Human Reproduction vol.14 no.5 pp.1307–1311, 1999

Successful fertilization and pregnancy following ICSI and electrical oocyte activation

K.Yanagida[1,3], H.Katayose[1], H.Yazawa[1], Y.Kimura[1], A.Sato[1], H.Yanagimachi[2] and R.Yanagimachi[2]

[1]Department of Obstetrics and Gynecology, Fukushima Medical College, l Hikarigaoka Fukushima, Fukushima 960–1295, Japan, and [2]Department of Anatomy and Reproductive Biology, School of Medicine, University of Hawaii, Honolulu, Hawaii, USA

[3]To whom correspondence should be addressed

In a total of 1048 intracytoplasmic sperm injection (ICSI) cycles, motile spermatozoa from four out of 424 patients (0.9%) failed to fertilize oocytes, despite an apparently successful ICSI procedure. No activation was observed in these injected oocytes. The spermatozoa from three of the four patients were injected into unfertilized mouse oocytes by ICSI (mouse test) to evaluate their oocyte activating ability. The oocyte activation rate of the spermatozoa of patients A, B, and C in the mouse test was 46, 100, and 86% respectively (control: 100%). Simultaneous injection of two spermatozoa from patient A into the mouse oocytes increased the oocyte activating rate to 89% (sham control: 29%). 100% fertilization rates were obtained for patients A and B by combining ICSI and electrical stimulation, and this resulted in pregnancy and the birth of healthy twins for the partner of patient A. Thus, it is considered that the spermatozoa of these patients are not lacking sperm factors but that the activity of these factors is depressed. The combination of ICSI and electrical stimulation is effective in these cases.

Key words: electrical stimulation/infertility/intracytoplasmic sperm injection/oocyte activation/spermatozoa

Introduction

Intracytoplasmic sperm injection (ICSI) has become the method of choice to overcome severe male infertility (Palermo *et al*., 1992; Van Steirteghem *et al*., 1993). However, there are still many couples who have not received the benefits of ICSI. It has been reported that round-headed human spermatozoa used in ICSI are unable to activate human oocytes (Rybouchkin *et al*., 1997). When ICSI and oocyte activation (ionophore treatment) were combined, this resulted in pregnancy and the birth of a healthy baby. While performing ICSI over the past 6 years, we have encountered four men whose spermatozoa were unable to fertilize oocytes despite their apparently successful introduction into the oocytes. Here we report that ICSI following oocyte activation with electrical stimulation in these cases resulted in normal pregnancy.

Materials and methods

Patients

A total of 1048 treatment cycles of ICSI were carried out in 424 couples at the Fukushima Medical College Hospital between January 1992 and January 1998. Criteria for admission to the ICSI programme were severe oligozoospermia or lack of fertilization in the preceding standard in-vitro fertilization (IVF). The cases in which no motile spermatozoa were available were not included in this report. In four out of 424 couples (0.9%), none of their oocytes were fertilized after two ICSI attempts with more than two oocytes injected on two different days. Written consent was obtained from the male partners of three out of four of these couples, who donated their semen samples for this research. The study was in accord with guidelines approved by the internal review board of research ethics.

Preparation of human gametes

Ovarian stimulation and oocyte collection were performed as described previously (Yanagida *et al*., 1998). Oocytes were incubated for 3–8 h in human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA), supplemented with 6% plasmanate cutter (Bayer Pharmaceutical Co., Osaka, Japan). Immediately before ICSI, cumulus cells were removed by pipetting the oocytes in HEPES-buffered HTF medium (mHTF; Irvine Scientific) containing 0.25 mg/ml hyaluronidase (type 8, H-3757, Sigma Chemical, St Louis, MO, USA). Oocytes were examined and only those with the first polar body were used for ICSI.

Semen samples were allowed to liquefy for 30 min at room temperature. As many spermatozoa as possible were collected by the swim-up method using HTF medium. When no motile spermatozoa were collected by this method, the semen was diluted with HTF medium, centrifuged at 350 *g* for 10 min, and motile spermatozoa were individually picked up using a micropipette immediately before ICSI.

Part of a semen sample from patient A was fixed with 2.5% glutaraldehyde and processed for electron microscopy.

Sperm injection into oocytes (ICSI) in human

Conventional ICSI or piezo ICSI was performed in a total of 1048 treatment cycles. ICSI using the conventional method (Palermo *et al*., 1992; Van Steirteghem *et al*., 1993) was performed between January 1992 and December 1996. Thin-wall injection pipettes with flush tips were prepared according to a previously described method (Perreault and Zirkin, 1982; Yanagida *et al*., 1991). The outer diameter of the injection needle was 5–6 μm and the inner diameter of the holding pipette was 15 μm. A motile spermatozoon with a morphologically normal head was selected and was immobilized immediately before ICSI. Immobilization was achieved by repeatedly drawing a spermatozoon in and out of an injection needle in mHTF containing 10% polyvinylpyrrolidone (PVP-360; Sigma). The immobilized spermatozoon was drawn tail-first into the injection needle. The oocyte was punctured by the needle and a small amount of cytoplasm was sucked into the needle to confirm rupture of the membrane. The spermatozoon was expelled into the oocyte.

Piezo ICSI using a piezo-electric actuator (model PMM-MB-A; Prime Tech Ltd, Tuchiura City, Japan) was carried out between January 1997 and January 1998. The procedure was essentially the same as that described for mouse ICSI (Kimura and Yanagimachi, 1995; Huang *et al*., 1996), except that all operations were carried out at 37°C. The outer diameter of the thin-wall injection pipette, with flush end, was 5–6 μm at the tip. Spermatozoa were suspended in HTF medium containing 8% PVP. A slowly moving spermatozoon was drawn, tail first, into the pipette and immobilized by applying a few piezo pulses to the midpiece of the spermatozoon. Zona pellucida drilling and sperm injection were carried out as described previously (Huang *et al*., 1996; Yanagida *et al*., 1999).

Table I. Semen parameters of three patients whose spermatozoa totally failed to fertilize after intracytoplasmic sperm injection (ICSI)[a]

Patient	Concentration ($\times 10^6$/ml)	Motility (%)	Normal morphology (%)
A	7.9	20.0	50
B	67.8	32.5	10
C	72.4	44.9	10

[a]ICSI was carried out twice for each patient, with the semen parameters shown here being determined in the first attempt.

Table II. Results of mouse tests – a single spermatozoon was injected into each mouse oocyte

Patient	No. (%) of surviving mouse oocytes			
	Injected with patient's spermatozoa	Surviving, with sperm tail	Activated	Fertilized
A	65	46 (71)	21 (46)	20 (43)
B	29	24 (81)	24 (100)	20 (83)
C	23	14 (87)	12 (86)	12 (86)
Control[a]	21	17 (85)	17 (100)	15 (88)

[a]A man of proven fertility.

Table III. Results of mouse tests – one or two spermatozoa from patient A were injected into each mouse oocyte

No. of spermatozoa injected into each oocyte	No. (%) of surviving mouse oocytes		
	Total	Surviving	Activated
1	65	46 (71)	21 (46)[d,f]
0[a]	28	20 (71)	3 (15)[d]
2	31	27 (87)	24 (89)[e,f]
0[b]	45	31 (69)	9 (29)[e]
0[c]	31	31 (100)	1 (3)

[a]Sham operation; each oocyte was injected with a bolus (~5 pl) of human tubal fluid (HTF) medium without spermatozoa.
[b]Sham operation; each oocyte was injected with a bolus (~10 pl) of HTF medium without spermatozoa.
[c]Control; oocytes were left untouched in the medium.
Values with the same superscripts were significantly different (Fisher's exact test): [d]$P < 0.05$; [e]$P < 0.001$; and [f]$P < 0.01$.

Electrical activation of injected human oocyte

Some oocytes were stimulated (activated) electrically (Yanagida *et al*., 1997) ~30 min after ICSl. Oocytes, suspended in a phosphate-buffered saline (PBS, P0261; Sigma) were placed between two parallel electrodes (2 mm apart) in an electric chamber (Model FTC-03; Shimazu Co, Tokyo, Japan), and were subjected to a single, square DC pulse (1.5 kV/cm, 100 μs). Stimulated oocytes were immediately transferred back to HTF medium.

Human oocyte culture and embryo transfer

Oocytes undergoing ICSI (with or without electric stimulation) were cultured in HTF medium containing 6% plasmanate cutter for 18 h. Oocytes with the second polar body and two pronuclei were considered normally fertilized. They were further cultured for 26 h to allow them to develop to 2–4-cell embryos which were then transferred to the patients' uteri.

Mouse oocyte activation assay (mouse test)

Female mice (B6D2F1), 6–12 weeks old, were stimulated to ovulate by consecutive i.p. injections 48 h apart of 7.5 IU pregnant mare's serum gonadotrophin (PMSG; Teikokuzoki Co, Tokyo, Japan) and 7.5 IU human chorionic gonadotrophin (HCG; Mochida Pharmaceutical Co, Tokyo, Japan). Oocyte–cumulus complexes were treated for 3–5 min with mHTF containing 1 mg/ml hyaluronidase (from bovine testis, 825 IU/mg; Sigma) to disperse cumulus cells. Cumulus-free oocytes were kept in HTF with 6% SSS (synthetic serum substitute; Irvine Scientific) at 37°C under 5% CO_2, 5% O_2 and 90% N_2 for up to 1 h before ICSI. Some oocytes were each injected with two spermatozoa simultaneously. Spermatozoa from a man of proven fertility served as controls. Sperm-injected oocytes were cultured in HTF medium for 5 h before they were fixed, stained and examined cytologically by phase-contrast microscopy (Yanagida *et al*., 1991). An oocyte with a sperm tail within the cytoplasm was considered to have been successfully injected. An oocyte with the second polar body and a female pronucleus was recorded 'activated' regardless of the status of the sperm nucleus. An oocyte with the second polar body and two pronuclei was considered to be 'normally fertilized'.

Table IV. Results of clinical intracytoplasmic sperm injection (ICSI), including electrostimulation

Patient	ICSI cycle no.	Electrical stimulation[a]	No. of ICSI oocytes			No. of embryos transferred	Pregnancy achieved
			Total	Survived	Fertilized		
A	1	–	6	5	0	–	–
	2	–	5		0	–	–
	3	+	3	3	3	2	Yes (twin)
B	1	–	3	3	0	–	–
	2	–	5	4	0	–	–
	3	+	1	1	1	1	No
C	1	–	3	2	0	–	–
	2	–	3	3	0	–	–

[a]Oocytes were stimulated with a single DC pulse ~30 min after ICSI.

Statistical significance was assessed using Fisher's exact test; $P < 0.05$ was considered to be statistically significant.

Results

Semen parameters of patients A, B and C are shown in Table I; their spermatozoa fertilized none of the 19 oocytes during previous ICSI cycles. When spermatozoa were individually injected into mouse oocytes, high proportions of the oocytes were activated and fertilized (Table II). The ability of the patients' spermatozoa to activate and fertilize mouse oocytes was comparable with that of the donor's spermatozoa.

Table III summarizes the results of experiments in which one or two spermatozoa of patient A were injected into mouse oocytes. A higher proportion of oocytes were activated after injection of two spermatozoa than when only one spermatozoon was injected, the difference being statistically significant ($P < 0.001$). Although the spermatozoa were apparently deficient in their ability to activate human oocytes, mouse oocyte activation tests failed to demonstrate activating factor deficiency. We were convinced, based on the clinical data that this deficiency, was real, we proceeded with artificial activation of human oocytes after ICSI.

When three oocytes from the wife of patient A were electrostimulated 30 min after ICSI, all were fertilized successfully. Two 4-cell embryos were transferred into the uterus, resulting in a dizygotic twin pregnancy. Prenatal diagnosis by amniocentesis was performed at week 15 of gestation. The fetal karyotypes were normal (46XX and 46XY). Two healthy babies were delivered after 38 weeks gestation (Table IV). One oocyte from the partner of patient B was similarly stimulated after ICSI and was fertilized, but no pregnancy resulted.

Figures 1 and 2 are light and electron micrographs of spermatozoa of patient A. Although some of the spermatozoa were motile and appeared normal (Figure 1), none were entirely normal when viewed by electron microscopy (Figure 2). Abnormalities in the head structure and in chromatin compaction were noted in all spermatozoa examined.

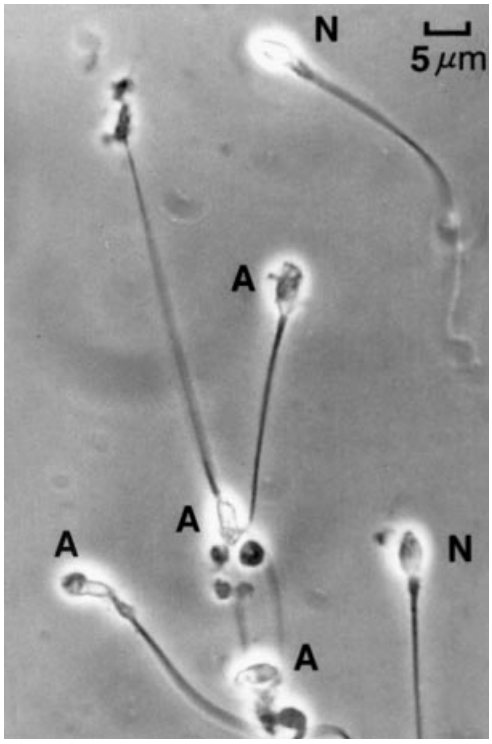

Figure 1. Phase-contrast micrograph; spermatozoa from patient A with amorphous heads (A) or normal heads (N).

Discussion

Pregnancy and delivery of healthy twins were achieved by a woman whose husband's spermatozoa failed to fertilize after conventional ICSI. None of the oocytes of this woman were activated despite apparent success in sperm injection. Spermatozoa of three other patients also failed to fertilize by ICSI. This failure of fertilization may have been due to either the lack or deficiency of the oocyte-activating capacity of spermatozoa, or to the inability of the oocytes to respond to penetrated spermatozoa. The high rate of mouse oocyte activation (50–100%) by the spermatozoa from three of these patients

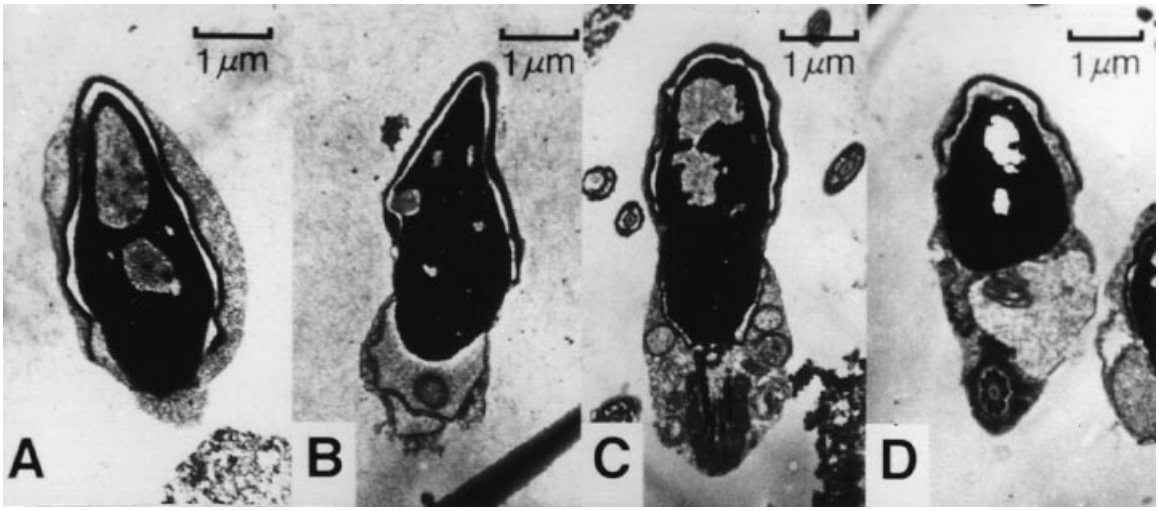

Figure 2. Transmission electron microscopy of spermatozoa of patient A. Longitudinal sections of spermatozoa with quasi-normal (A,B) and abnormal heads (C,D).

(Tables II and III) suggested that their spermatozoa were potentially capable of activating oocytes. Round-headed human spermatozoa are unable to activate, by ICSI, either human oocytes or mouse oocytes (Rybouchkin *et al*., 1997). In a comparative study, donor spermatozoa activated all the mouse oocytes, suggesting that the round-headed spermatozoa lacked oocyte-activating abilitity. Defects in the oocytes may also have contributed because some patients' oocytes failed to respond to artificial activation stimuli (e.g. calcium ionophore) following sperm injection (Rybouchkin *et al*., 1997).

How mammalian spermatozoa activate oocytes is still the subject of debate. Some investigators believe that ligand-receptor interactions between gamete membranes trigger oocyte activation (e.g. Schultz and Kopf, 1995), while others maintain that spermatozoa bring some proteins into oocytes to initiate activation (Swann and Lai, 1997). As ICSI by-passes gamete membrane interaction, oocyte activation following ICSI seems to be induced by the action of a non-membranous factor or factors in the spermatozoon (Yanagimachi, 1997). At least part of the sperm-borne oocyte-activating factors (perhaps proteins) reside in/with the perinuclear material (Kimura *et al*., 1998) which makes direct contact with the oocyte's cytoplasm upon sperm–oocyte fusion (Yanagimachi, 1994). Physiologically, the oocyte-activating factor of human spermatozoa exists in spermatids and spermatozoa and does not exist in spermatocytes (Sousa *et al*., 1996). As for the disorder of spermiogenesis, this factor may be deficient in many round spermatids recovered from men with complete spermiogenesis failure (Tesarik *et al*., 1998), in spite of the presence of this factor in round spermatids from healthy men.

None of the spermatozoa of patient A were strictly normal in their morphology as determined by electron microscopy (Figure 2). Structural abnormalities are caused by a disorder of spermiogenesis and may be associated with abnormality or deficiency of oocyte-activating factors. The fact that spermatozoa from patient A could not activate his partner's oocytes, but were capable of activating mouse oocytes (Table II), suggests that either, mouse oocytes are much more sensitive to the sperm-borne oocyte-activating proteins than human oocytes or that, in this case, human oocytes had some defects in responding to sperm proteins. The smaller volume of mouse oocytes in comparison with human oocytes may have contributed to this species difference.

Although a single spermatozoon activates an oocyte of the homologous species under normal conditions, many artificial reagents or conditions can induce oocyte activation as well. Although the process and mechanism of oocyte activation by spermatozoa and artificial agents may differ in minor details, it is currently believed that a temporal rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key event in oocyte activation (Cuthbertson *et al*., 1981; Miyazaki and Igusa, 1981). Typically, $[Ca^{2+}]_i$ rises repetitively (Miyazaki, 1991; Kline and Kline, 1992; Tesarik *et al*., 1994) until oocytes reach the pronuclear stage (Jones *et al*., 1995). In contrast, some artificial agents (e.g. ethanol, calcium ionophore, or a single electric pulse) activate oocytes by provoking a single, long-lasting $[Ca^{2+}]_i$ rise (Kline and Kline, 1992; Rickords and White, 1992; Sun *et al*., 1992; Tesarik and Testart, 1994). It is not known why the pattern of $[Ca^{2+}]_i$ rise is important for oocyte activation and embryonic development. In the present study, we activated oocytes by a single electric pulse. It is known that this causes a rapid rise in $[Ca^{2+}]_i$ which decreases gradually to the original value in ~300 s (Yanagida *et al*., 1997). This may or may not be the most efficient and safest way of activating human oocytes, but two oocytes stimulated this way did develop into healthy offspring. Possibly, the native sperm-borne oocyte-activating factor (protein) is the ideal substance to activate human oocytes. However, the clinical use of sperm extract including oocyte-activating factor is not accepted because of the risk of infectious agent transmission (Tesarik, 1998). Tesarik (1998) suggested that pharmacological stimulators of calcium oscillations might be considered for boosting oocyte activation in ICSI involving abnormal spermatozoa. Until such agents have been identified and become readily available commercially, electrical pulses instead of pharmacological stimulators may be used as a simple, yet effective method of activating human oocytes. Electrical stimulation of oocytes was carried out 30 min after ICSI in this study. Swollen nuclei with fragmented pieces of chromosomes were observed in 51% of unfertilized (inactivated) oocytes after ICSI, and these were probably degenerating nuclei (Dozortsev *et al*., 1994). So it is important that the electrical stimulation is carried out as soon as possible after ICSI.

The 'mouse oocyte activation assay', which was first proposed by Rybouchkin *et al*. (1995) and used in the present study, may be useful in informing patients whether they have a chance of a successful pregnancy after an initial failure in an ICSI attempt. Of course, there is no guarantee that success in the mouse oocyte activation assay will ensure that spermatozoa have the ability to trigger Ca^{2+} release in the oocyte (Swann, 1998). Our data indicate that the activating factor deficiency cannot always be detected by using the mouse oocyte activation assay. Nevertheless, in such cases, artificial activation of human oocytes may prove to be an effective and useful procedure to achieve pregnancy.

Acknowledgements

We thank to Mrs Charlotte Oser for her assistance in the preparation of the manuscript.

References

Cuthbertson, K.S.R., Whittingham, D.G. and Cobbold, P.H. (1981) Free Ca^{2+} increases in exponential phases during mouse oocyte activation. *Nature*, **294,** 754–757.

Dozortsev, D., De Sutter, P. and Dhout, M. (1994) Behaviour of spermatozoa in human oocytes displaying no or one prenucleus after intracytoplasmic sperm injection. *Hum. Reprod.*, **9,** 2139–2144.

Huang, T., Kimura, Y. and Yanagimachi, R. (1996) The use of piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. *J. Assist. Reprod. Genet*., **13,** 320–328.

Jones, K.T., Carroll, J., Merriman, J.A. *et al.* (1995) Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development*, **121,** 3259–3266.

Kimura, Y. and Yanagimachi, R. (1995) Intracytoplasmic sperm injection in the mouse. *Biol. Reprod*., **52,** 709–720.

Kimura Y., Yanagimachi R., Kuretake S. *et al.* (1998) Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. *Biol. Reprod*., **58,** 1407–1415.

Kline, D. and Kline, J.T. (1992) Repetitive calcium transients and the role of

calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol*., **149,** 80–89.

Miyazaki, S. (1991) Repetitive calcium transients in hamster oocytes. *Cell Calcium*, **12,** 205–216.

Miyazaki, S. and Igusa, Y. (1981) Fertilization potential in golden hamster eggs consists of recurring hyperpolarizations. *Nature*, **290,** 702–704.

Palermo, G., Joris, H., Derde, M.P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340,** 17–18.

Perreault, S. and Zirkin, B.R. (1982) Sperm nuclear decondensation in mammals: role of sperm-associated proteinase *in vivo*. *J. Exp. Zool*., **224,** 253–257.

Rickords, L.F. and White, K.L. (1992) Electrofusion-induced intracellular Ca^{2+} flux and its effect on murine oocyte activation. *Mol. Reprod. Dev.*, **31,** 152–159.

Rybouchkin, A., Dozortsev, D., de Sutter, P. *et al* (1995) Intracytoplasmic injection of human spermatozoa into mouse oocytes: a useful model to investigate the oocyte-activating capacity and the karyotype of human spermatozoa. *Hum. Reprod.*, **10,** 1130–1135.

Rybouchkin, A.V., Van der Straeten, F., Quatacker, J. *et al.* (1997) Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. *Fertil. Steril.*, **68,** 1144–1147.

Schultz, R.M. and Kopf, G.S. (1995) Molecular basis of mammalian egg activation. *Curr. Topics Dev. Biol.*, **30,** 21–62.

Sousa, M., Mendoza, C., Barros, A. *et al.* (1996) Calcium responses of human oocytes after intracytoplasmic injection of leukocytes, spermatocytes and round spermatids. *Mol. Hum. Reprod.*, **2,** 853–857.

Sun, F.Z., Hoyland, J., Huang, X. *et al.* (1992) A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. *Development*, **115,** 947–956.

Swann, K. and Lai, F.A. (1997) A novel signalling mechanism for generating Ca^{2+} oscillations at fertilization in mammals. *Bioessays*, **19,** 371–378.

Swann, K., Parrington, J. and Jones, K.T. (1998) On the search for the sperm oscillogen. *Mol. Hum. Reprod.*, **4,** 1010–1012.

Tesarik, J. (1998) Oscillin—reopening the hunting season. *Mol. Hum. Reprod.*, **4,** 1007–1009.

Tesarik, J. and Testart, J. (1994) Treatment of sperm-injected human oocytes with Ca^{2+} ionophore supports the development of Ca^{2+} oscillations. *Biol. Reprod.*, **51,** 385–391.

Tesarik, J., Sousa, M. and Testart, J. (1994) Human oocyte activation after intracytoplasmic sperm injection. *Hum. Reprod.*, **9,** 511–518.

Tesarik, J., Sousa, M., Greco, E. *et al.* (1998) Spermatids as gametes: indications and limitations. *Hum. Reprod.*, **13** (Suppl. 3), 89–107.

Van Steirteghem, A.C., Liu, J. and Joris, H. (1993) Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum. Reprod.*, **8,** 1061–1066.

Yanagida, K., Yanagimachi, R., Perreault, S.D. *et al.* (1991) Thermostability of sperm nuclei assessed by microinjection into hamster oocytes. *Biol. Reprod.*, **44,** 440–447.

Yanagida, K., Katayose, H., Hoshi, K. *et al*. (1997) Effect of electrical stimulation on oocyte activation after intracytoplasmic sperm injection. *J. Mamm. Ova Res*., **14,** 132–138.

Yanagida, K., Yazawa, H., Katayose, H. *et al*. (1998) Influence of oocyte preincubation time on fertilization after intracytoplasmic sperm injection. *Hum. Reprod.*, **13,** 2223–2226.

Yanagida, K., Katayose, H., Yazawa, H. *et al*. (1999) The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans. *Hum. Reprod*., **14**, 448–453.

Yanagimachi, R. (1994) Mammalian fertilization. In Knobil, E. and Neill, J.D. (eds), *The Physiology of Reproduction.* 2nd edn. Raven Press, New York, USA, pp. 189–317.

Yanagimachi, R. (1997) The mammalian oocyte's response to intracytoplasmic sperm injection (ICSI) In Motta, P.M. (ed.), *Microscopy of Reproduction and Development: A Dynamic Approach.* Antonio Delfino Editore, Rome, Italy, pp. 187–195.

Received on August 17, 1998; accepted on February 2, 1999