation. Evidence increasingly supports the notion that ectopic methylation may play a broad role in gene inactivation and mutation in mammals (Oberle *et al.*, 1991; Verdine, 1994). Recent experimental studies have provided evidence that monoallelic expression of several imprinted genes depends on DNA methylation. It has been shown that mice with a deficiency of DNMTase die in the early post-implantation period. In the absence of DNMTase, the monoallelic expression of four imprinted genes (IGF2, IGF2/MPR300; H19, and XIST) is lost in post-implantation embryos because of gametic methylation imprinting and failure of the expected role of methylation in silencing gene expression (Li *et al.*, 1992). Moreover, overexpression of H19 in transgenic mice leads to late gestational death, suggesting that the transcriptional dosage of this gene may have to be regulated carefully (Brunkow and Tilghman, 1991). The maternal duplication in regions containing the *Snrpn* gene (paternal imprinted allele) causes post-natal lethality in mice (Cattanach *et al.*, 1992; Leff *et al.*, 1992). In view of these findings, it could be suspected that the injection of spermatids into oocytes may induce problems in the development of the embryo, and may be linked to the quality of parental DNA. Indeed, careful genetic investigation is still needed.

Y-chromosome deletion

It has been shown that non-obstructive azoospermia and idiopathic oligozoospermia are commonly caused by Sertoli cell-only syndrome or by a maturation arrest of spermatogenesis. These conditions are usually genetically inherited on the long arm of chromosome Y (Silber *et al.*, 1995). Recent studies have demonstrated that ~13% of cases with non-obstructive azoospermia are caused by deletion of a particular region called azoospermia factor (AZF), a gene, or gene complex, normally located on the long arm of Y chromosome (Yq11). The deletion of the AZF region also causes a severe defect in sperm production, resulting in oligozoospermia in ~3–4% infertile men. The nature and the severity of the spermatogenic disorder in any given individual depends upon which genes in the AZF cluster are deleted. Such findings alone are insufficient to assert that the Y-chromosome deletion is the cause of azoospermia, since many Y-DNA sequence variants are polymorphisms, readily passed from one generation to the next, with little or no functional consequence (Nakahori *et al.*, 1994). The cause of azoospermia may be due to a new mutation in the Y-chromosome variants of infertile men, not present in the affected individuals from whom they inherited the Y-chromosome variants (Chandley and Cooke, 1994; Reijo *et al.*, 1996). The promising candidate for deletion of the AZF region is the DAZ gene (Deletion in Azoospermia). This gene

appears to encode an RNA binding motif, as reported by Reijo *et al.* (1995). More recent studies (Reijo *et al.*, 1996) reported that the human DAZ gene is absent in oligozoospermic and azoospermic patients with the Yq deletion. If the absence of DAZ is the cause of oligozoospermia, it cannot be excluded that a point mutation may also cause oligozoospermia. The finding of such a point mutation in oligozoospermia or in azoospermic men could prove that the DAZ gene is the AZF (Reijo *et al.*, 1995, 1996). The latest studies reported the identification of the DAZ gene as the Y-chromosome azoospermia factor. This is expressed exclusively in the testis and has an essential meiotic function in human spermatogenesis. In individuals with the deleted Yq DAZ-containing region, the postmeiotic stages of spermatogenesis are rare or absent. Some affected men display maturation arrest, others have the Sertoli cell-only syndrome. This provides strong support for the hypothesis that the DAZ gene is the AZF. However, further investigation to the function of DAZ might provide further insight into both regulation of the meiotic cell cycle and the physiological basis of a significant determinant of human infertility (Eberhart *et al.*, 1996).

As ICSI is widely used to treat azoospermic men with maturation arrest, the AZF-deletion or DAZ mutation may be transmitted from father to son. A comparative study of fertile and infertile men indicates that mutations in the DAZ gene are extremely frequent, occurring in at least 1 in 8000 men, and that the DAZ deletion causes oligozoospermia, as well as azoospermia (Reijo *et al.*, 1995, 1996).

Both current and previous clinical results (Fishel *et al.*, 1995; Tesarik *et al.*, 1995, 1996) support the biological studies reported by Ogura *et al.* (1994) and Kimura and Yanagimachi (1995a). In more recent works by Kimura and Yanagimachi (1995b), fertilization and normal birth were obtained after electroactivation of injected oocytes with secondary spermatocytes (2N haploid), indicating that essential imprinting occurred at the secondary spermatocyte stage. These studies, reporting birth of normal animal and human offspring after the injection of immature spermatozoa or spermatid nuclei into oocytes, may indicate that gametic imprinting is completed at secondary spermatocyte stage in mice and at the round spermatid stage in humans.

However, in view of the concerns discussed above about genetic aberration related to genomic imprinting and DNA methylation, further studies are needed. On the other hand, the couples included in spermatid injection programs must be checked thoroughly in order to detect the presence of genetic abnormalities responsible for defects in sperm maturation and offspring must be monitored before and after conception. Evaluation of the methylation status of the known imprinted gene must also be extended to the offspring. Since Reijo *et al.* (1996) suggested the possibility that genetic aberration related to Y-chromosome deletion could be transmitted to the sons of azoospermic patients undergoing ICSI treatment, extensive examination and counselling of parents should be a necessary requirement of the treatment. Such counselling should underline the risk of transmitting and developing the new mutation factor in the DAZ gene of the offspring. As the data are still insufficient it is really too early to consider the use of immature

sperm cells as the definitive cure for azoospermia, and further genetic investigations are needed.

References

- Baldou, F., Grillo, J.M., Rossi, D. *et al.* (1991) Epididymal sperm aspiration in conjunction with in-vitro fertilization and embryo transfer in cases with obstructive azoospermia. *Hum. Reprod.*, **6**, 1284–1287.
- Barlow, D.P. (1995) Gametic imprinting in mammals. *Science*, **270**, 1610–1613.
- Brandeis, M., Kafri, T., Ariel, M. *et al.* (1993) The ontogeny of allele-specific methylation associated with imprinted genes in mouse. *EMBO J.*, **12**, 3669–3677.
- Brunkow, M.E. and Tilghman, S.M. (1991) Ectopic expression of the H19 gene in mice causes prenatal lethality. *Genes Dev.*, **5**, 1092–1101.
- Cattanach, B.M. and Beechey, C.V. (1990) Autosomal and X-chromosome imprinting. *Development*, (Suppl.), 63–72.
- Cattanach, B.M., Barr, J.A., Evans, E.P. *et al.* (1992) A candidate mouse model for Prader–Willi syndrome which shows an absence of *Snrpn* expression. *Nature Genet.*, **2**, 270–274.
- Chandley, A.C. and Cooke, H.G. (1994) Human male infertility: Y-linked genes and spermatogenesis. *Hum. Mol. Genet.*, **3**, 1449–1452.
- Chen, S.U., Tsai, T.C., Ho, H.N. *et al.* (1996) Fertilization and embryo cleavage after intracytoplasmic spermatid injection in an obstructive azoospermic patient with defective spermiogenesis. *Fertil. Steril.*, **66**, 157–160.
- Craft, I., Bennett, V. and Nicholson, N. (1993) Fertilizing ability of testicular spermatozoa. *Lancet*, **342**, 864.
- Devroey, P., Liu, J., Nagy, Z. *et al.* (1995) Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum. Reprod.*, **10**, 1457–1460.
- Eberhart, C.G., Maines, J.Z. and Wasserman, S.A. (1996) Meiotic cell cycle requirement for fly homologue of human deleted azoospermia. *Nature*, **381**, 783–785.
- Edwards, R.G., Tarín, J.J., Dean, N. *et al.* (1994) Are spermatid injections into human oocytes now mandatory? *Hum. Reprod.*, **9**, 2217–2219.
- Fishel, S., Aslam, I. and Tesarik, J. (1996) Spermatid conception: a stage too early, or a time out? *Hum. Reprod.*, **11**, 1371–1375.
- Fishel, S., Timsom, J., Lisi, F. and Rinaldi, L. (1992) Evaluation of 225 patients undergoing subzonal insemination for fertilization *in vitro*. *Fertil. Steril.*, **57**, 840–849.
- Fishel, S., Antinori, S., Jackson, P. *et al.* (1990) Twin birth after subzonal insemination. *Lancet*, **342**, 1237–1238.
- Fishel, S., Green, S., Bishop, M. *et al.* (1995) Pregnancy after intracytoplasmic injection of spermatid. *Lancet*, **345**, 1641–1642.
- Grimes, S.R. (1986) Nuclear proteins in spermatogenesis. *Comp. Biochem. Physiol. (B)*, **83**, 495–500.
- Hall, J.G. (1990) Genomic imprinting: review and relevance to human diseases. *Am. J. Hum. Genet.*, **46**, 857–873.
- Hecht, N.B. (1986) Regulation of gene expression during mammalian spermatogenesis. In Rossant, J. and Pedersen, R.A. (eds), *Experimental Approaches to Mammalian Embryonic Development*. Cambridge University Press, Cambridge, pp. 151–193.
- Hecht, N.B. (1987) Gene expression during spermiogenesis. *Ann. N.Y. Acad. Sci.*, **513**, 90–101.
- Hecht, N.B. (1988) Post meiotic gene expression during spermiogenesis. In Hase Hihc, F. (ed.), *Meiotic Inhibition: Molecular Control of Meiosis*. Alan R. Liss, New York, pp. 291–313.
- Hecht, N.B. and Williams, J.L. (1978) Synthesis of RNA by separated heads and tails from bovine spermatozoa. *Biol. Reprod.*, **19**, 573–579.
- Hirsh, A., Montgomery, K., Mohan, P. *et al.* (1993) Fertilization by testicular sperm with standard IVF techniques. *Lancet*, **342**, 1237–1238.
- Hovatta, O. and Von Smitten, K. (1993) Sperm aspiration from vas deferens and in-vitro fertilization in cases of non-treatable anejaculation. *Hum. Reprod.*, **8**, 1689–1691.
- Kimura, Y. and Yanagimachi, R. (1995a) Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development*, **121**, 2397–2405.
- Kimura, Y. and Yanagimachi, R. (1995b) Development of normal mice from oocytes injected with secondary spermatocyte nuclei. *Biol. Reprod.*, **53**, 855–862.
- Leff, S.E., Brannan, C.I., Reed, M.L. *et al.* (1992) Maternal imprinting of the mouse *Snrpn* gene and conserved linkage homology with the human Prader–Willi syndrome region. *Nature Genet.*, **2**, 259–264.

- Li, E., Bestor, T.H. and Jaenisch, R. (1992) Targeted mutation of DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.
- Loir, M. and Courtens, J.L. (1979) Nuclear reorganization in ram spermatids. *J. Ultrastruct. Res.*, **67**, 309–324.
- Loir, M. and Lanneau, M. (1984) Structural function of the basic nuclear proteins in ram spermatids. *J. Ultrastruct. Res.*, **86**, 262–276.
- Lyon, M.F. and Glenister, P.H. (1977) Factors affecting the observed number of young resulting from adjacent-2 disjunction in mice carrying a translocation. *Genet. Res.*, **29**, 83–92.
- Malter, H.E. and Cohen, J. (1989) Partial zona dissection of human oocytes: a nontraumatic method using micromanipulation to assist zona pellucida penetration. *Fertil. Steril.*, **51**, 139–148.
- Meistrich, M.L., Brock, W.A., Grimes, S.R. *et al.* (1978) Nuclear protein transitions during spermatogenesis. *Fed. Proc.*, **37**, 2522–2525.
- Nakahori, Y., Kobayashi, K., Komaki, R. *et al.* (1994) Locus of the candidate gene family for azoospermia factor (YRRM2) is polymorphic with a null allele in Japanese males. *Hum. Mol. Genet.*, **3**, 1709.
- Oberle, I., Rousseau, F., Heitz, D. *et al.* (1991) Instability of a 55-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science*, **252**, 1097–1102.
- Ogura, A. and Yanagimachi, R. (1993) Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. *Biol. Reprod.*, **48**, 219–225.
- Ogura, A., Yanagimachi, R. and Usui, N. (1993) Behaviour of hamster and mouse round spermatid nuclei incorporated into mature oocytes by electrofusion. *Zygote*, **1**, 1–8.
- Ogura, A., Matsuda, J. and Yanagimachi (1994) Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA*, **91**, 7460–7462.
- Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17.
- Razin, A. and Cedar, H. (1991) DNA methylation and gene expression. *Microbiol. Rev.*, **55**, 451–458.
- Razin, A. and Cedar, H. (1994) DNA methylation and genomic imprinting. *Cell*, **77**, 473–476.
- Reijo, R., Lee, T.Y., Salo, P. *et al.* (1995) Diverse spermatogenic defects in human caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nature Genet.*, **10**, 383–393.
- Reijo, R., Algappan, R.K., Patrizio, P. and Page, D.C. (1996) Severe oligozoospermia resulting from deletion of azoospermia factor gene on Y chromosome. *Lancet*, **347**, 1290–1293.
- Schoysman, R., Vanderzwalmen, P., Nijs, M. *et al.* (1993a) Successful fertilization by testicular spermatozoa in an in-vitro fertilization programme. *Hum. Reprod.*, **8**, 1339–1340.
- Schoysman, R., Vanderzwalmen, P., Nijs M. *et al.* (1993b) Pregnancy after fertilization with human testicular spermatozoa. *Lancet*, **342**, 1237.
- Silber, S.J., Balmaceda, J., Borreo, C. *et al.* (1988) Pregnancy with sperm aspiration from proximal head of epididymis: a new treatment for congenital absence of vas deferens. *Fertil. Steril.*, **50**, 525–528.
- Silber, S.J., Ord, T., Balmaceda, J. *et al.* (1990) Congenital absence of vas deferens: the fertilizing capacity of human epididymal sperm. *N. Engl. J. Med.*, **323**, 1788–1792.
- Silber, S.J., Nagy, Z.P., Liu, J. *et al.* (1994) Conventional IVF versus ICSI (intracytoplasmic sperm injection) for patients requiring microsurgical sperm aspiration (MESA). *Hum. Reprod.*, **9**, 1705–1709.
- Silber, S.J., Nagy, Z., Liu, J. *et al.* (1995a) The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility. *Hum. Reprod.*, **10**, 2031–2043.
- Silber, S.J., Van Steirteghem, A.C. and Devroey, P. (1995b) Sertoli cell only revisited. *Hum. Reprod.*, **10**, 1031–1032.
- Sofikitis, N., Zavos, P., Koutselinis, A. *et al.* (1994a) Achievement of pregnancy after injection of round spermatid (RS) nuclei into rabbit oocytes and embryo transfer: a possible mode of treatment for men with spermatogenic arrest at the spermatid stage. *J. Urol.*, **5** (Suppl.), 151.
- Sofikitis, N., Miyagawa, I., Agapitos, E. *et al.* (1994b) Reproductive capacity of the nucleus of male gamete after completion of meiosis. *J. Assist. Reprod. Genet.*, **11**, 335–341.
- Sofikitis, N., Toda, T., Miyagawa, I. *et al.* (1996) Beneficial effects of electrical stimulation before round spermatid nuclei injections into rabbit oocytes on fertilization and subsequent embryonic development. *Fertil. Steril.*, **65**, 176–185.
- Solter, D. (1988) Differential imprinting and expression of maternal and paternal genomes. *Ann. Rev. Genet.*, **22**, 127–146.
- Surani, M.A. (1991) Genomic imprinting: developmental significance and molecular mechanism. *Curr. Opin. Genet. Dev.*, **2**, 241–246.
- Surani, M.A.H., Barton, S.C. and Norris, M.L. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, **308**, 548–550.
- Temple-Smith, P.D., Southwick, C.J. and Yates, C.A. (1985) Human pregnancy by in-vitro fertilization (IVF) using sperm aspirated from epididymis. *J. In Vitro Fertil. Embryo Transfer*, **4**, 298–303.
- Tesarik, J. and Mendoza, C. (1996) Spermatid injection into human oocytes. Laboratory techniques and special features of zygote development. *Hum. Reprod.*, **11**, 772–779.
- Tesarik, J., Mendoza, C. and Testart, J. (1995) Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med.*, **333**, 525.
- Tesarik, J., Rolet, F., Brami, C. *et al.* (1996) Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum. Reprod.*, **11**, 780–783.
- Tournaye, H., Devroey, P., Liu, J. *et al.* (1994) Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of vas deferens. *Fertil. Steril.*, **61**, 1045–1051.
- Vanderzwalmen, P., Lejeune, B., Nijs, M. *et al.* (1995) Fertilization of an oocyte microinseminated with spermatid in an in-vitro fertilization programme. *Hum. Reprod.*, **10**, 502–503.
- Van Steirteghem, A., Nagy, Z., Joris, H. *et al.* (1993) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8**, 1061–1066.
- Verdine, G. (1994) The flip side of DNA methylation. *Cell*, **76**, 197–200.

Received on August 8, 1996; accepted on November 15, 1996