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Recent scientific and medical advances in assisted human conception

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Introduction

It is a pleasure to contribute to Denis New's valedictory issue of The International Journal of Developmental Biology by describing some recent advances in the science and medicine of assisted human reproduction. During the early days of this field, in the 1970's and 1980's, he and I occupied adjacent rooms, and I watched with admiration his work on the culture of the post-implantation mammalian embryo. On several occasions, his methods of tissue culture helped me to test new methods of growing human embryos *in vitro*, and I shall always be grateful for his advice so freely given.

It is impossible both for want of time and because there is an immense of material to do anything other than describe three current topics of great interest in assisted human reproduction. Even then, their scientific and clinical aspects can be covered only briefly. The topics include the intracytoplasmic injection of a spermatozoon into human eggs (ICSI), studies on the growth of cleaving human embryos *in vitro*, and the preparation of the hatched blastocyst for implantation.

ICSI and male infertility

Development of the intracytoplasmic injection of spermatozoa into oocytes (ICSI)

Some advances in assisted human conception are breathtakingly simple in design, yet touch upon profound scientific and clinical aspects of a particular topic. ICSI is such an advance. The concept of microinjecting a single spermatozoon into the perivitelline space or into the ooplasm, in order to achieve a form of fertilization, had earlier been achieved in a few and uncontrolled animal studies. The clinical advantages of helping infertile men to conceive using this approach were apparent to most investigators working on *in vitro* fertilization (IVF). IVF itself had already opened novel opportunities of alleviating some moderate forms of male infertility, and by 1990, when the possibilities of ICSI were beginning to emerge, more than 150,000 IVF babies had been delivered world-wide. The methods had spread into most countries, providing a source of technology that could adapt new advances in research. The weakness of IVF for the treatment of male infertility lay in dealing with severe oligozoospermia, where insufficient spermatozoa of normal morphology and good activity were available to achieve successful fertilization *in vitro*.

Several clinics had already turned to micromanipulation, to drill holes in the zona pellucida in order to help spermatozoa gain access to the oolemma. Others had injected one or more spermatozoa into the perivitelline space, so bypassing the difficulties of achieving normal sperm passage through this membrane. Success with these methods was moderate, and many eggs were fertilized abnormally. The succeeding explosive events leading to the introduction of ICSI would show once again how research with an applied motive can produce rapid scientific and clinical advances in a particular field.

Curiously, initial attempts to apply ICSI to human eggs met with virtually no success at all (Lanzendorf *et al.*, 1988). Pessimism about its prospects hampered further attempts at ICSI for some years, yet in retrospect there seems to be little doubt that these researchers were using insufficient force to penetrate the oolemma. One laboratory, headed by Andre Van Steirteghem and Paul Devroey in Brussels, nevertheless persisted with micromanipulative fertilization. Apparently, they were attempting to improve fertilization rates by injecting spermatozoa into the perivitelline space, when one day the injection pipette was pushed accidentally into the ooplasm and the spermatozoa were injected there. To their delight, pronuclei formed in the egg, and the procedure was repeatable. If the initial breakthrough had a slice of

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fortune about it, the subsequent development of ICSI by this team was achieved with a single-minded purposefulness.

The large-scale introduction of ICSI began in 1991/2. Individual spermatozoa prepared from the semen of men with severe oligozoospermia and other disorders were drawn tail-first into the injection pipette and injected directly into the ooplasm. Many pronucleate eggs and embryos were produced as the micromanipulation technique was mastered (Table 1). Two pronuclei formed in more than two-thirds of the injected oocytes, and >70% of the embryos undergo cleavage. Embryos grew excellently *in vitro*, just as well as those achieved by IVF, and transfers produced implantations, pregnancies and births (Palermo *et al.*, 1992). ICSI instantly offered far more scope than other micromanipulative approaches, and within 12 months large amounts of data were accruing in the Brussels clinic (Van Steirteghem *et al.*, 1993a,b). Severe – even very severe forms of infertility were treated as confidence grew, as I shall describe below.

ICSI and fundamental studies on fertilization

It was also apparent that this simple clinical procedure had upset some previous concepts on the processes of fertilization. ICSI had apparently by-passed the closely-knit interactions occurring during fertilization, including sperm-egg fusion, membrane hyperpolarization and calcium discharges in the oocyte and the activation of the egg.

Perhaps the success of ICSI was nevertheless a narrow victory over some problems involving the cell cycle. The successive events of activation and pronuclear formation during fertilization after ICSI proceeded quite normally in most eggs. Some oocytes nevertheless displayed a disordered activation, and pronuclei formed abnormally in one-quarter of them. Approximately 5% of the resulting fertilized eggs were evidently digynic, since they contained three pronuclei and no extruded second polar body. The frequency of digynic tripronucleate eggs, which are very rare after IVF, indicated that both chromosome sets on the anaphase spindle had somehow entered the egg instead of separating at anaphase (Edwards, 1995). A few fertilized eggs with a single pronucleus were androgenic or gynogenetic in origin. The formation of androgenic eggs with a single (male) pronucleus also indicated that the spindle was functioning abnormally, in this case to expel all chromosomes into polar bodies. Other unipronucleate eggs may have been gynogenetic, with an inert spermatozoon. Nor was the condensation of the pronuclei and their disappearance at syngamy always synchronous.

TABLE 1

INITIAL OBSERVATION ON FERTILIZED HUMAN EGGS AFTER ICSI, USING SPERM SAMPLES FROM OLIGOZOOSPERMIC MEN

	Numbers of spermatozoa		
	$<1\times10^6$	$<5\times10^6$	$>5\times10^6$
No. of cycles	97	128	684
Oocytes injected	1038	1386	6981
Intact oocytes (%)	92.1	88.7	88.7
Two pronuclei (%)	64.5	70.4	70.7
One pronucleus (%)	5.1	3.9	3.7
Three pronuclei (%)	4.8	4.3	4.2
Percentage of eggs that cleaved	69.3	69.4	70.7
Clinical pregnancies	24	27	190
(%) of transfers	(25.8)	(22.0)	(30.0)

(Nagy *et al.*, 1995).

Anomalies were perhaps to be expected during ICSI in view of the artificial nature of the process. Existing knowledge had also illustrated a need to coordinate the cell cycles of an oocyte and a cell undergoing experimental fusion with it. There was clear evidence from cloning that two cells undergoing fusion should preferably be at the same stage of the cell cycle, otherwise an M-phase cell could drive a G1 or G2 cell into a premature mitosis. In fact, the number of anomalies proved to be modest, and not too different from those occurring after normal fertilization or IVF. Unipronucleate eggs are rare during IVF, perhaps forming 1% of the total undergoing fertilization. Their formation indicates that cell-cycle anomalies have arisen which prevent the formation of the other pronucleus or restrict its growth. Some unipronucleate eggs are gynogenetic, as the sperm nucleus fails to decondense, and others are androgenic because all oocyte chromosomes have been expelled into polar bodies (Edwards *et al.*, 199). Still others are formed because the centrosome of the fertilizing spermatozoon is unable to organise a normal first cleavage spindle (Asch *et al.*, 1995; Van Blerkom, 1996). Many androgenic oocytes contain a single pronucleus and a set of prematurely condensed chromosomes, or two sets of condensed chromosomes (Schmiady and Kentenich, 1993).

These anomalies arising after ICSI indicated that the processes of fertilization had become defective in some oocytes. During normal fertilization, capacitated, acrosome-reacted spermatozoa bind to ZP3 on the zona pellucida, then pass through this investment into the perivitelline space. Adhesion peptides such as integrin and disintegrin on the sperm surface and oolemma, or the acrosomal adhesion protein PH-30, mediate sperm-oocyte binding (Bronson and Fusi, 1990; Blobel *et al.*, 1992). Spermatozoa become less motile as their plasma membrane fuses with oocyte membrane. The resulting membrane hyperpolarization possibly activates G proteins and inositoltriphosphate, to result in the formation of membrane phospholipases (Moore *et al.*, 1993).

Membrane hyperpolarization or the action of polypeptides released from the fertilising spermatozoon invoke a propagating wave of calcium release (Dozortsev *et al.*, 1995b). Inositoltriphosphate is generated and an oscillatory calcium release occurs; ryanodine receptors may also be involved (Homa *et al.*, 1992; Swann, 1992; Whitaker and Swann, 1993). Calcium itself evokes this pulsatile calcium-induced calcium release (CICR), recognizable in repetitive calcium transients (spikes) in the oocyte over a period of hours after fertilization (Cuthbertson and Cobbold, 1985; Swann, 1990; Whitaker and Patel, 1990). These oscillations begin 20-35 min after the insemination of human eggs.

Calcium spiking evidently stimulates mitosis promoting factor (MPF), re-initiates the cell cycle, invokes the discharge of cortical granules and activates metaphase 2 by invoking the formation of enzymes that destroy mos protein and other proteins (Babcock *et al.*, 1992). In *Xenopus*, the enzyme calpain is responsible for destruction of mos protein within a few minutes of sperm entry, and this leads to the disassociation of the MPF/cyclin/mos complex which holds oocytes at metaphase 2. Calpain has not yet been found in mammalian eggs. The sperm head then enters the

ooplasm, apparently guided by its own limited movement and independently of microfilaments (van Blerkom *et al.*, 1995). Isolated sperm centrioles have been transferred into human oocytes, and shown to be capable of enucleating tubulin and organizing a normal-looking sperm aster (Van Blerkom and Davis, 1995). The second polar body is extruded, sperm chromatin decondenses as the nuclear envelope disintegrates, and disulphide bonds between protamines are degraded by proteolysis. The protamines are replaced by nucleohistones synthesized by the egg (Tesarik, 1992).

During normal fertilization, the sperm nucleus therefore begins to decondense 3-4 h after sperm entry, and nucleohistones form in the male pronucleus. Protamines or the condensed nature of sperm DNA may protect it from the M factors in the oocyte until the MPF/cyclin/mos complex is destroyed. The rapidly-forming male pronucleus may thus avoid exposure to M-phase factors during normal fertilization.

A quite different situation could arise after ICSI. Sperm/oocyte binding and presumably membrane hyperpolarization do not occur. A persisting MPF/cyclin/mos protein complex in the oocyte could create conditions which impair the growth of the decondensing sperm head or drive it to a precocious metaphase. The absence of membrane polarization could also distort the normal process of polar body extrusion and lead to the digynic tripronuclear eggs typical of ICSI.

The passage of the pipette in the ooplasm during ICSI produces only a modest single calcium transient, judged as insufficient to induce activation (Tesarik *et al.*, 1994). After a lag period of 4-12 h, a sequence of oscillatory or non-oscillatory spikes of calcium are released, the former being similar to those occurring during normal fertilization. 'Mock' ICSI operations involving medium alone rarely activate metaphase 2 oocytes (Flaherty, 1995). When ICSI is performed with a spermatozoon, polar body extrusion and pronucleus formation are not usually delayed (Nagy *et al.*, 1994). The sperm nucleus swells 2 h after ICSI whether the oocyte is activated or not, but further pronucleus development depends on the oocyte having been activated (Dozortsev *et al.*, 1995a).

The events occurring during ICSI must be clarified in order to understand these events of egg activation and other aspects of the procedure. Some technical aspects are still unclear, such as the advantage gained by damaging the sperm tail by touching or excising part of it, which seems to improve the success of ICSI (Fishel *et al.*, 1993; Gerris *et al.*, 1995). This procedure may indeed result in a highly significant increase in the incidence of fertilization, and a reduction in cytoplasmic fragmentation among blastomeres of the resulting embryo (Fishel *et al.*, 1993). It may help the sperm pronucleus to form and migrate in the oocyte (Van Blerkom *et al.*, 1995). Fortunately, eggs with two pronuclei (the overwhelming majority) do not appear to be affected by any disorders in pronucleus formation or syngamy, and grow and implant as well as embryos conceived by IVF.

Clinical development of ICSI

The introduction of ICSI has virtually enabled every man with severe oligozoospermia or oligoteratozoospermia to have his own child. The majority of ICSI cases involve the injection of a washed, ejaculated spermatozoon, but other more remarkable cases have been described and are now treated routinely. Among these are the successful fertilizations achieved in couples where the husband produces as few as 10-20 spermatozoa in all, and their wives may actually produce more eggs than the husbands' spermatozoa. Ejaculated spermatozoa are not essential for ICSI. Epididymal and testicular spermatozoa, fresh or cryopreserved, can be used successfully and have given good rates of fertilization and many human births. Testicular spermatozoa are extracted from a testis biopsy or from testis material withdrawn in a syringe, an approach successful in cases of obstructive or non-obstructive azoospermia (Devroey *et al.*, 1995a,b; Tsigoritis *et al.*, 1995). Spermatids can be used instead of spermatozoa (Vanderzwalnen *et al.*, 1995), and a few living human offspring have been born (Fishel *et al.*, 1995; Tesarik *et al.*, 1995). Spermatids can be obtained from ejaculates or testis preparations, and can be sucked off Sertoli cell membranes if necessary (Silber, 1995a,b).

TABLE 2

ANOMALIES IN 130 CHILDREN CONCEIVED BY ICSI COMPARED WITH 130 CONCEIVED BY IVF

ICSI	IVF
1 preauricular tag	1 entropion
1 sacral dimple	2 congenital naevi
5 congenital hemangioma	2 congenital angioma
3 hydroceles	1 congenital hip subluxation
3 umbilical hernias	1 congenital hip dysplasia
1 congenital hip dislocation	1 umbilical hernia
1 ventricular septum defect (closed)	1 pes equinovarus
3 atrium septum defects (closed)	1 metatarsus adductus
	1 club foot
	7 atrium septum defects (closed)
	2 ductus arteriosus (closed)
	1 veso-urethral reflux

(Bonduelle *et al.*, 1995)

Some clinical risks with ICSI have arisen unexpectedly. There is apparently a higher risk of sex chromosome trisomies in embryos derived from ICSI, perhaps arising from men who are gonadal mosaics for these trisomies (In't Veld *et al.*, 1995; Van Steirteghem, 1996). The gene causing congenital absence of the vas has proved to be a variant of cystic fibrosis, so considerable care is needed to ensure that the wife is free of this disease, and counselling is essential (Patrizio *et al.*, 1993). There are also risks with ICSI in the inheritance of Y chromosome deletions in severely oligozoospermic men (Reijo *et al.*, 1995). Subject to these somewhat considerable reservations, the overall frequency of anomalies in children is nevertheless similar to those arising in IVF or after natural conception (Table 2) (Bonduelle *et al.*, 1995; Wisanto *et al.*, 1995).

By now, hundreds of children have been conceived by ICSI. It is practised worldwide in IVF clinics. Andrologists are overjoyed with its success, and wonder what forms of male infertility are left to be cured (Silber, 1995a). Unfortunately, the procedures are expensive, since a series of additional technical sequences are added on to the cost of IVF. Totally different approaches will doubtless be needed to most of the world's infertile men, and these will depend on a deeper understanding of andrology and fertilization. Nevertheless, ICSI has led to sharp reductions in the need for donor insemination, raised the possibility of achieving fertilization using a single spermatozoon of a single type (e.g. with an X or a Y chromosome) to avoid the birth of children with genetic disease,

and has raised the hopes of patients where IVF fails in several successive cycles. It has helped men with spinal injury to conceive, since it is easier to extract testicular spermatozoa from them than to obtain semen by means of electroejaculation.

In vitro growth of the human preimplantation embryo

The second topic of this review concerns the growth of human embryos *in vivo*, and the applications of various experimental procedures to them.

The human preimplantation embryo in culture

Many different culture media sustain the growth of the human embryo *in vitro*, such as Earle's medium which contains pyruvate when a protein such as albumin is added. Ham's F10, minimal essential medium (MEM), and others including Whitten's, Ham's F12 and media based on human tubal fluid (Bavister, 1995). One-half of the embryos grow to the blastocyst stage. The advantages of avoiding the use of glucose in culture media during the early post-fertilization stages of development is a topic of current interest. Serum or other biological fluids are added to culture media in many clinics, at a time when defined solutions are urgently needed to standardize the conditions of growth (Leese, 1995).

There are no apparent differences between embryos growing in culture and those of equivalent stage that were flushed from the reproductive tract of women volunteers (Edwards and Brody, 1995). Growth rates *in vitro* have been quantified, and most human embryos reach 2 cell on day 2-3 post-insemination, and 8-16 cell on day 3-4 (Fishel *et al.*, 1985) (Table 3). Many embryos developing *in vitro* have abnormally nucleated or anucleated blastomeres, abnormal cytology, cytoplasmic debris, necrosis, uneven-sized blastomeres or other pathological signs. Rare embryos are still pronucleate on day 2.

Various protocols have been devised to assess the growth of embryos *in vitro*. These include cleavage rates or cleavage numbers, using log-transformed cell numbers, the numbers of uneven blastomeres, fragments and other anomalies. There is no obvious block to development, as found in rodent embryos *in vitro*. Embryos may be arrested in 2 cell by 2.5 days after fertilization (Edwards and Brody, 1995) yet are apparently capable of developing to full term when replaced in their mother (Fishel *et al.*, 1982). Timing the formation of morulae or early blastocysts is another guide to human embryonic development *in vitro*. The quality of growth has been assessed in single embryos cultured in 5 ul microdrops by measuring the uptake of metabolites or the secretion of various factors (Leese, 1995).

TABLE 3

TIMING OF THE GROWTH OF HUMAN EMBRYOS *IN VITRO*

Mid-stage of development	Hours post-insemination
2 cell	33.2 ± 1.3
4 cell	49.0 ± 1.9
8 cell	64.8 ± 1.8
16 cell	80.7 ± 2.4
Morulae	96.8 ± 1.9
Blastocyst	112.7 ± 2.9
Hatched blastocyst	ca 178

Blastocysts form *in vitro* at the 32-64 cell stage, usually on day 5 after a mean of 4.6-6.5 cleavages (Steptoe *et al.*, 1971; Hardy *et al.*, 1989). Cells may be allocated to lines which develop into inner cell mass or trophectoderm early in growth, even in 4-8 cell stages, and more human cells are allocated to inner cell mass than in other species (Hardy *et al.*, 1989). The inner cell mass can be very distinct or diffuse and contain varying numbers of cells, yet even abnormal blastocysts contain a few ICM cells. Some human blastocysts with slow cleavage possess only 5 or 6 cells on day 5. Others contain several small blastocoelic cavities, membranous folds or septae traversing the blastocoelic cavity, a diffuse inner cell mass and localized areas of necrosis. Some blastocysts have two inner cell masses, no inner cell mass, or display various forms of damage or necrosis.

Many human embryos are chromosomally aneuploids, perhaps as many as one-third in total. These conditions arise through various errors in meiosis, fertilization and cleavage. Anomalous growth may be due to chromosomal imbalance such as monosomy, trisomy, triploidy, tetraploidy, structurally-changed chromosomes and other anomalies. Many other embryos, including some developing normally, are apparently complex chromosomal mosaics. The frequency of these abnormalities is twice as high in poor-quality embryos (Pellestor, 1995).

Chromosomal anomalies such as trisomy or monosomy arise as an unbalanced complement inherited from the gametes, especially the oocyte. Dispermy leading to the formation of fertilized eggs with three pronuclei occurs in approximately 5-6% of eggs. Many tripronucleate eggs undergo a normal syngamy and grow as triploids, whereas others display various pronuclear or abnormalities at syngamy which lead to complex haploid mosaics, diploid/triploid mosaics and complex heteroploids. Mosaicism, haploidy, triploidy (and higher levels of euploidy) stem from parthenogenesis, high-order polyspermy or digyny, and chromosome fragmentation. Most tetraploid embryos probably arise through the suppression of the first or a later cleavage division, or as a consequence of trispermy. The chromosomes in a few embryos have structural damage or fragmentation. Many high-quality embryos, and even more of those of poor-quality, carry a chromosomal abnormality (Ederisinghe *et al.*, 1992).

Fluorescence *in situ* hybridization (FISH) using chromosome primers enables at least four different chromosome pairs to be identified in a single blastomere nucleus (Munne *et al.*, 1993). It has revealed how abnormal embryos arise through errors in syngamy or a later cleavage division. It also reveals that many embryos with a good morphological quality carry a chromosomal abnormality (Ederisinghe *et al.*, 1992). Anomalies afflict 70% of embryos with arrested cleavage. Multiple nuclei within a blastomere arise through a failure of cytokinesis, and may result in groups of cells composed as a syncytium. These syncytia could be precocious syncytiotrophoblast cells. Many embryos (perhaps >30%) are mosaic, including diploid/heteroploid mosaics which presumably arise through non-disjunction during cleavage (Zenzes *et al.*, 1992; Delhanty and Handyside, 1995). Five or more polyploid cells were identified in 30% of human blastocysts, during tests for chromosomes X, Y and 18; this proportion will presumably rise much higher when other chromosome pairs are included in the scores for mosaicism (Benkhalifa *et al.*, 1993). The lack of cell-cycle checkpoints, and a short G1 phase, have been suggested as potential causes of the high incidence of chromosomal anomalies in human preimplantation embryos (Delhanty and Handyside, 1995).

Various structural disorders arise in individual cells of human embryos growing *in vitro*. Two-thirds display some form of anomaly by the 4-8 cell stage (Winston *et al.*, 1991), including blastomeres with a normal ultrastructure yet containing multiple nuclei which incorporate DNA precursors. Blastomeres with no obvious signs of damage may divide to produce descendants containing one or more subnuclei, and multinucleated cells may produce cells with a single nucleus; these events clearly cause many chromosomal and other anomalies in human embryos (Pickering *et al.*, 1995). Other blastomeres possess extranuclear chromatin with a low precursor uptake, or many pseudonuclei; these cells may be expelled into extracellular spaces. Micronuclei or extruded chromatin could be a consequence of chromosomal non-disjunction and they could result in mosaicism. Cytoplasmic fragments or abnormal cells may be excluded from inner cell mass into the blastocoelic cavity, or from trophectoderm to the perivitelline space.

Two-fifths of pronucleate eggs cultured *in vitro* form blastocysts with between <10->60 nuclei on day 5, and only one-half of embryos develop normally from fertilization (Winston *et al.*, 1991). Reactive oxygen species such as H_2O_2 could impair the growth of human embryos. H_2O_2 is synthesized by preimplantation embryos and may act as a second messenger. Its levels treble in mouse pronucleate eggs exposed to gas phases containing 20% or 40% oxygen or excess light, and mouse embryos grow more rapidly if superoxide dismutase, EDTA or thioredoxin, a protein disulphide reductase, are included in culture medium (Nasr-Esfahani *et al.*, 1990). Such anomalous forms of growth are recognized today as being due to apoptosis or necrosis.

Apoptosis may be activated by reactive oxygen species, growth factors and high intracellular levels of calcium, and results in the formation of cytoplasmic fragments typical of cleaving human embryos and blastocysts (Parchment, 1993; Pierce *et al.*, 1993). The end-labeling of DNA, identified by combined fluorochrome/terminal transferase (TUNEL), combined with analyses of ultrastructure, indicate that apoptosis occurs in cells of many cleaving human embryos (Juriscova *et al.*, 1995). Other blastomeres appear to undergo necrosis. These changes may be due to the actions of the gene for c-*myc*. Its transcripts and those for TNF-α and APOI/FAS antigen have been identified in mammalian oocytes and preimplantation embryos (Chen *et al.*, 1993; Pal *et al.*, 1993; Guo *et al.*, 1994). TNF-α is released from cleaving human embryos *in vitro*, but not from blastocysts, in the range 2-117 pg/ml/24 h (Lachapelle *et al.*, 1993).

Experimental studies on cleaving human embryos and blastocysts

Feeder layers (co-cultures) of various cell types may sustain the growth of human embryos *in vitro*, by 'conditioning' medium or absorbing harmful components in it (Bongso *et al.*, 1993). Many of the beneficial effects claimed for co-cultures must be non-specific, since kidney epithelium sustains embryonic growth as effectively as oviductal epithelial cells. Benefits claimed for co-cultures include less blastomere fragmentation, more blastomeres at transfer, better rates of blastocyst formation, expansion and hatching and embryos with higher implantation rates. Unfortunately, an equal number of reports contradict this finding.

Micromanipulation has been applied to animal embryos for many years. It forms the basis of preimplantation genetic diagnosis, designed to help to avoid the birth of children with inherited disease. This topic has been discussed in detail elsewhere (Edwards and Brody, 1995). To perform these diagnoses, one or more blastomeres are excised from cleaving human embryos or small pieces of trophectoderm from blastocysts. Preimplantation diagnosis is being applied clinically to relatively simple situations such as sickle-cell anemia where the mutation involves an A→T change in codon 6 of the ß-globin gene. It is also being applied to more complex systems such as deletions or *de novo* mutations arising in different exons of the Duchenne and Becker muscular dystrophy genes. As with amniocentesis and chorionic villus sampling (CVS), knowledge of the exact nature of the genetic risk in a particular family is helpful but not always essential. Typing embryos for sex using X- or Y-linked markers can avoid the birth of boys afflicted with X-linked mutant genes.

Today, many embryos are diagnosed using fluorescent *in situ* hybridization (FISH), or DNA methods based on the polymerase chain reaction (PCR). Amplification by PCR is successful in 90% of single blastomeres (Pickering and Muggleton-Harris, 1995), and two of the most frequent mutations for Tay-Sachs disease were amplified in >90% of single blastomeres (Sermon *et al.*, 1995). Embryos can be sexed by PCR using a 149 bp sequence repeated 800-5000 times on the long arm of the Y chromosome and Y sequences on the pseudoautosomal boundary; by combining a 130 bp sequence on the X chromosome with a 500 bp Page sequence on the Y in a duplex assay, and by identifying the two amelogenin alleles present on X and Y chromosomes respectively (Levinson *et al.*, 1992). Fluorescent PCR, in which different electrophoretic bands are stained in a variety of colors, offers a variant of PCR (Findlay *et al.*, 1995). FISH carried out on an excised blastomere or on pieces of trophectoderm enables embryos to be sexed by visualizing the sex chromosomes. Chromosomes 13, 18 and 21 can also be scored simultaneously with the X/Y pair using different-colored fluorochromes (Delhanty and Handyside, 1995). As yet, preimplantation diagnosis has been confined to relatively few clinics. The total number of children born whose genotypes were correctly diagnosed cannot exceed 50, and at least three errors in diagnosis have been reported (Handyside, 1996).

The hatching human blastocyst and its preparations for implantation

Finally, I would like to discuss the preparation of the human blastocyst for implantation. Much of these data have accumulated during the past five years.

Hatching of the blastocyst

Human blastocysts prepare for implantation by escaping from their enclosing zona pellucida by days 6-7 post-fertilization. *In vitro*, enzymes digest a hole in the zona pellucida, and blastocysts then escape by a limited series of contractions and expansions – perhaps 5-6 in all. Internal pressure from the blastocysts may assist hatching. Human blastocysts can become semi-constricted during hatching *in vitro*, trapped temporarily half-in and half-out of the slit zona pellucida and so resemble a figure 8. Drilling small incisions in the zona pellucida during cleavage is reported to overcome problems in hatching and improve implantation rates, but no prospective trials have been reported (Cohen *et al.*, 1990). Embryos failing to hatch perish within their zona pellucida *in vitro*, which may represent a significant form of embryonic loss *in vivo*.

The empty zona pellucida contains living and dead cells and cell debris.

Many growth factors, inducers, cytokines and paracrine factors including IGF-II, TGF-α, FGF and PDGF are synthesized by hatched blastocysts as implantation approaches. Receptors for insulin, EGF, PDGF, TGF-α and EGF, and IL-1ß, IL-1r and IL-r are expressed in human blastocysts (Mühlhauser *et al.*, 1993; Simon, 1995). Differentiating trophoblast of the preimplantation blastocyst expresses regulatory factors, substrates and adhesion molecules including cytokeratin, placental-type alkaline phosphatase, plasminogen activator (PA) and its inhibitors (PAI-1 and PAI-2), high affinity uPA receptors, HLA class I framework antigens, NODG5 antigen, receptors for integrins and LIF (especially in blastocysts), and IGF-II mRNA and protein (Bhatt *et al.*, 1991; Sharkey *et al.*, 1995). Integrins expressed in human preimplantation embryos include a3, a5, av, b1, b3, and b4, plus the E-cadherins, ICAM-1 and NCAM (Fusi *et al.*, 1992; Turpeenniemi-Hujanen *et al.*, 1992; Campbell *et al.*, 1995). The gene for u-PA may confer invasiveness on trophoblast, as in somatic cells. Many of these cytokines and substrates are expressed in the endometrium, and a correct functioning of blastocyst-endometrial relationships is essential for implantation to occur (Stewart *et al.*, 1992).

Human blastocysts may send various signals to the mother (Table 4). They may secrete embryo-derived platelet activating factor (EDPAF) (O'Neill *et al.*, 1985), and early pregnancy factor (EPF), another elusive factor. Steroid signals include estradiol and perhaps progesterone, in amounts correlated with embryonic HCG secretion (Edgar *et al.*, 1993). Human blastocysts secrete HCG especially after hatching (Fishel *et al.*, 1984). *In vivo*, they produce 16,000 mIU daily by day 10 and 160,000 mIU by day 14, more than most blastocysts growing *in vitro* although some can release 19,500 mIU into culture medium (Lopata and Oliva, 1993). Insulin, transferrin and PDGF seem to be involved in initiating HCG secretion from human blastocysts by 7-8 days *in vitro*.

It is now recognized that the blastocyst is highly active genetically. In addition to preparing for implantation, its embryonic tissue also differentiates rapidly. The successive changes occurring in human blastocysts during differentiation, germ layer formation and organogenesis have been described elsewhere (Edwards and Brody, 1995).

Conclusions

This brief review has concentrated on ICSI and the growth of human embryos *in vitro*. ICSI is a new technique with immense consequences for the clinical treatment of very severe male infertility. Studies on cleaving human embryos *in vitro* have illustrated the existence of an astonishing number of variations and anomalous forms of growth. Micromanipulations on embryos to remove one or more cells for genetic diagnosis promise to introduce new approaches to prenatal diagnosis. More knowledge is needed on hatching and hatched human blastocysts. These stages are of major importance in achieving a successful implantation, and implantation rates per embryo still remain very low during assisted human reproduction (<20%). An improvement in this rate would be among the most important advances in IVF at the present time.

These advances encompass a variety of studies: from advanced molecular biology to the improvement of culture media by assessing their physical and chemical properties. Assisted human conception thus offers numerous opportunities for investigators who specialize in clinical or fundamental studies.

TABLE 4

POTENTIAL PRE-IMPLANTATION SIGNALS FROM THE EMBRYO TO THE MOTHER

EDPAF	Pregnancy-specific plasma protein C
EPF	Histamine-releasing factors
Estradiol 17ß	Prostaglandins
Progesterone	Inhibins
HCG	TGF-α, IGF-2

(Edwards and Brody, 1995)

KEY WORDS: *sperm injection, oocytes, human, fertilization, embryos*

References

ASCH, R.H., SIMERLY, C., ORD, V.A. and SCHATTEN, G. (1995). The stages at which human fertilization arrests: microtubule and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in humans. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 1897-1906).

BABCOCK, D.F., BOSMA, M.M., BATTAGLIA, D.E. and DARSZON, A. (1992). Early persistent activation of sperm K^+ channels by the egg peptide speract. *Proc. Natl. Acad. Sci. USA 89:* 6001-6005.

BAVISTER, B.D. (1995). Culture of preimplantation embryos: facts and artefacts. *Hum. Reprod. Update 1:* 91-148.

BENKHALIFA, M., JANNY, L., VYE, P., MALET, P., BOUCHER, D. and MENEZO, Y. (1993). Assessment of polyploidy in human morulae and blastocysts using coculture and fluorescent *in-situ* hybridization. *Hum. Reprod. 8:* 895-902.

BHATT, H., BRUNET, L.J. and STEWART, L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc. Natl. Acad. Sci. USA 88:* 11408-11412.

BLOBEL, C.P., WOLSBERG, T.G., TURCK, C.W., *et al.* (1992). A potential fusion peptide and an integrin legend domain in a protein active in sperm-egg fusion. *Nature 356:* 248-252.

BONDUELLE, M., LEGERN, J., DERDE, M-P., BUYSSE, A., WISANTO, A., DEVROEY, P., VAN STEIRTEGHEM, A. and LIEBARS, J. (1995). Comparative follow-up study of 130 children born after intracytoplasmic sperm injection and 130 children born after *in vitro* fertilization. *Hum. Reprod. 10:* 3327-3331.

BONGSO, A., FONG, C.Y., NG, S.C. and RATNAM, S. (1993). The search for improved *in vitro* systems should not be ignored: embryo co-culture may be one of them. *Hum. Reprod. 8:* 1155-1160.

BRONSON, R.A. and FUSI, F. (1990). Evidence that the Arg-Gly-Asp (RGD) adhesion sequence plays a role in mammalian fertilization. *Biol. Reprod. 43:* 1019-1025.

CAMPBELL, S., SWANN, H.R., SEIF, M.W., KIMBER, S.J. and APLIN, J.D. (1995). Cell adhesion molecules on the oocyte and the preimplantation human embryo. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 1571-1578).

CHEN, H., MARCINKIEWICZ J., SANCHO-TELLO, M., HUNT, J. and TERRANOVA, P. (1993). Tumour necrosis factor-a expression in mouse oocytes and follicular cells. *Biol. Reprod. 48:* 707-714.

COHEN, J. *et al.* (1990). Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Hum. Reprod. 5:* 7-13.

CUTHBERTSON, K.S.R. and COBBOLD, P.H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillation in cell Ca^{++}. *Nature 316:* 541-542.

DELHANTY, J.D.A. and HANDYSIDE, A.H. (1995). The origin of genetic defects in the human and their detection in the preimplantation embryo. *Hum. Reprod. Update 1:* 201-215.

DEVROEY, P., LIU, J., NAGY, Z., GOOSSENS, A., TOURNAYE, H., CAMUS, M., VAN STIERTEGHEM, A. and SILBER, S. (1995a). Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum. Reprod. 10:* 1457-1460.

DEVROEY, P., SILBER, S., NAGY, Z., LIU, J., TOURNAYE, H., JORIS, H., VERHEYEN, G. and VAN STEIRTEGHEM, A. (1995b). Ongoing pregnancies and birth after intracytoplasmic sperm injection with frozen-thawed epididymal spermatozoa. *Hum. Reprod. 10:* 903-906.

DOZORTSEV, D., DE SUTTER, P., RYBOUCHKIN, A. and DHONT, M. (1995a). Timing of sperm and oocyte nuclear progression after intracytoplasmic sperm injection. *Hum. Reprod. 10:* 3012-3017.

DOZORTSEV, D., RYBOUCHKIN, A., DESUTTER, P., QIAN, C. and DHONT, M. (1995b). Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. *Hum. Reprod. 10:* 403-407.

EDERISINGHE, W.R., MURCH, A.R. YOVICH, J.L. (1992). Cytogenetic analysis of human oocytes and embryos in an *in-vitro* fertilization programme. *Hum. Reprod. 7:* 230-236.

EDGAR, D.H., JAMES, G.B. and MILLS, J.A. (1993). Steroid secretion by early human embryos in culture. *Hum. Reprod. 8:* 277-278.

EDWARDS, R.G. and BRODY S. (1995). *Principles and Practice of Assisted Human Reproduction.* WB Saunders, Philadelphia.

EDWARDS, R.G., CROW, J., DALE, S., MACNAMEE, M.C., HARTSHORNE, G.M. and BRINSDEN, P. (1992). Pronuclear, cleavage and blastocysts histories in the attempted preimplantation diagnosis of the human hydatidiform mole. *Hum. Reprod. 7:* 994-998.

FINDLAY, I., URQUHART, A., QUIRKE, SULLIVAN, K., RUTHERFORD, A. and LILFORD, R. (1995). Simultaneous DNA fingerprinting, diagnosis of sex and single-gene defect status from single cells. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 1609-1618).

FISHEL, S., COHEN, J., FEHILLY, C., PURDY, J.M., WALTERS, D.E. and EDWARDS, R.G. (1985). Factors affecting human embryonic development *in vitro.* In *In Vitro Fertilization and Embryo Transfer* (Eds. M. Seppala and R.G. Edwards). Annals of the New York Academy of Sciences 442, pp. 564-576.

FISHEL, S.B., EDWARDS, R.G. and EVANS, C.J. (1984). Human chorionic gonadotrophin secreted by preimplantation embryos cultured *in vitro. Science 223:* 816-818.

FISHEL, S.B., EDWARDS, R.G. and PURDY, J.M. (1982). Births after a prolonged delay between oocyte recovery and fertilization *in vitro. Gamete Res. 9:* 175-181.

FISHEL, S., GREEN, S., BISHOP, M. *et al.* (1995a). Pregnancy after intracytoplasmic injection of a spermatid. *Lancet 345:* 1641-1642.

FISHEL, S.B., LISI, F., RINALDI, L., GREEN, S., HUNTER, DOWELL, K. and THORNTON, S. (1995b). Systematic examination of immobilising spermatozoa before intracytoplasmic sperm injection in the human. *Hum. Reprod. 10:* 497-500.

FLAHERTY, S., PAYNE, D., SWANN, N.J. and MATTHEWS, C.D. (1995). Assessment of fertilization failure and abnormal fertilization after intracytoplasmic sperm injection (ICSI). *Reprod. Fertil. Dev. 7:* 197-210.

FUSI, F.M., VIGNALLI, M., BUSACCA, M. and BRONSON, R.A. (1992). Evidence for the presence of an integrin on the oolemma of unfertilised human oocytes. *Mol. Reprod. Dev. 31:* 215-222.

GERRIS, J., MANGELSCHOTS, K., VAN ROYEN, E., JOOSTENS, M., EASTERMANS, W. and RYCHAERT, G. (1995). ISCI and severe male factor infertility: breaking the sperm tail prior to injection. *Hum. Reprod. 10:* 484-485.

GUO, M.W., MORI, E., XU, J.P. and MORI, T. (1994). Identification of FAS antigen associated with apoptotic cell death in murine ovary. *Biochem. Biophys. Res. Commun. 203:* 1438-1446.

HARDY, K., HANDYSIDE, A.H. and WINSTON, R.M.L. (1989). The human blastocyst: cell number, death and allocation during late preimplantation development *in vitro. Development 107:* 597-604.

HOMA, S.T., CARROLL, J. and SWANN, K. (1993). The role of calcium in mammalian oocyte maturation and egg activation. *Hum. Reprod. 8:* 1274-1281.

IN'T-VELD, P.A. *et al.* (1995). Increased incidence of cytogenetic abnormalities in chorionic vilus samples from pregnancies established by *in vitro* fertilization and embryo transfer (IVF-ET). *Prenat. Diagn. 15:* 975-980.

JURISKOVA, A., VARMUZA, S. and CASPER, R. (1995). Involvement of programmed cell death in preimplantation embryo demise. *Hum. Reprod. Update 1:* 558-566.

LACHAPELLE, M.H., MIRON, P., HEMMINMGS, R., *et al.* (1993). Embryonic resistance to tumour necrosis factor-α mediated cytotoxicity: novel mechanisms underlying maternal immunological tolerance to the fetal allograft. *Hum. Reprod. 8:* 1032-1038.

LANZENDORF, S., MALONEY, M., VEECK, L.L., SLUSSER, J., HODGEN, G. and ROSENWAKS, Z. (1988). A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil. Steril. 49:* 835-842.

LEESE, H.J. (1995). Metabolic control during preimplantation development. *Hum. Reprod. Update 1:* 63-72.

LEVINSON, G., KEYVANFAR, K., WU, J.C., *et al.* (1995). DNA-based X-enriched sperm separation as an adjunct to preimplantation diagnosis testing for the prevention of X-linked disease. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 979-982).

LOPATA, A. and OLIVA, K. (1993). Chorionic gonadotrophin secretion by human blastocysts. *Hum. Reprod. 8:* 932-938.

MOORE, G.D., KOPF, G.S. and SCHULTZ, R.M. (1993). Complete mouse egg activation in the absence of sperm by stimulation of an exogenous G protein-coupled receptor. *Dev. Biol. 159:* 669-678.

MÜHLHAUSER, J., CRESCIMANNO, C., KAUFMANN, P., HÖFLER, H., ZACCHEO, D. and CASTELLUCCI, M. (1993). Differentiation and proliferation pattern in human trophoblast revealed by c-*erb* ß-2 oncogene product and EGF-R. *J. Histochem. Cytochem. 41:* 165-173.

NAGY, Z.P., LIU, J., JORIS, H., DEVROEY, P. and VAN STEIRTEGHEM, A. (1994). Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum. Reprod. 9:* 1743-1748.

NAGY, Z.P., LIU, J., JORIS, H., VERHEYEN, G., TOURNAYE, H., CAMUS, H., DERGE, M-P., 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.

NASR-ESFAHANI, M.N., AITKEN, J.R. and JOHNSON, M.H. (1990). Hydrogen peroxidase levels in mouse oocytes and early cleavage stage embryos developed *in vitro* or *in vivo. Development 109:* 501-507.

O'NEILL C. (1985). Partial characterization of the embryo-derived platelet-activating factor in mice. *J. Reprod. Fertil. 75:* 375.

PAL, S., CROWELL, R., KIESSLING, A. and COOPER, G. (1993). Expression of proto-oncogenes in mouse eggs and preimplantation embryos. *Mol. Reprod. Dev. 35:* 8-15.

PALERMO, G., JORIS, H., DEVROEY, P. and VAN STEIRTEGHEM, A.C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoa into an oocyte. *Lancet 340:* 17-18.

PARCHMENT, R.E. (1993). The implications of a unified theory of PCD, polyamines, oxyradicals and histogenesis in the embryo. *Int. J. Dev. Biol. 37:* 75-83.

PELLESTOR, F., GIRARDET, A., ANDREO, B., ARNAL, F. and HUMEAU, C. (1994). Relationship between morphology and chromosomal constitution in human preimplantation embryo. *Mol. Reprod. Dev. 39:* 141-146.

PICKERING, S.J. and MUGGLETON-HARRIS, A.L. (1995). Reliability and accuracy of polymerase chain reaction amplification of two unique target sequences from biopsies of cleavage-stage and blastocyst-stage human embryos. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 1021-1029).

PICKERING, S. and MUGGLETON-HARRIS, A.L. (1995). Reliability and accuracy of polymerase chain reaction amplification of two unique target sequences from biopsies of cleavage-stage and blastocyst-stage human embryos. *Hum. Reprod. 10:* 1912-1922 .

PIERCE, G.B., PARCHMENT, R.E. and LLEWELLYN, A.L. (1991). Hydrogen peroxide as a mediator of PCD in the blastocyst. *Differentiation 46:* 181-186.

REIJO, R., LEE, T-Y., SALO, P., *et al.* (1995). Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein. *Nature Genet. 10:* 383-393.

SCHMIADY, H. and KENTENICH, H. (1993). Cytological studies of human zygotes exhibiting developmental arrest. *Hum. Reprod. 8:* 744-751.

SERMON, K., LISSENS, W., NAGY, P., VAN STEIRTEGHEM, A. and LIEBARS, I. (1995). Simultaneous amplification of the two most frequent mutations of infantile Tay-sachs disease in single blastocysts. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 2214-2217.

SHARKEY, A.M., DELLOW, K., BLAYNEY, M., MACNAMEE, M., CHARNOCK-JONES, S. and SMITH, S. (1995). Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. *Biol. Reprod. 53:* 974-981.

SILBER, S. (1995). What forms of male infertility are there left to cure CHECK. *Hum. Reprod. 10:* 503-504.

SILBER, S.J. and LENAHAN, K. (1995). Sertoli cell surgery: spermatid retrieval and ICSI in azoospermic patients with maturation arrest. *Hum. Reprod. Update 1:* No. 6, Item 26, Video.

SIMON, C., PELLICER, A. and POLAN, M.L. (1995). Interleukin crosstalk between embryo and endometrium in implantation. In *Regulators of Human Implantation* (Eds. C. Simon and A. Pellicer). Human Reproduction Suppl. 2. pp. 43-54.

STEPTOE, P.C., EDWARDS, R.G. and PURDY, J.M. (1971). Human blastocysts grown in culture. *Nature 229:* 132-133.

STEWART, C.L., KARPUR, P., BRUNET, L.J., BHATT, H., GADI, I, KÖNTGEN, F. and ABBONDANZO, S.J. (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature 359:* 76-79.

SWANN, K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive store. *Biochem. J. 287:* 79-84.

TESARIK, J. (1992). Metabolism of human preimplantation embryos. In *Preconception and Preimplantation Diagnosis of Human Genetic Disease* (Ed. R.G. Edwards). Cambridge University Press, Cambridge, pp. 43-79.

TESARIK, J. *et al.* (1995). Viable embryos from injection of round spermatids into oocytes. *New Eng. J. Med. 333:* 525.

TESARIK, J., SOUSA, M. and TESTART, J. (1994). Human oocyte activation after intracytoplasmic sperm injection. *Hum. Reprod. 9:* 511-518.

TSIGORITIS, M. and CRAFT, I.L. (1995). Sperm retrieval methods and ICSI for obstructive azoospermia. *Hum. Reprod. 10:* 758-760.

TURPEENHEIM-HUJANEN, T., RONNBERG, L., KAUPILA, A. and PUISTOLA, V. (1992). Laminin in human embryo implantation: analogy to the invasion by malignant cells. *Fertil. Steril. 58:* 105-113.

VAN BLERKOM, J. (1996). Sperm centrosome dysfunction: a possible new class of male factor infertility in the human. *Mol. Hum. Reprod. 2:* 349-354.

VAN BLERKOM, J. and DAVIS, P. (1995). Evolution of the sperm aster after the injection of isolated sperm centrosomes into meiotically mature human oocytes. *Mol. Hum. Reprod. 1* (see *Hum. Reprod. 10:* 2179- 2182).

VAN BLERKOM, J., DAVIS, P., MERRIAM, J. and SINCLAIR, J. (1995). nuclear and cytoplasmic dynamics of sperm penetration, pronuclear formation and microtubule organization during fertilization and early preimplantation development in the human. *Hum. Reprod. Update 1:* 429-461.

VAN STEIRTEGHEM, A. (1996). Proceedings of the Bourn Hall Meeting. *Hum. Reprod. (Suppl.).* (In press).

VAN STEIRTEGHEM, A.C., LIU, J., JORIS, H., NAGY, Z., *et al.* (1993a). Higher success rate by intracytoplasmic sperm injection than by subzonal insertion. Report of a second series of 300 successive treatment cycles. *Hum Reprod. 8:* 1055-1060.

VAN STEIRTEGHEM, A.C., NAGY, Z., JORIS, H., LIU, J., STAESSEN, C., SMITZ, J., WISANTO, A. and DEVROEY, P. (1993b). High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod. 8:* 1061-1068.

VANDERZWALNEN, P., LEJEUNE, B., NIJS, M., SEGAL-BERTIN, G., VANDAMME, B. and SCHOYSMANN, R. (1995). Fertilization of an oocyte microinseminated with a spermatid in an *in-vitro* fertilization programme. *Hum. Reprod. 10:* 502-503.

WHITAKER, M. and PATEL, R. (1990). Calcium and cell cycle control. *Development 108:* 525-542.

WHITAKER, M. and SWANN, K. (1993). Lighting the fuse at fertilization. *Development 117:* 1-12.

WINSTON, N.J., BRAUDE, P.R., PICKERING, S.J., *et al.* (1991). The incidence of abnormal morphology and nucleocytoplasmic ratios in 2-, 3- and 5-day human pre-embryos. *Hum. Reprod. 6:* 17-24.

WISANTO, A., MAGNUS, M., BONDUELLE, M., LIU, J., CAMUS, M., TOURNAYE, H., LIEBARS, I., VAN STEIRTEGHEM, A.C. and DEVROEY, P. (1995). Obstetric outcome of 424 pregnancies after intracytoplasmic sperm injection. *Hum. Reprod. 10:* 2713-2718.

ZENZES, M.T., WANG, P. and CASPER, R.F. (1992). Chromosome status of untransferred (spare) embryos and probability of pregnancy after *in-vitro* fertilization. *Lancet 340:* 391-394.