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
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 bypassed 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.

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 (Schmida and Kcntenich, 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

| TABLE 1 |
| INITIAL OBSERVATION ON FERTILIZED HUMAN EGGS AFTER ICSI, USING SPERM SAMPLES FROM OLIGOZOOSPERMIC MEN |
| Numbers of spermatozoa |
| <1×10⁶ | <5×10⁶ | >5×10⁶ |
| 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).
oolplasm, 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 dyginic triplo-nuclear 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 (Flaterty, 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 twists 2 h after ICSI whether the oocyte is activated or not, but further pronucleus development depends on the oocyte having been activated (Dозортцев 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, and have been used successfully in cases of obstructive or non-obstructive azoospermia (Devroey et al., 1995a, b; Tsigorilis et al., 1995). Spermaticuds can be used instead of spermatozoa (Vanderzwalwen et al., 1995), and a few living human offspring have been born (Fishel et al., 1995; Tesarik et al., 1995). Spermaticuds can be obtained from ejaculates or testis preparations, and can be sucked off Sertoli cell membranes if necessary (Silber, 1995a, b).

Some clinical risks with ICSI have arisen unexpectedly. There is a 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 those 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 *vitro*, 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 unovulated 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 ml microdrops by measuring the uptake of metabolites or the secretion of various factors (Leese, 1995).

**TABLE 3**

<table>
<thead>
<tr>
<th>Mid-stage of development</th>
<th>Hours post-insemination</th>
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<tr>
<td>2 cell</td>
<td>33.2 ± 1.3</td>
</tr>
<tr>
<td>4 cell</td>
<td>49.0 ± 1.9</td>
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<tr>
<td>8 cell</td>
<td>64.8 ± 1.8</td>
</tr>
<tr>
<td>16 cell</td>
<td>80.7 ± 2.4</td>
</tr>
<tr>
<td>Morulae</td>
<td>96.8 ± 1.9</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>112.7 ± 2.9</td>
</tr>
<tr>
<td>Hatched blastocyst</td>
<td>ca 178</td>
</tr>
</tbody>
</table>

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 blastocoel 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 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 triploid 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 dipiny, and chromosome fragmentation. Most tetraploid embryos probably arise through the suppression of the first or a later cleavage division, or as a consequence of triploidy. 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 syncytiotrophoblast. 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 chromatid 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 blastocellular 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$_2$O$_2$ could impair the growth of human embryos. H$_2$O$_2$ 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 thioridoxin, 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 (Jurisicova 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 APO/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 β-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 Handside, 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 (Handside, 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 persist 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-1β, 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, placentaly-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 α3, α5, αv, β1, β3, and β4, plus the E-cadherins, ICAM-1 and NCAM (Fusi et al., 1992, Turpeenemi-Hujanen et al., 1992; Campbell et al., 1995). The gene for uPA 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 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.

**KEYWORDS:** sperm injection, oocytes, human, fertilization, embryos

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**References**


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**TABLE 4**

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<tr>
<th>POTENTIAL PRE-IMPLANTATION SIGNALS FROM THE EMBRYO TO THE MOTHER</th>
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<tr>
<td><strong>EDPAF</strong></td>
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<tr>
<td><strong>EPF</strong></td>
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<tr>
<td><strong>Estradiol 173</strong></td>
</tr>
<tr>
<td><strong>Progesterone</strong></td>
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<td><strong>HCG</strong></td>
</tr>
</tbody>
</table>

(Edwards and Brody, 1995)


