—Review—

Limits to the Clinical Application of ICSI

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Microinsemination is a revolutionary and promising technique for the treatment of severe male infertility, such as severe oligozoospermia, asthenozoospermia, and the case of unexplained unfertilization in which the conventional IVF is ineffective even if semen parameters are normal. Several different microinsemination techniques, such as zona opening [1–3], subzona sperm injection [4, 5], and intracytoplasmic sperm injection (ICSI) [6], have been introduced. ICSI is considered the most effective technique, because ICSI has the highest fertilization rate and can be performed with the smallest number of spermatozoa. In addition, this technique is effective for functional disorders, such as disorders of capacitation, acrosome reaction, and sperm-egg fusion. ICSI was first reported in 1992 [6], and for the above reasons became a powerful fertilization tool in only three or four years [7, 8].

At first, ICSI was indicated for the treatment of severe infertility in which the probability of pregnancy was nonexistent or very law. Its indications were then expanded to include azoospermia, and it has been fairly effective [9–11], but in such cases chromosomal and genetic abnormalities may be passed on to children [12–14]. In addition, it has been clarified that ICSI is not effective in some cases, such as necrozoospermia [15], in which there are no viable spermatozoa; immotile spermatozoa [16], in which viable spermatozoa cannot always be picked up; cases in which spermatozoa have the disorder of oocyte activation factor; and azoospermia, in which mature spermatozoa cannot be found in the testes. The present paper concerns issues that must be considered in the performance of ICSI, as well as limitations to the effectiveness of ICSI.

Received: August 30, 2000
Accepted: September 29, 2000
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Necrozoospermia

In necrozoospermia, every spermatozoa on in semen is found to be dead when analyzed by vital staining (eosin stain [17], FertiLight stain (Molecular Probes, Eugene, OR) [18]). Sperm-injected oocytes are less likely to develop when injected with dead spermatozoa than with motile spermatozoa. The rate of fertilization for ICSI ranges from 0 to 11% [15]. Since 1992, 659 patients have been treated for infertility, a total of 1,317 treatment cycles at our hospital, and there had been six treatment cycles for necrozoospermia (3 patients). Among these cases, the rate of fertilization was 33.3% (5/15 oocytes), but there were no pregnancies. Since the fertilization rate was very low, dead (nonviable) spermatozoa may reduce the oocyte activating factor or have fragmented nuclear DNA or have chromosome disintegration [19]. Even when the sperm factor is injured and nuclear DNA is damaged, fertilization is possible with ICSI, but we cannot get well developed embryos from these spermatozoa, so that there is no hope of pregnancy. In humans, the development of fertilized oocytes to 4-cell stage embryos is controlled by the maternal genes rather than the paternal ones [20], so that fertilized oocytes can develop to this stage regardless of the state of the paternal DNA. The results of our basic studies on mice have shown that, when ICSI is performed with killed spermatozoa, the longer the interval of time from the killing to ICSI is, the lower will be the fertilization and development rates of the oocytes. The rate of development to the blastocyst stage was decreased in a couple of hours from spermatozoa killing. Human sperm nuclei are less stable than mice sperm nuclei, because even nucleoproteins in ejaculated spermatozoa are not completely converted from histone to protamine [21]. In addition, in necrozoospermia, all spermatozoa have been dead for an extended period of time, so when ICSI is performed with these cells, there is only a remote possibility of embryogenesis and preg-

nancy. In such cases, ICSI should be performed by testicular sperm extraction (TESE) [22].

Immotile Spermatozoa

With immotile spermatozoa, the motility of spermatozoa in semen is 0%, but this condition differs from necrozoospermia in that both viable and dead spermatozoa are found in semen. Of the 1317 treatment cycles (659 patients) at our hospital, there have been 42 treatment cycles with immotile spermatozoa (30 patients). The rate of fertilization was about 50%, but there were no pregnancies. The ratio of viable to dead spermatozoa in semen could be determined by vital staining, and the ratio of viable spermatozoa had a wide range, the lowest of which was 1% in our cases. The rate of pregnancy for ICSI with spermatozoa collected from patients with immotile spermatozoa was lower than that for ICSI with motile spermatozoa. This was due to the fact that dead spermatozoa could be used in ICSI. Viable spermatozoa can be identified by the abovementioned sperm staining technique, but spermatozoa that have been stained by this technique are not appropriate for injection due to safety concerns. Therefore, the hypoosmotic swelling test (HOST) was devised as a means of selecting viable spermatozoa[23]. In HOST, ballooning of the tail of spermatozoa in a hypoosmotic solution is morphologically observed in order to assess the function of the cell membrane, that is, the fertilizing ability of spermatozoa. When viable spermatozoa are placed in a hypoosmotic solution, their tails become noticeably swollen as a sign of properly functioning membranes. Spermatozoa with swollen tails are collected and transferred to a normal solution. After the swelling goes down, they can be used in ICSI. The fertilization rate for ICSI with HOST ranges from 42 to 76%, and the pregnancy rate is comparable to that when motile spermatozoa are used [24, 25]. Nonetheless, caution should be exercised, as the nuclear DNA of spermatozoa may be damaged in the hypoosmotic solution if nucleoproteins have few SS bonds [26]. When this technique is not effective, microsurgical epididymal sperm aspiration (MESA) or TESE is indicated [27].

Azoospermia

If spermatozoa can be retrieved from the epididymis [28] or testis [10, 11] in azoospermia cases, ICSI can be performed to fertilize oocytes and achieve pregnancy by embryo transplantation. In obstructive azoospermia, when the degree of the disorder is severe or seminal duct reconstruction is not feasible, MESA or percutaneous epididymal sperm aspiration (PESA) [29] is employed. In addition, with non-obstructive azoospermia caused by a severe spermatogenesis disorder, testicular sperm extraction (TESE) is performed. The fertilization and pregnancy rate for this technique is comparable to that when ejaculated spermatozoa are used [30], but because in obstructive azoospermia, pregnancy can be achieved by seminal duct reconstruction in many cases [31, 32], the above techniques should be performed when seminal duct reconstruction is not effective. In TESE, spermatozoa are retrieved from testicular biopsy samples. Since information as to whether spermatozoa can be obtained is important in this method, serum FSH, Jhonsen's score, testicular volume etc., as markers are investigated. But at present there is no good marker that could predict the existence of spermatozoa beforehand [33–35]. Even under a diagnosis of aspermatogenesis or Sertoli cell only syndrome, spermatozoa can be retrieved in some cases. At our department spermatozoa were retrieved in 50% of the former cases, and in 17% of the latter cases. Testicular spermatozoa are often less motile than ejaculated spermatozoa, but when motile spermatozoa are used in ICSI, the fertilization and pregnancy rates are comparable to those with ejaculated spermatozoa.

Spermatid Injection

When spermatozoa cannot be obtained despite TESE, a decision must be made regarding whether spermatids should be used.

Spermatids are cells that have undergone two meiotic divisions. These cells have the same type of haploid and the same number of DNA as mature spermatozoa, and contain centrioles. Spermatids are therefore physiologically comparable to spermatozoa. When spermatids are used to fertilize oocytes by electric fusion or microinjection, newborns have been obtained in experiments on mice [36, 37] and rabbits [38], thus clarifying that spermatids have the ability to fertilize and develop. There are reports on infertility treatments with spermatids for human subjects. Round or elongated spermatids were collected from patients with non-obstructive azoospermia who did not have mature spermatozoa in their testes, and some of their partners became pregnant and delivered babies. Table 1 summarizes the major reports on round spermatid injection [39–48]. As can be seen in the table, the rates of fertilization and delivery had been low, but the cause of these poor results had not yet been clearly identified. The Japanese Infertility Association Ethics Committee cautions

Table 1. Reports of round spermatid injection in the literature (1995–2000)

Authors	Fertilization rate	Pregnancy	Delivery
Tesarik et al. (1995, 96) [39–40]	36% (14/39)	2	2
Shoysman et al. (1997) [41]	26% (10/32)	none	none
	64% (7/11)	1	
Antinori et al. (1997) [42]	56% (75/135)	2	–
Antinori et al. (1997) [43]	47% (7/15)	1	–
Sofikitis et al. (1997) [44]	38.5% (47/122)	3	–
Vanderzwalmen et al. (1997) [45]	22% (57/260)	1	1
Amer et al. (1997) [46]	25% (63/251)	4	none*
Kaharaman et al. (1998) [47]	26% (51/199)	1	none*
Barak et al. (1998) [48]	27% (10/37)	1	1

none means "no". "–" means "no report. *: chemical pregnancy.

against the clinical use of round spermatids in ICSI due to insufficient basic research, including animal studies. And fertilization could also be hindered by the etiologic factor of non-obstructive azoospermia, which is caused by chromosomal abnormality (Kleinfelter syndrome) [49] or abnormalities of spermatogenesis-related genes (AZF: azoospermic factor) [50–53]. There are several reports mentioning the transmission of Y chromosome deletion from father to son [54]. Microinsemination offers some hope for patients with this type of severe oligospermia, but these abnormalities can be passed on to male children in the next generation.

In a classification proposed by de Kretser, spermatids are electron-microscopically classified into six groups: Sa, Sb1, Sb2, Sc, Sd1 and Sd2. Sa and Sb1 are round spermatids; Sb2 are elongating spermatids; and Sc, Sd1 and Sd2 are elongated spermatids[51]. In addition, round spermatids (Sa and Sb1) are sometimes referred to as "early" spermatids, and other types of spermatids are referred to as "late" spermatids [52]. Some researchers refer to Sd2 as "mature spermatid [53]" or "late elongated spermatid [54]" to distinguish it from the other late spermatids. This leads to some confusion when their results are evaluated. There are cells that have the same morphology as mature spermatozoa retrieved from the testis: some are elongated spermatids that are about to detach from Sertoli cells and spermatids that have already separated from Sertoli cells. In wet preparations, elongated spermatids are sometimes mechanically separated from Sertoli cells, thus making it impossible to distinguish a Sd2 from a spermatozoon. In terms of their cellular functions, Sd2 spermatids are comparable to testicular spermatozoa. Therefore, when the fertilizing function of Sd2 spermatids is discussed, free Sd2 spermatids should be considered as testicular spermatozoa.

Among the abovementioned spermatids, the result of fertilization therapy is particularly poor when round spermatids are used (Table 1). The reason for this is not clear, but the factors specified below may contribute to the poor results.

Nucleoproteins

At the round-spermatid stage, nucleoproteins are histone. Starting at this point, this nucleoprotein is gradually replaced by protamine, which is more stable than histone and contains more SH bonds, thus making it possible for SS bonds to form by oxidation. This practice can improve nuclear condensation and stability, and suppress genetic transcription until sperm genes are expressed in the oocyte. This structure protects sperm's DNA, which transmits genetic information to the next generation[55]. Therefore, with round spermatids, the DNA is fragile and is more likely to be damaged by the micro-manipulation, resulting in DNA fragmentation or even chromosomal abnormalities.

Centriole

During fertilization, aster leads the male nucleus toward female nucleus, and its center is known as a "centriole" (a pair of centrioles are known as a "centrosome"). In addition, during division, a centriole acts as the center of two spindle poles. As spermatids are larger than spermatozoa, they are more likely to be physically damaged during microinjection. In other words, loss of or damage to the centriole could also contribute to unsuccesful fertilization when round spermatids are injected. Because there are no reports mentioning the centriole of spermatids, further research on this matter is needed.

Oocyte activating factor

In microinjection, because sperm-egg fusion does not

occur, the oocyte activating factor (also known as the "sperm factor") in spermatozoa is believed to activate oocytes that remain inactive during the metaphase of the second meiotic division[56]. This factor has not been identified, and it is not clear whether it not only activates an oocyte, but also induces calcium oscillations. When a round human spermatid is injected into a mouse oocyte, the oocyte is activated approximately 50% of the time, but normal Ca^{2+} oscillations are induced in only 50% of activated oocytes [57, 58]. Artificial oocyte activation is therefore probably necessary when round spermatids are used in microinjection. Excessive ooplasm aspiration [39, 40, 42, 43, 45], calcium ionophore treatment [41, 45], and electric stimulation [59] for artificial oocyte activation have been introduced when round human spermatids are used. But with these artificial oocyte activation methods, Ca^{2+} oscillation is not induced. When Ca^{2+} oscillation fails to occur in mouse oocytes, the number of cells in the inner cell mass is reduced during embryogenesis [60]. Therefore, a lack of Ca^{2+} oscillation can adversely affect the development of early embryos.

Genomic imprinting

A genome consists of a set of alleles and, in many cases, complementing genes express their function to a comparable degree, but in some cases only one gene is selectively expressed to control the development of an organism. This type of selective inactivation of a gene is referred to as "genomic imprinting," and inactivated genes are referred to as "genomic-imprinting genes." Thus far, approximately 20 genomic-imprinting genes have been identified in human subjects and mice, and many of these genes are involved in the growth and development of embryos and the onset of genetic and chromosomal abnormalities. During differentiation into reproductive cells, this imprinting is eliminated, and when male reproductive cells differentiate into spermatogonia, genomic imprinting is established to control genetic expression.

In mice, live newborns can be obtained by using primary spermatocytes [61], thus suggesting that genomic imprinting is mostly completed at this stage, but these cells are by no means comparable to mature spermatozoa. Genomic imprinting is not completed at the time of fertilization and, in mature mouse spermatozoa, some genes, such as the Xist gene, are still demethylated [62].

Future Problems

Fragmentation of sperm DNA

In ICSI, fertilization is said not to be affected by the sperm concentration, motility, or morphological abnormalities (general parameters) [63]. In the past most studies investigated the mean motility of spermatozoa in semen, rather than the motility of individual spermatozoa. There have been some reports on the motility of individual spermatozoa with emphasis on the straight-line velocity (SLV) in CASA (computed assisted semen analysis). The tendency was that the higher the SLV, the greater the fertilization rate [64]. The motility of spermatozoa correlates to the degree of DNA fragmentation, and there is a negative correlation between the degree of DNA fragmentation and the IVF fertilization rate [65]. In addition, the incidence of mitochondrial DNA deletions is high in spermatozoa with low motility [66]. When unfertilized oocytes are analyzed after ICSI, the incidence of DNA fragmentation in spermatozoa with a decondensed head is lower than that for those with a condensed head [67]. Therefore, when spermatozoa with low motility are used in ICSI, high-quality fertilization cannot be expected, so that in order to improve the quality of fertilization, the most motile spermatozoa should be selected.

The same applies to obstructive azoospermia. The motility of testicular spermatozoa obtained by TESE is lower than that of spermatozoa obtained by MESA, but there are no differences between fertilization rates for ICSI [68]. In general, sperm concentration, motility, and morphological abnormalities are important factors in the selection of spermatozoa for infertility therapy. But we chose spermatozoa with poor semen parameters for ICSI procedures. There have recently been reports of an increased risk of sex chromosomal abnormalities among children who were born by means of ICSI [68]. In addition, when epididymal and testicular spermatozoa are compared in obstructive azoospermia, DNA fragmentation is seen more frequently in the former [69]. According to this report, fragmentation was seen in 25% of epididymal spermatozoa, so that spermatozoa retrieved by TESE should be used in patients with obstructive azoospermia. It will be important to conduct research to investigate the quality of gametes.

Oocyte factor

Approximately 85 to 90% of retrieved oocytes are mature oocytes in metaphase II, but the older the woman, the lower the quality of the oocytes is likely to be. In addition, the greater the age, the higher the incidence of oocyte chromosomal abnormalities (95.8% for women above the age of 40) [70]. Furthermore, in mice, fragmentation was seen in 25% of aged oocytes (40–48 weeks) [71]. These and other issues associated with aging can no longer be ignored and there are no solutions to these problems. This is, therefore, the limitation

of ART at this point, making further research necessary.

Conclusion

The ICSI method has now been established. Since ICSI started without sufficient support in basic animal experiments, we need follow up studies on babies after the ICSI procedure. ICSI might prove to be a good quality procedure, and the key point for future ICSI might be the idea of methods for selecting good quality zygotes (genetically safe embryos). And we are making every effort to obtain good results.

References

1) Gordon, J.W. and Taransky, B.E. (1986): Assisted fertilization by zona drilling; a mouse model for correction of oligospermia. J. Exp. Zool., 239, 347–354.
2) Cohen, J., Malter, H., Wright, G., Kort, H., Massey, J. and Mitchell, D. (1989): Partial zona dissection of human oocyte when failure of zona pellucida penetration in anticipated. Hum. Reprod., 4, 435–442.
3) Odawara, Y. and Lapata, A. (1989): A zona opening procedure for improving in vitro fertilization at low sperm concentrations: a mouse model. Fertil. Steril., 51, 139–148.
4) Ng, S.C., Bongso, A. and Ratnum, S.S. (1988): Pregnancy after transfer of sperm under zona pellucida. Lancet, 2, 790.
5) Redgment, C.J. and Yang, D., Tsirigotis, M., Yazdani, N., Shawaf, T., Craft, I.L. (1994): Experience with assisted fertilization in severe male factor infertility and unexplained failed fertilization in vitro. Hum.Reprod., 9, 680–683.
6) Palermo, G., Joris, H., Devroy, P. and Van Steirteghem, A.C. (1992): Pregnancies after intracytoplasmic injection of single spermatozoa into an oocyte. Lancet. 340. 17–18.
7) Van Steirtegehem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. (1993): High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod., 8, 1061–1066.
8) Van Steirteghem, A.C., Nagy, P., Joris, H., Verheyen, G., Smitz, J., Camus, M., Tournaye, H., Ubaldi, F., Bonduelle, M., Silber, S., Liebaers, I. and Devroey, P. (1996): The development of intracytoplasmic sperm injection. Hum.Reprod., 8(Supple.1), 59–72.
9) Silber, S.J., Nagy, Z.P., Liu, J., Godoy, H., Devroey, P. and Van Steirteghem, A.C. (1994): Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. Hum. Reprod., 9, 1705–1709.
10) Kahraman, S., Ozgur, S., Alatas, C., Aksoy, S., Balaban, B., Evrenkaya, T., Nuhoglu, A., Tasdemir, M., Biberoglu, K., Schoysman, R., Vanderzwalmen, P. and Nijs, M. (1996): High implantation and pregnancy rates with testicular sperm extraction and intracytoplasmic sperm injection in obstructive and non obstructive azoospermia. Hum. Reprod., 11, 673–676.
11) Silber, S.J., Van Steirteghem, A.C., Liu, J., Nagy, Z., Tournaye, H. and Devroey, P. (1995): High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. Hum. Reprod., 10, 148–152.
12) Bourne, H., Stern, K., Clarke, G., Pertile, M., Speirs, A. and Baker, H.W. (1997): Delivery of normal twins following the intracytoplasmic injection of spermatozoa from a patient with 47, XXY Klinefelter's syndrome. Hum. Reprod.. 11. 2447–2450.
13) McDonough, P.G. (1998): The Y-chromosome and reproductive disorders. Reprod. Fertil. Dev, , 10, 1–16.
14) Silber, S.J., Alagappan, L.G. and Page, D.C. (1998): Y chromosome deletions in azoospermic and severely oligozoospermic men undergoing intracytoplasmic sperm injection after testicular sperm extraction. Hum. Reprod.13, 3332–3337.
15) Vandervorst, M., Tournaye, H., Camus, M., Nagy, Z.P., Van Steirteghem, A. and Devroey, P. (1997): Patients with absolutely immotile spermatozoa and intracytoplasmic sperm injection. Hum. Reprod., 12, 2429–2433.
16) Kahraman, S., Tasdemir, M., Tasdemir, I., Vicdan, K.. Ozgur. S.. Polat. G.. Islk. A.Z.. Biberoglu. K.. Vanderzwalmen, P., Nijs, M. and Schoysman, R. (1996): Pregnancies achieved with testicular and ejaculated spermatozoa in combination with intracytoplasmic sperm injection in men with totally or initially immotile spermatozoa in the ejaculate. Hum. Reprod., 11, 1343–1346.
17) World Health Organisation (1987): Laboratory Manual for Examination of Human Semen—Cervical Mucus Interaction. Cambridge: Press Syndicate of the University of Cambridge.
18) Garner, D.L. and Johnson, L.A. (1995): Viability assessment of mammalian sperm using SYBR-14 and Propidium Iodide. Biol.Reprod.. 53, 276–284.
19) Rybouchkin, A., Benijts, J., De Sutter, P. and Dhont, M. (1997): Disintegration of chromosomes in dead sperm cells as revealed by injection into mouse oocytes. Hum. Reprod. 12, 1693–1698.
20) Braude, P., Bolton, V. and Moore, S. (1988): Human gene expression first occurs between the four and eight cell stages of preimplantation development. Nature, 332, 459–461.
21) Kosower, NS., Katayose, H. and Yanagimachi, R. (1992): Thioldisulfide status and acridine orange fluorescence of mammalian sperm nuclei. J. Androl., 13, 342–348.
22) Tournaye, H., Liu, J., Nagy, Z., Verheyen, G., Van Steirteghem, A. and Devroey, P. (1996): The use of testicular sperm for intracytoplasmic sperm injection in patients with necrozoospermia. Fertil.Steril., 66, 331–334.
23) Casper, R.F., Meriano, J.S., Jarvi, K.A., Cowan, L.

and Lucato, M.L. (1996): The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. Fertil. Steril., 65, 972–976.

24) Liu, J., Tsai, Y.L., Katz, E., Compton, G., Garcia, J.E. and Baramki, T.A. (1997): High fertilization rate obtained after intracytoplasmic sperm injection with 100% nonmotile spermatozoa selected by using a simple modified hypo-osmotic swelling test. Fertil.Steril., 68, 373–375.

25) Ved, S., Montag, M., Schmutzler, A., Prietl, G., Haidl, G. and van der Ven, H. (1997): Pregnancy following intracytoplasmic sperm injection of immotile spermatozoa selected by the hypo-osmotic swelling-test: a case report. Andrologia, 29, 241–242.

26) Yanagimachi, R., Katayose, H., Matsuda, J. and Yanagida, K. (1992): Stability of mammalian sperm nuclei. In: Molecular and Cell Biology of Reproduction (Spera, G., Fabbrini, A., Gnessi, L., Bardin, C.W., eds.), pp 157–176, Raven Press, New York.

27) Kahraman, S., Isik, A.Z, Vicdan, K., Ozgur, S. and Ozgun, O.D. (1997): A healthy birth after intracytoplasmic sperm injection by using immotile testicular spermatozoa in a case with totally immotile ejaculated spermatozoa before and after Percoll gradients. Hum. Reprod., 12, 292–293.

28) Silber, S.J., Nagy, Z.P., Liu, J., Godoy, H., Devroey, P. and Van Steirteghem, A.C. (1994): Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. Hum.Reprod., 9, 1705–1709.

29) Tsirigotis, M., Pelekanos, M., Beski, S., Gregorakis, S., Foster, C. and Craft, I.L. (1996): Cumulative experience of percutaneous epididymal sperm aspiration (PESA) with intracytoplasmic sperm injection. J. Assist. Reprod. Gene., 13, 315–319.

30) Ubaldi, F., Camus, M., Tournaye, H., Clasen, K., Nagy, Z., Smitz, J., Van Steirteghem, A. and Devroey, P. (1996): Results of microsurgical epididymal sperm aspiration (MESA) and testicular sperm extraction (TESE) in azoospermic men using intracytoplasmic sperm injection (ICSI). Andrologia, 1, 71–75.

31) Belker, A.M., Thomas, A.J.Jr., Fuchs, E.F., Konnak, J.W. and Sharlip, I.D. (1991): Results of 1469 microsurgical vasectomy reversals by the Vasovasostomy Study Group. J. Urol., 145, 505–511.

32) Matuda, T., Horii, Y., Muguruma, K., Komatz, Y. and Yoshida, O. (1994): Microsurgical epididymovasostomy for obstructive azoospermia. Eur. Urol., 26, 322–326.

33) Chen, C.S., Chu, S.H., Lai, Y.M., Wang, M.L. and Chan, P.R. (1996): Reconsideration of testicular biopsy and follicle-stimulating hormone measurement in the era of intracytoplasmic sperm injection for non-obstructive azoospermia? Hum. Reprod., 11, 2176–2179.

34) Mulhall, J.P., Burgess, C.M., Cunningham, D., Carson, R., Harris, D. and Oates, R.D. (1997): Presence of mature sperm in testicular parenchyma of men with nonobstructive azoospermia: prevalence and predictive factors. Urology, 49, 91–95.

35) Kim, E.D., Gilbaugh, J.3., Patel, V.R., Turek, P.J. and Lipshultz, L.I. (1997): Testis biopsies frequently demonstrate sperm in men with azoospermia and significantly elevated follicle-stimulating hormone levels. J. Urology, 157, 144–146.

36) Ogura, A., Matsuda, J. and Yanagimachi, R. (1994): Birth of normal young after electrofusion of mouse oocytes with round spermatids. Pro. Nat. Acad. Sci. U.S.A., 91, 7460–7462.

37) Kimura, Y. and Yanagimachi, R. (1995): Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development. 121. 2397–2405.

38) Sofikitis, N.V., Toda, T., Miyagawa, I., Zavos, P.M., Pasyianos, P. and Mastelou, E. (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.

39) Tesarik, J., Mendoza, C. and Testart, J. (1995): Viable embryos from injection of round spermatids into oocytes. New Eng. J. Med., 333, 525, 1995.

40) Tesarik J., Rolet, F., Brami, C., Sedbon, E., Thorel, J., Tibi, C. and Thebault, A. (1996): Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. Hum. Reprod., 11, 780–783.

41) Schoysman, R., Vanderzwalmen, P., Zech, H., Yemini, M., Birkenfield, A., Chan, M., Vandamme, B. and Lejeune, B. (1996): Results of spermatid injection in human oocytes in relation to previous testis biopsy and oocytes treatment. Fertil. Steril. Supple., S259.

42) Antinoi, S., Versaci, C., Dani, G., Antinori, M., Pozza, D. and Selman, H.A. (1997): Fertilization with human testicular spermatids: four successful pregnancies. Hum. Reprod., 12, 286–291.

43) Antinori, S., Versaci, C., Dani, G., Antinori, M. and Selman, H.A. (1997): Successful fertilization and pregnancy after injection of frozen-thawed round spermatids into human oocytes. Hum. Reprod., 12, 554–556.

44) Sofikitis, N.V., Mantzavinos, T., Loutradis, D., Antypas, S., Miyagawa, I. and Tarlatzis, V. (1997): Treatment of male infertility caused by spermatogenic arrest at the primary spermatocyte stage with ooplasmic injections of round spermatids or secondary spermatocytes isolated from foci of early haploid male gametes. Hum. Reprod., 12 (Supple.), 81–82.

45) Vanderzwalmen, P., Zech, H., Birkenfeld, A., Yemini, M., Bertin, G., Lejeune, B., Nijs, M., Segal, L., Stecher, A., Vandamme, B., van Roosendaal, E. and Schoysman, R. (1997): Intracytoplasmic injection of spermatids retrieved from testicular tissue: influence of testicular pathology, type of selected spermatids and oocyte activation. Hum. Reprod., 12, 1203–1213.

46) Amer, M., Soliman, E., El-Sadek, M., Mendoza, C. and Tesarik, J. (1997): Is complete spermiogenesis failure a good indication for spermatid conception?

Lancet, 350, 116.
47) Kahraman, S., Polat, G., Samli, M., Sozen, E., Ozgun, O.D., Dirican, K. and Ozbicer, T. (1998): Multiple pregnancies obtained by testicular spermatid injection in combination with intracytoplasmic sperm injection. Hum. Reprod., 13, 104–110.
48) Barak, Y., Kogosowski, A., Goldman, S., Soffer, Y., Gonen, Y. and Tesarik, J. (1998): Pregnancy and birth after transfer of embryos that developed from single-nucleated zygotes obtained by injection of round spermatids into oocytes. Fertil. Steril., 70, 67–70.
49) Kruse, R., Guttenbach, M., Schartmann, B., Schubert, R., van der Ven, H., Schmid, M. and Propping, P. (1998): Genetic counseling in a patient with XXY/XXXY/XY mosaic Klinefelter's syndrome: estimate of sex chromosome aberrations in sperm before intracytoplasmic sperm injection. Fertil. Steril., 69, 482–485.
50) Ma, K., Sharkey, A., Kirsch, S., Vogt, P., Keil, R., Hargreave, T.B., McBeath, S. and Chandley, A.C. (1992): Towards the molecular localisation of the AZF locus mapping of microdeletions in azoospermic men within 14 subintervals of interval 6 of the human Y chromosome. Hum. Mol. Genet., 1, 29–33.
51) Vogt, P., Chandley, A.C., Hargreave, T.B., Keil, R., Ma, K. and Sharkey, A. (1992): Microdeletions in interval 6 of the Y chromosome of males with idiopathic sterility point to disruption of AZF, a human spermatogenesis gene. Hum. Genet., 89, 491–496.
52) Silber, S.J., Alagappan, L.G. and Page, D.C. (1998): Y chromosome deletions in azoospermic and severely oligozoospermic men undergoing intracytoplasmic sperm injection after testicular sperm extraction. Hum. Reprod., 13, 3332–3337.
53) McDonough, P.G. (1998): The Y-chromosome and reproductive disorders. Reprod. Fertil. Dev., 10, 1–16.
54) Page, D., Silber, S. and Brown, L.G. (1999): Men with infertility caused by AZFc deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. Hum. Reprod., 14, 1722–1726.
55) Yanagida, K., Yanagimachi, R., Perreault, S.D. and Kleinfeld, R.G. (1991): Thermostability of sperm nuclei assesed by microinjection into hamster oocytes. Biol. Reprod., 44, 440–447.
56) Swann, K. (1990): A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development, 110, 1295–1302.
57) Yazawa, H., Hayashi, S., Hashimoto, S., Yamada, H., Katayose, H., Yanagida, K. and Sato, A. (2000): Oocyte activation and Ca^{2+} oscillation inducing abilities of some rodents and human spermatids. —Assessed by mouse oocyte activation assay (mouse test)—. J. Fert. Implant., 17, 20–23.
58) Yanagida, K., Yazawa, H., Katayose, H., Kimura, Y., Hayashi, S. and Sato, A. (2000): Oocyte activation induced by spermatids and the spermatozoa. Int. J. Androl., 23(Supple.), 63–65.
59) Yanagida, K., Kimura, Y., Katayose, H., Yazawa, H., Konnai, K. and Sato, A. (1997): Fertilization using male germ line-cells. Hum. Cell, 10, 255–262.
60) Bos-Mikich, D., Whittingham, D.G. and Jones, K.T. (1997): Meiotic and mitotic Ca2+ oscillations affect cell composition in resulting blastocysts. Dev. Biol., 182, 172–179.
61) Sasagawa, I., Kuretake, S., Eppig, J.J. and Yanagimachi, R. (1998): Mouse primary spermatocytes can complete two meiotic divisions within the oocyte cytoplasm. Biol. Reprod., 58, 248–254.
62) Norris, D.P., Patel, D., Kay, G.F., Penny, G.D., Brockdorff, N., Sheardown, S.A. and Rastan, S.O. (1994): Evidence that random and imprinted Xist expression is controlled by preemptive methylation. Cell. 77. 41–51.
63) Nagy, Z.P., Liu, J., Joris, H., Verheyen, G., Tournaye, H., Camus, M., Derde, M.C., Devroey, P. and Van Steirteghem, A.C. (1995): The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. Hum. Reprod., 10, 1123–1129.
64) Van den Bergh, M., Emiliani, S., Biramane, J., Vannin, A.S. and Englert, Y. (1998): A first prospective study of the individual straight line velocity of the spermatozoon and its influences on the fertilization rate after intracytoplasmic sperm injection. Hum. Reprod., 13, 3103–3107.
65) Sun, J.G., Jurisicova, A. and Casper, R.F. (1997): Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol. Reprod., 56, 602–607.
66) Kao, S.H., Chao, F.T. and Wei, Y.H. (1995): Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. Biol. Reprod., 52, 729–736.
67) Lopes, S., Jurisicova, A. and Casper, R.F. (1998): Gamete-specific DNA fragmentation in unfertilized human oocytes after intracytoplasmic sperm injection. Hum. Reprod., 13, 703–708.
68) Bonduelle, M., Camus, M., De Vos, A., Staessen, C., Tournaye, H., Van Assche, E., Verheyen, G., Devroey, P., Liebaers, I. and Van Steirteghem, A. (1999): Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. Hum. Reprod., 14 (Supple. 1), 243–264.
69) Steele, E.K., McClure, N., Maxwell, R.J. and Lewis, E.M. (1999): A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. Mol. Hum. Reprod., 9, 831–835.
70) Lim, A.S. and Tsakok, M.F. (1997): Age-related decline in fertility: a link to degenerative oocytes? Fertil. Steril., 68, 265–271.
71) Fujino, Y., Ozaki, K., Yamamasu, S., Ito, F., Matsuoka, I., Hayashi, E., Nakamura, H., Ogita, S., Sato, E. and Inoue, M. (1996): DNA fragmentation of oocytes in aged mice. Hum. Reprod., 11, 1480–1483.
72) Cohen, J., Scott, R., Schimmel, T., Levron, J. and Willadsen, S. (1997): Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. Lancet, 350, 186–187.