On Fragmentation: Origin and Consequences of Abnormal Cell Division in Human Embryos *in vitro*

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To Jacques, Giulia, and Lucas

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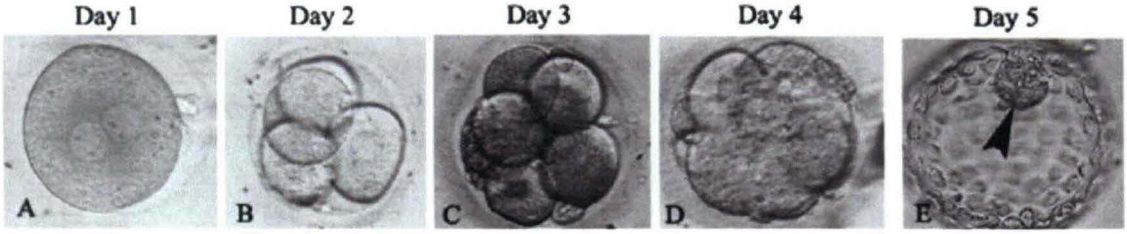
Notice 1

Addendum

Chapter 2

Representative embryos with normal morphology on five consecutive days in culture are shown in the figure below. The arrowhead in E points to the inner cell mass of the blastocyst.



On page 29, the volume of culture droplets should be corrected to 100-200µL.

On page 34, the protocol for fixation of single cells and whole embryos should be modified as follows: Fixation of single cells should include a first step of brief exposure of the cell to a hypotonic solution (sodium citrate in 0.2mg/mL bovine serum albumin)> For fixation of day-2 to morula stage whole embryos for FISH, embryos were exposed to hypotonic solution for approximately 2 minutes, then transferred to the fixative solution where the zona pellucida dissolved. The embryo was then transferred to a slide and small drops of fixative were added while increasing humidity (to 35%-40%) until the cells lysed, and the nuclei became fixed to the slide. For blastocyst fixation, the embryo was exposed to hypotonic solution for approximately 30-60 seconds and transferred to the fixative solution for approximately 2-3 minutes. The blastocyst, now with "transparent" cells, was picked up in a capillary tube filled with 10µL of 70% acetic acid and 20 µL of methanol/acetic acid/water, 3:3:1, and placed on the glass slide in a small volume of the solution; before complete evaporation, a drop of the methanol/acetic acid (3:1) was added directly onto the blastocyst. Once the nuclei were spread properly, a second drop of fixative was added, while humidity was kept at 40% or more. Immediately after the cells in the blastocyst lysed, air was blown over the embryo to push the remains of cytoplasm away from the nuclei and to force the nuclei to expand, avoiding clumping.

Chapters 3 and 4

The definition of homogeneous transfers on page 42 is an error; it should read *more than half* rather than *half or more embryos in the same category*.

Because implantation was the most important outcome measure in the study, this definition was chosen with the aim of eliminating or minimizing correlative uncertainties when multiple embryos were transferred, but without restricting the analyses to too few cases. According to the definition, homogeneous transfers included all single embryo transfers, 2-embryo transfers in which both embryos were in the same category, 3-embryo transfers in which 2 or 3 of the embryos were in the same category, etc. The majority of transfers involved 2-4 embryos; the range was between 1 and 6 embryos.

During routine clinical practice, day-3 evaluations were assigned to (often two) senior embryologists whose decisions were further reviewed by a laboratory director. Scoring consistency was evaluated through proficiency tests, administered periodically, using videotaped images and photographs of embryos. ANOVA and correlation analysis of the data showed that the values for variables, cell number, degree of fragmentation, and fragmentation type, scored by four different embryologists were statistically comparable both in their overall mean levels and in their degree of association.

The primary objective of embryo transfer was to establish a clinical pregnancy; therefore the best embryos available were transferred. Among the 1727 cases, 894 cases had no fragment removal (no fragmentation or minimal fragmentation); all others (833 cases) had embryos from which fragments were removed.

On page 39, the phrase, *largely but not entirely*, refers to higher pregnancy rate in group W1 compared to groups W4 and W5, and in groups W2, W3, and W4 compared to group W5. It also refers to higher implantation rate in group W1 compared to groups W3 and W5, and in groups W2, W3, and W4 compared to group W5. It is true that the most significant clinical effect occurred when embryos with more than 35% fragmentation were transferred. However, this was under the conditions described in the study, i.e., after application of selective assisted hatching and removal of fragments. *C. elegans* was noted on page 44 not to suggest it as a good model for humans, but as an example of a species in which cell corpses are engulfed by neighboring cells thus removed from the body.

Chapter 5

The experiments in the mouse did not support the notion that cell fragments *per se* limit developmental capacity of viable cells in an embryo. However, the extent to which these findings can be extended to the human is questionable since this experimental model did not mimic spontaneous fragmentation in the human in several respects, as noted. It has been suggested that the "packing pressure" within the intact embryo may be higher than that in the experimental embryos and this may explain reduced negative effect of fragmentation in both the experimental mouse embryos and the human embryos from which fragments were removed. This is a possibility, however, the experiments in Chapter 6 suggest that packing pressure is not a significant factor in the success (or failure) of development. Moreover, Chapters 4 and 6 suggest a secondary effect of spontaneous fragmentation in the human. Fragment removal, while unlikely to address other problems represented by fragmentation, may address such a secondary effect (e.g., of failed compaction due to disorganization), by bringing the viable cells in close proximity.

Chapter 7

As advised by the manufacturer, the mouse monoclonal anti-e-cadherin antibody was raised against human placental e-cadherin and reacts strongly and specifically with human e-cadhrin. Although the study clearly demonstrates a link between disorganization of the embryo and disrupted distribution of e-cadherin, the question of whether disruption in e-cadherin distribution occurred as a result of embryo disorganization or was the cause of it remains to be resolved.

TABLE OF CONTENTS

Synopsis

Cytoplasmic fragmentation is the most common cause of embryonic loss after *in vitro* fertilization and culture of human eggs. The occurrence of fragmentation often coincides with the presence of other abnormalities, including low cell number, uneven blastomere size, disrupted cell-cell contact, blastomere multi-nucleation, and chaotic chromosome mosaicism. As commonly as fragmentation is observed during clinical practice of *in vitro* fertilization and embryo transfer, the phenomenon itself rarely has been studied. The occasional investigative attempts have narrowly focused on linking fragmentation to apoptosis. Undoubtedly, one reason for this limited inquiry is the practical difficulties and obstacles associated with the use of human eggs and embryos for experimentation. The lack of a suitable animal model and the relative unimportance of fragmentation in experimental mouse embryology have also contributed to its dismissal as a worthy research subject. The present studies aim to reach beyond these imposed and inherent limitations and uncover the causes and mechanisms of cytoplasmic fragmentation, and delineate its developmental consequences. Chapter 3 describes a fragmentation classification system which was developed through non-invasive morphological evaluation of large numbers of embryos and was based on fragmentation patterns: the size and distribution of fragments relative to the size and position of nucleated cells. These patterns along with (but independent of) the degree of fragmentation were shown to be of predictive value for clinical outcome of *in vitro* fertilization and embryo transfer and therefore useful for selection of embryos for transfer. The loss of more than one third of the cytoplasmic volume to fragmentation or formation of large fragments was found to lead to a significant decrease in implantation and pregnancy rates following intrauterine transfer on day-3 of development. In Chapter 4, embryos with fragmentation and other abnormalities were followed through day-5 of development in culture; it was discovered that the processes of compaction, cavitation, and blastulation were often abnormal in such embryos. In Chapters 5 and 6, attempts were made to discover whether (and how) fragments interfered with development. It was shown in a mouse model that continued development of blastomeres was generally unaffected by the presence of fragments. In the human, isolation of intact blastomeres from several fragmented embryos and their aggregation within a host zona pellucida led regularly to formation of chimaeric blastocysts. This work demonstrated that viable cells with apparently normal regulatory capacity can be found within non-viable embryos. In Chapter 7, the distribution of a vital cell adhesion protein, E-cadherin, was investigated in abnormal embryos by immunocytochemistry and confocal

fluorescence microscopy. The results suggested that the characteristic distribution pattern of E-cadherin is perturbed and erratic in such embryos, providing one explanation for their failure to compact, cavitate or blastulate normally. Finally, the experiments described in Chapter 8 revealed the key to the nature of fragmentation: its resemblance to cytokinesis in its requirement for activation, its timing in the cell cycle, and its mediation by reorganization of the cytoskeleton. This was investigated in a mouse model, using enucleation as a way to make eggs and embryos "fragmentation-prone". In meiotic cells, the timing of fragmentation coincided with second polar body extrusion, and in mitotic cells, it coincided with mitosis and cell division. Therefore, far from being random, fragmentation occurred only during the M phase of the cell cycle. By taking into consideration the observation that the non-activated mature eggs neither divide nor fragment and that such eggs are arrested in metaphase, it was also possible to point to cytokinesis as the phase during which fragmentation occurs. The close association of fragmentation with the M phase of the cell division cycle, and in particular with the failing coordination of microtubule function would account for the numerous chromosomal and nuclear abnormalities that accompany fragmentation. Furthermore, by firmly establishing a link between fragmentation and cell division, both nuclear and cytoplasmic, the present work has definitively opened an important area for future research, most critically, into strategies to minimize or prevent fragmentation and its associated abnormalities.

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Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I, Mina Alikani, hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes **five** original papers published in peer reviewed journals and **four** unpublished papers. The core theme of the thesis is fragmentation in human embryos *in vitro*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Institute for Reproduction and Development and Monash Immunology and Stem Cell Laboratories under the supervision of Professor Alan O. Trounson.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

For each and every chapter of the thesis, one through nine, my own contribution to the work as well as those of others, when applicable, have been detailed in a chapter declaration.

		Date
Signature	[redacted]	24 DECEMBER, 2005

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Declaration for Thesis Chapter 1

In the case of Chapter 1, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	100	Literature review, compilation of figures, writing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

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Chapter 1

Literature overview

1.1 Background

1.1.1 Fertilization and culture of mammalian eggs in vitro

Efforts to fertilize mammalian eggs outside the female reproductive tract date back to the late 1800s. Success and rapid progress, however, seem to have been out of reach until the discovery of sperm capacitation by Austin (1951; 1952) and Chang (1951). Working independently, these pioneers discovered that in the rat and the rabbit, epididymal or ejaculated sperm had to undergo some changes within the female tract before they could penetrate the egg zona pellucida (ZP). Austin and Chang subsequently used sperm recovered from the rabbit uterus to successfully induce fertilization of rabbit eggs *in vitro*. Prior to reports by Austin and Chang, there were other claims of *in vitro* fertilization (IVF), most notably one by Moricard (1950). However, the validity of such claims before the discovery of capacitation has been vigorously disputed (Chang, 1968; Austin, 1961).

For several years after the success of IVF in the rabbit, as Chang (1968) describes, "it was felt that unless living young could be obtained after transplanting such fertilized eggs into recipient rabbits, successful fertilization *in vitro* could not be held to be proven, since such eggs could be abnormally fertilized or may not be fertilized at all." This final confirmation was obtained by Chang in 1959 (Chang, 1959). He incubated newly ovulated eggs with capacitated sperm for four hours then cultured the eggs in 50% rabbit serum for another 18 hours before transferring the eggs into recipient females. These experiments resulted in the first live births following IVF and embryo transfer (ET) in mammals. This outcome was later confirmed by Bedford and Chang (1962).

It was over a decade after the discovery of *in vivo* capacitation in the rabbit that sperm capacitation *in vitro* was first achieved in the hamster (Yanagimachi and Chang, 1963). After another few years, IVF was achieved in the mouse (Whittingham, 1968), opening a tremendous avenue for research into early mammalian development.

For experimental embryology in mammals to move forward, however, a reliable embryo culture method was imperative so that the fertilized eggs could be maintained *in vitro* through the cleav-

age stages. Hammond (1949) had discovered that mouse embryos collected at the 8-cell stage, but not the 2-cell stage, could be cultured to the blastocyst stage in a physiological saline solution that was supplemented with hen egg yolk and white. Although the inability to culture 2-cell embryos was and remained for some time a formidable challenge, Hammond's discovery was a significant one and set the stage for abandonment of biological fluids as culture media for embryos. In 1956, Whitten (1956) replaced Hammond's medium with a modification of Krebs-Ringer bicarbonate solution. He supplemented the latter with glucose, antibiotics, and bovine serum albumin (BSA) and showed that it could support development of 2-cell and 8-cell mouse embryos to the blastocyst stage. Two years later, McLaren and Biggers (1958) obtained normal young following transfer of blastocysts grown in Whitten's medium, proving that viable blastocysts could be produced *in vitro.*

At this juncture, it was the inability to obtain large numbers of eggs at a time (and at will) that hampered research efforts. According to Edwards (1980), the dogma of the time dictated that ovaries of adult females would not respond to gonadotrophic hormones. However, Fowler and Edwards (1957) successfully challenged this dogma. They followed the work of Gates (Runner and Gates, 1954) who had artificially induced ovulation in pre-pubertal mice using a regimen that included injection of pregnant mare serum followed two days later by serum from a pregnant woman. Fowler and Edwards (1957) used the same method and induced superovulation and pregnancy in mature mice. Later, it was shown that superovulation could be achieved in the human using pituitary gonadotrophins (Gemzell, 1962).

But another hurdle had to be overcome before production, fertilization, culture, and transfer of mammalian eggs would become routine practice. A reliable and efficient embryo culture system had to be devised. In 1963, Brinster (1963) introduced culture of eggs and embryos in small drops of culture medium under a layer of paraffin oil. With only minor modifications, this "micro-drop" method has become the most widely used and successful system for culture of mammalian embryos *in vitro* today.

The basic principles of and experience gained in experimental animal embryology, including oocyte maturation *in vitro* (Edwards, 1965), were first successfully applied to the human in March 1968: Edwards and Bavister, using a modification of Tyrode's solution devised by Yanagimachi and Chang (1963) for hamster IVF, added sperm to nine human eggs and, eleven hours later, recorded the presence of a sperm tail within one egg and the presence of pronuclei in another. This was indisputable evidence of fertilization *in vitro* in the human (Edwards et al., 1969; Bavister et al., 1969; Bavister, 1969).

What followed was a series of dogged attempts, particularly on the part of Edwards, Steptoe, and Purdy, to perfect the procedure and establish a human pregnancy through *in vitro* fertilization,

culture, and intrauterine transfer of human eggs (Edwards et al., 1970; Edwards and Steptoe, 1974; 1976). The first birth following human IVF/ET was announced in 1978 by Steptoe and Edwards (1978). A new era was dawning.

1.2 Events following activation of a human egg: an overview

1.2.1 Resumption of meiosis II and the mitoses beyond

The mature, non-activated human egg is arrested in the modified prophase of the second meiotic division. It contains a typically barrel-shaped meiotic spindle positioned in the cortex of the egg (Pickering et al., 1988). This is similar to the mouse, although the difference in size between the meiotic spindle of a mouse egg (about 20μm long) and that of a human spindle (about 12μm long) relative to the size of their eggs (80μm and 120μm, respectively) is quite interesting (Figure 1.1). In a recently matured egg, shortly after first polar body (PB1) extrusion, the spindle would be expected to be close to where PB1 has been extruded. However, in human eggs, this is often not the case (Silva et al., 1999; Hardarson et al., 2000; Cook et al., 2003). This has been attributed to the "highly movable property" of PB1 (Cook et al., 2003) rather than the movement of the spindle (Gardner, 1997). At the same time, it should be kept in mind that spindle movement is quite common in the mouse as the mature egg ages, though this is an inward and not a lateral movement (Eichenlaub-Ritter et al., 1986). Therefore, the location of the spindle in the human eggs examined may have in some cases reflected ageing of the egg by the time of examination.

The egg chromosomes are held within the spindle in metaphase of meiosis II. Activation of the egg, whether sperm-induced, artificial, or spontaneous, involves a local increase in cytosolic calcium concentration, which spreads wavelike through the cell and is followed by prolonged calcium oscillations. Exocytosis of cortical granules and release of their enzymes then induce changes in two specific protein components of the zona pellucida (ZP)— ZP2 and ZP3. The activation alteration of the ZP is generally termed zona reaction and normally has the effect of blocking entry of additional sperm.

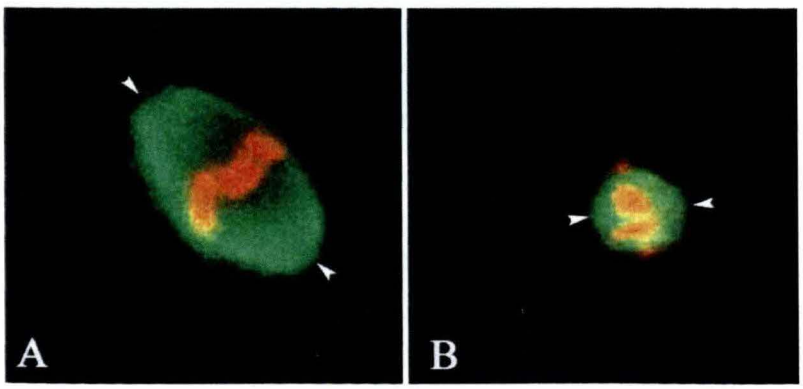


Figure 1.1 Meiotic spindles in non-activated eggs. (A) Mouse MII spindle in an oocyte collected at 16 hrs post hCG. Distance between arrowheads is approximately 20μm. (B) Human MII spindle in an oocyte collected at 35.5 hrs post hCG and matured *in vitro* overnight. Distance between arrowheads is approximately 12μm.

Activation changes set the egg on a developmental course which begins with progression through anaphase and telophase of meiosis II. Activation becomes morphologically obvious some hours later (after fertilization, about 4-5 hours in the mouse and 9-11 hours in the human), when a highly asymmetric cytoplasmic division occurs and a small second polar body is produced. The sister chromatids of each of 46 chromosomes, joined at their centromeres, are separated by the spindle microtubules, leaving 23 daughter chromosomes in each resulting cell. The completion of meiosis II thus results in reduction of the chromosome number of the egg from a diploid to a haploid complement, before incorporation of the male haploid genome.

Successful fertilization is marked by the emergence of two (one female and one male) pronuclei within the egg, while artificial or spontaneous activation results in formation of a single (female) pronucleus. The fertilized egg is called a zygote. Human pronuclei can be visualized in zygotes as early as 11 hours post insemination; but eggs are routinely checked for fertilization between 16 and 19 hours following insemination. Within the pronuclei, the nucleoli can be visualized (Figure 1.2). The size and position of the pronuclei as well as the alignment of the nucleoli have been shown to have predictive value for embryo viability (Wright et al., 1990; Sadowy et al., 1998; Scott and Smith, 2000). A role as a determinant of developmental competence has also been suggested for "inter-pronuclear synchrony" (Tesarik and Greco, 1999).

Roughly 24 hours after insemination, the pronuclear membranes break down and the zygote is said to be in syngamy; development of the syngamic zygote continues with the first mitotic cell cycle.

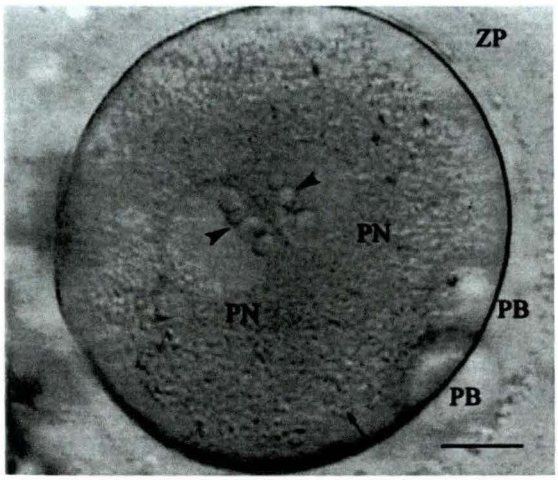


Figure 1.2 A human zygote with two closely apposed pronuclei (PN). The nucleoli (arrowheads) are visible in each pronucleus, aligned symmetrically. The cytoplasm is typically slightly contracted, leaving a small area devoid of organelles (arrow) in the cortex. The zona pellucida (ZP) is also visible. Scale bar is approximately 20μm.

1.2.2 The mitotic cell cycle

The mitotic cell cycle is controlled by a number of cyclin-dependent kinases (Cdks), which are activated/inactivated in a specific sequence. The cycle includes an interphase and a mitotic phase or an M phase. Interphase (shown in the human in Figure 1.3A) is the period between two M phases where the cell spends most of its time. In rapidly dividing cells, interphase includes two gap phases,

G1 and G2, during which the cell grows. However, during early cleavage in mammals, there is no growth between successive mitoses, so the size of the cells is reduced—essentially halved—with each division. Protein synthesis, chromosome duplication, and centrosome (microtubule organizing center or MTOC) duplication occur during the S phase of interphase. In the human, as in several other mammals, the major MTOC has been shown to be paternal in origin (Sathananthan, 1992; Palermo et al., 1995) while in the mouse, the maternal MTOC play this critical role (Maro et al., 1985; Schatten et al., 1985). Nuclear and cytoplasmic divisions occur during the M phase of the cell cycle. Nuclear division or karyokinesis is driven by the tubulin-based mitotic spindle apparatus while cytoplasmic division or cytokinesis is driven by an actin and myosin-based contractile ring. The location of the cleavage furrow is determined by the position of the spindle.

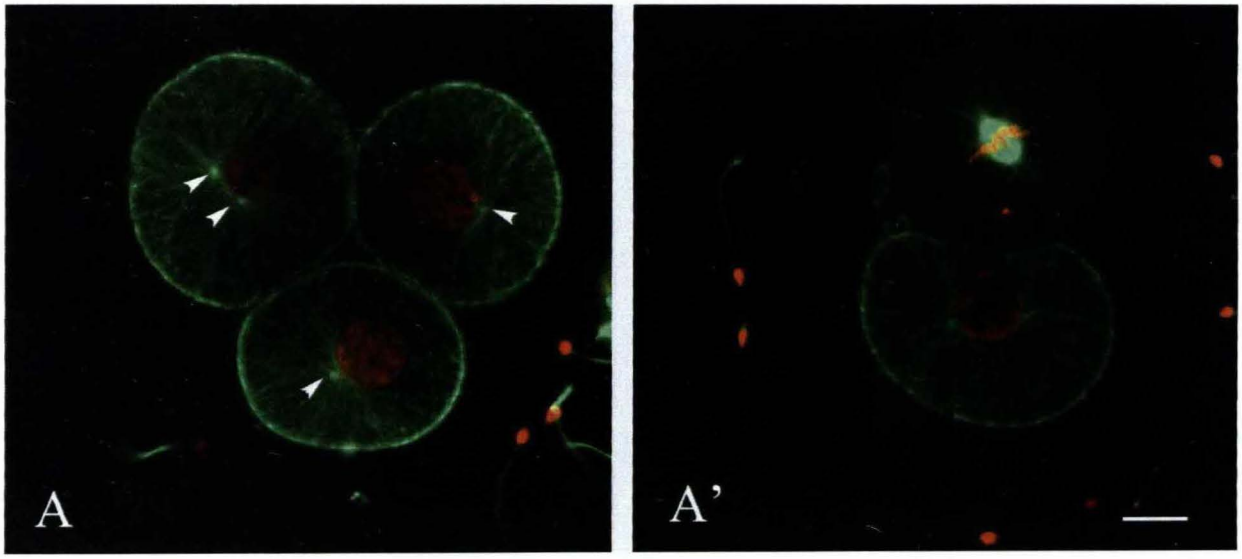


Figure 1.3 A 5-cell, day-3 human embryo, stained for tubulin (green) and DNA (red). Images are projections of multiple 3-4μm thick optical sections obtained on the laser scanning confocal microscope. (A) microtubule organizing centers (arrows) are visible closely associated with nuclei in three cells. (A') A metaphase spindle is visible in a fourth cell. Scale bar is 20μm.

1.2.3 Mitosis

Mitosis (Reviewed by Nigg, 2001) is dependent on the proper assembly and function of the mitotic spindle. The mitotic spindle is an infinitely intriguing machine, the workings of which, despite having been studied at the molecular level at a dizzying pace in recent years, seem to remain a mystery. The assembly of the spindle begins in prophase with the duplicated centrosomes already positioned on/near the nuclear envelope (Figure 1.3A and 1.4A, B). Nuclear envelope breakdown (NEBD) (shown in the mouse in Figure 1.4A), provides microtubule access to the chromosomes (and vice versa) and thus further elaboration of the spindle. In metaphase, the spindle microtubules attach to the condensed chromosomes at the kinetochore (Kinetochore microtubules) and align them in the metaphase plate (metaphase shown in the human in Figure 1.3A' and in the mouse in

Figure 1.4D). It is generally thought, though not known for certain, that in the absence of centrosomes, microtubule nucleation is coordinated by the chromosomes. Progression of the cell cycle into anaphase is dependent on proper alignment of all chromosomes along the metaphase plate. A spindle assembly checkpoint mechanism delays anaphase until the kinetochors of the sister chromatids in all pairs are attached to microtubules emanating from opposite poles of the spindle.

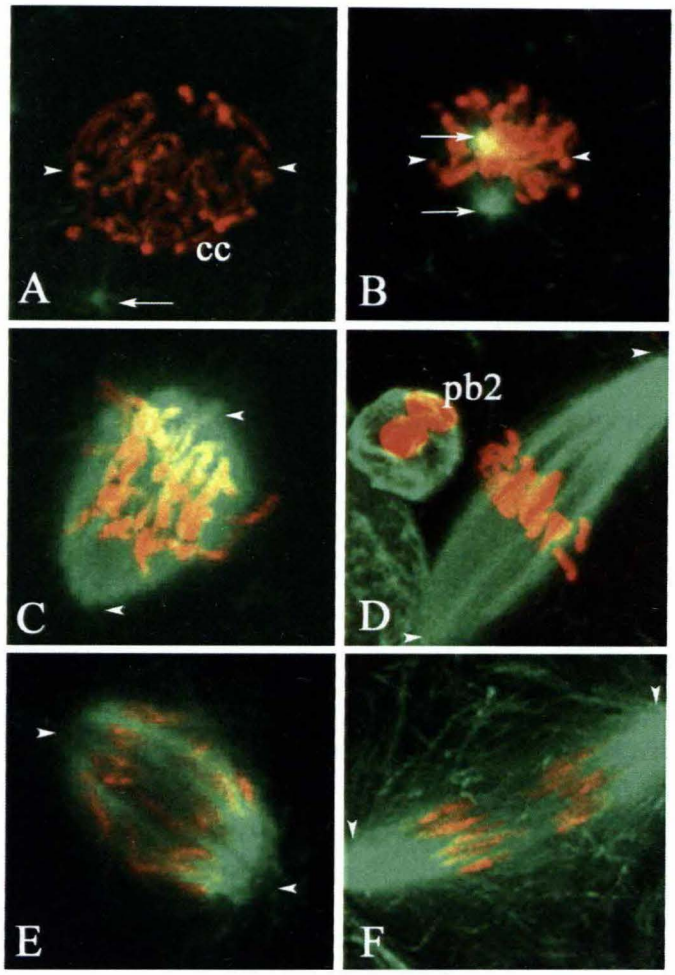


Figure 1.4 Three of four phases of mitosis in the mouse. Eggs have been stained for tubulin (green) and DNA (red). Images are projections of multiple 3-4μm thick optical sections obtained on the laser scanning confocal microscope. (A) Prophase; nuclear envelope breakdown and chromatin condensation (cc) into chromosomes in progress. Distance between arrowheads is approximately 20μm. (B) Prometaphase; nucleation of microtubules initiated by microtubule organizing centers (arrows). Distance between arrowheads is approximately 16μm. (C) Prometaphase; metaphase spindle formation in progress. Distance between arrowheads is approximately 20μm. (D) Metaphase; spindle fully formed, chromosomes aligned in the middle; the second polar body (pb2) is visible. Distance between the arrowheads is approximately 40μm. (E) Anaphase A; chromosomes being pulled toward the spindle poles. Distance between arrowheads is approximately 23μm. (F) Anaphase B; cytoplasmic microtubules starting to reform. Distance between arrowheads is approximately 42μm.

During anaphase A, the sister chromatids are separated through the action of proteases; these daughter chromosomes, as they are now labeled, are then moved toward the poles. The movement is mediated by the dynamic shortening of kinetochore microtubules. In anaphase B, the poles are separated through the "push" of elongation of the overlapping microtubules between the separating chromosomes and the "pull" of motor proteins in the poles (anaphase is shown in the mouse in Figure 1.4E, F). At the completion of anaphase, two groups of chromosomes are at the opposite poles of the cell and nuclear envelopes begin to form around them. Decondensation of the chromosomes and formation of the nuclear envelope mark the completion of mitosis.

1.2.4 Cytokinesis

Cytokinesis overlaps with the final two phases of mitosis—anaphase and telophase—and its completion marks the end of the M phase. The first overt sign of cytokinesis is the appearance of a cleavage furrow as a result of the inward movement of the plasma membrane at a site near the middle of the cell and perpendicular to the bipolar spindle axis.

The machinery driving the advancing furrow is the so-called contractile ring. This "ring" is assembled during anaphase from actin and myosin II filaments and generates the considerable force that is necessary to ultimately divide the cytoplasm. Once cleavage is complete, the ring is disassembled, and the remnants of the spindle form a midbody that joins the two daughter cells until the next division. The product of this division is an embryo, although it has been argued that the use of this term is misleading and incorrect (Johnson and Selwood, 1996) and that the word "embryogen" would more accurately describe the preimplantation stages (Johnson, 2001). The length of the second and following cell cycles in the human embryo is estimated to be 11.9 hours (Cummins et al., 1986), but there is considerable variation among IVF embryos, as will be discussed in the next section. The first cleavage occurs around 25 hours post insemination.

1.3 Division in human eggs fertilized *in vitro*

In vitro fertilization and culture of preovulatory human eggs (obtained by superovulatory treatment) often leads to atypical development for reasons that are largely unknown. Not surprisingly, many atypical features of embryos are associated with reduction in embryo viability. The question that needs to be answered is whether the major abnormalities are mechanistically inter-related.

1.3.1 Cleavage timing and cleavage rate

Several studies have suggested that the timing of the first cleavage is a strong marker of human embryo viability. Early cleaving (EC) zygotes, defined as those that have cleaved to two cells by 25-27 hours post-insemination, show an advantage over those with delayed cleavage (Edwards et al., 1984; Shoukir et al., 1997; Sakkas et al., 1998; 2001; Bos-Mikich et al., 2001; Lundin et al., 2001; Fenwick et al., 2002; Salumets et al., 2003).

Lundin et al. (2001) evaluated 10,798 embryos with respect to early cleavage and found that a significantly higher proportion of good quality embryos resulted from EC embryos and that following intracytoplasmic sperm injection (ICSI), early cleavage was an "independent predictor of birth."

In a study by Sakkas et al. (2001), 45% of patients undergoing IVF or ICSI had EC zygotes. Both implantation and pregnancy rates increased significantly as the number of embryos resulting

from EC zygotes in the transfer cohort increased from zero to two (the mean number of embryos replaced was 2.2).

Perhaps the most convincing study so far is that of Salumets et al. (2003). These authors evaluated 178 elective single embryo transfer (eSET) procedures in patients 37 years of age or younger with good quality embryos. All embryos in the study were checked for early cleavage, but this was not used as a criterion for embryo selection for transfer. They subsequently compared 72 transfers with EC embryos and 106 that did not include EC embryos and found that clinical pregnancy rate was significantly higher in the case of the former (50% vs. 26.4%, respectively).

Although the higher implantation potential of early cleaving embryos is clear from these studies, the biological basis of this observation is not well-understood or tested in the human. It has been speculated that early cleavage following ICSI reflects inherent advantages in the egg rather than accelerated completion of the fertilization process (Sakkas, 1998). One such advantage is suggested to be "ample protein and RNA stores in which case cytoplasmic and nuclear maturation are better synchronized" (Lundin et al., 2001). It will be interesting to see whether the apparently faster transition from interphase to M phase in EC embryos can be attributed to a low incidence of chromosomal abnormalities among such embryos. It is possible that early cleavage indicates the absence of checkpoint induced (Hartwell and Weinert, 1989) cell cycle deterrents such as non-replicated DNA, damaged DNA, or misaligned chromosomes. However, in view of implantation and development (even to term) of many chromosomally abnormal embryos, and the apparently "clonal" pattern of chromosomal mosaicism in cleavage stage embryos (Harrison et al., 2000), it is questionable whether these checkpoints are fully operational during early embryonic development.

Cleavage rate is another important predictor of embryo viability. Among the embryos transferred to patients who become pregnant, very slowly as well as very rapidly developing embryos are known to be underrepresented (Cummins et al., 1986). A review of ongoing twin pregnancies after 2-embryo transfers has shown that these embryos have 4 to 5 blastomeres on day-2 and at least 7 blastomeres on day-3 (Gerris et al., 1999; Van Royen et al., 1999).

We defined slow cleavage as fewer than 5 cells on day-3 of development, and fast cleavage as more than 10 cells on day-3 of development; our analysis of about 75,000 day-3 embryos with less than 15% fragmentation has shown that slow cleavage is far more common than fast cleavage among IVF embryos (M. Alikani and G. Tomkin, unpublished). While 1% show fast cleavage, 15% are slowly cleaving. In the mouse model, lack of a specific protein product (Qa-2) of the preimplantation development gene, or *ped* gene, consistently leads to slowly developing embryos that implant at the same rate as their fast developing counterparts, but are lost between mid-gestation and birth (Warner et al., 1998). A homologue of this gene in the human probably exists (Jurisicova et al.,

1996a; Cao et al., 1999), since a functional homologue of the mouse Qa-2 (identified as HLA-G) has been found in human 8-cell embryos and blastocysts (Comiskey et al., 2003). However, early pregnancy loss specifically attributable to transfer of slowly cleaving human embryos has not been reported. Our estimate of the implantation rate of 4-cell embryos transferred on day-3 is 10%.

Examination of unusually fast cleaving embryos, defined as those with nine or more cells at 62 hours post-insemination, has shown high levels of mosaicism and polyspermic fertilization (Harper et al., 1994; Magli et al.; 1998). Consistent with this finding, fewer fast embryos transferred on day-3 of development implant (Racowsky et al., 2003).

1.3.2 Cell size and symmetry

There is general agreement that transfer of embryos with unevenly sized cells results in lowered pregnancy and implantation rates (Ziebe et al., 1997; Hardarson et al., 2001; Racowsky et al., 2003). The position of the cleavage furrow is ordinarily determined by the position of the mitotic spindle. The pattern of the first two cleavage divisions and its functional significance in mammalian embryos has been the subject of intense study and controversy (Gulyas, 1975; Gardner, 1999; 2002; Hansis and Edwards, 2003; Edwards and Hansis, 2005). In the mouse (and also frequently in the human), the first cleavage is merdional relative to the position of the second polar body and produces two daughter cells, each of which inherit cytoplasm from both the animal pole (where the second polar body is located) and the vegetal pole (opposite to the polar body) of the egg. The pattern of cleavage during the second division gives the resulting 4-cell embryo its typical tetrahedral shape. Edwards and Hansis (2005) have suggested that during this division, one blastomere often divides meridionally again to produce two identical daughter cells, but the second blastomere divides equitorally, producing one daughter cell with mostly animal pole cytoplasm and another with mostly vegetal pole cytoplasm (Edwards and Hansis, 2005). They have proposed that the fate of the blastomeres is determined after the second division: the two blastomeres inheriting a full animal/vegetal axis would be precursors of the inner cell mass, the blastomere inheriting cytoplasm from the animal pole would be the precursor of the trophectoderm, and the blastomere with cytoplasm from the vegetal pole would be the originator of the germline (Edwards and Beard, 1997). If true, the disruption of this pattern would have profound implications for the embryo, but in view of the known plasticity of the mammalian embryo prior to compaction, this question is still unanswered.

Asymmetric or unequal cell division can result from unusual placement of the spindle and may compromise both the genetic and the cytoplasmic equality of the daughter cells. In an interesting study by Goyanes et al. (1990), quantitative evaluation of human embryos revealed that those with unevenly sized blastomeres had a total blastomere volume that was 8% lower than normal em-

bryos. At the same time, these authors found, as would have been expected, that total blastomere volume during cleavage stages did not differ from ooplasmic volume at the pronuclear stage. So, perhaps the reduced volume of the uneven embryos reflects a reduction in oocyte volume, and is an indication of subtle changes in oocyte development and maturation within the follicle.

Some representative embryos with unevenly sized blastomeres are shown in Figure 1.5. Cytoplasmic fragmentation can lead to uneven appearance of groups of cells (Figure 1.5A). Alternatively, unevenly sized cells may appear as a result of missed cytokinesis in one or more cells; in these instances, if karyokinesis did occur, the arrested cells may appear bi-nucleated or multi-nucleated (Figure 1.5B). Hardarson et al. (2001) found a significantly higher frequency of both multi-nucleation and aneuploidy in unevenly-sized blastomeres. An overt form of blastomere asymmetry is a single large cell among several smaller cells (Figure 1.5C). The larger cell is often multi-nucleated. These embryos have been found to be typically polyploid indicating failure of cytokinesis (Munné et al., 1994).

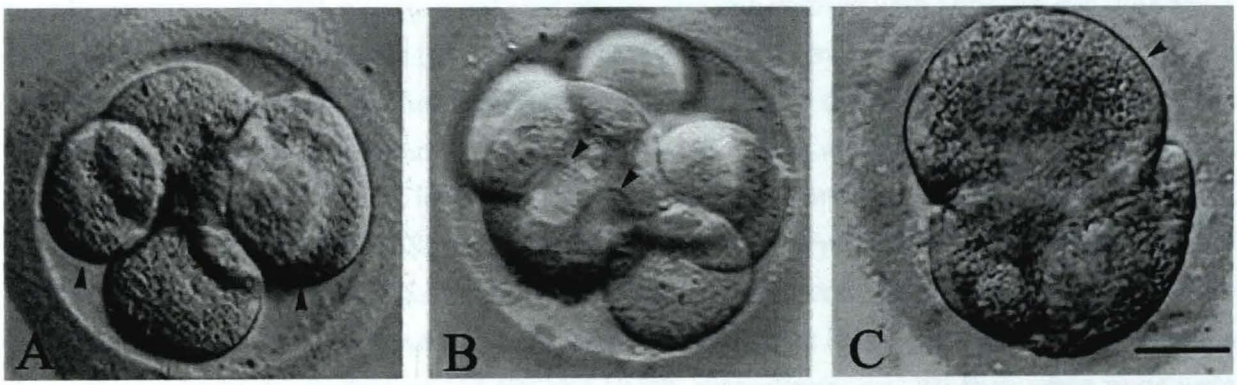


Figure 1.5 Human embryos with unevenly sized blastomers. (A) Loss of large volumes of cytoplasm through fragmentation leading to uneven appearance of cells (arrowheads); fragments have been removed. (B) Possible arrested cytokinesis and continued karyokinesis giving rise to blastomere bi-nucleation (arrowheads). (C) A dominant cell (arrowhead) among other smaller cells, usually associated with polyploidy. Scale bar is approximately 50μm.

1.3.3 Multi-nucleation

Following the first cleavage division, some blastomeres in human embryos can show multiple nuclei (Figure 1.6A). One proposed cause of multi-nucleation is follicular under-oxygenation (Van Blerkom et al., 1997). Accelerated follicular response to exogenous gonadotrophins has also been implicated in multi-nucleation (Jackson et al., 1998). The mechanisms of multi-nucleation include karyokinesis without cytokinesis, "fragmentation of nuclei", and abnormal migration of chromosomes during anaphase (Tesarik et al., 1987; Winston et al., 1991; Hardy et al., 1993). On day-2 of development, blastomere multi-nucleation has been shown to occur significantly more frequently in 2-cell embryos than 4-cell embryos (Balakier and Cadesky, 1997). The presence of multi-

nucleated blastomeres (MNB) in developing day-2, day-3, and day-4 embryos is correlated with extensive mosaicism and/or polyploidy (Kligman et al., 1996; Laverge et al., 1997; Magli et al., 2001).

Staessen and Van Steirteghem (1998) found that 2-cell embryos with bi- and multi-nucleation often cleaved further and gave rise to embryos containing mono-nucleated diploid blastomeres, mono-nucleated diploid and non-diploid blastomeres, or only non-diploid blastomeres. These investigators concluded that multi-nucleation may in some instances be temporary and reversible. In a study by Van Royen et al. (2003), blastomere multi-nucleation was observed in 34% of the embryos and was represented among the embryos of 87% of the patients—an unusually high incidence. This condition was found to be associated with faster response to exogenous gonadotrophins, higher yield of eggs, impaired cleavage, increased fragmentation, and lowered ongoing implantation rates.

Walmsley (2002) evaluated a large set of data on multi-nucleation and made a number of interesting observations. In total, 63.6% of patients had at least one embryo with MNB on day-2 or day-3 of development. Of all embryos (n=55,612), 17.6% had one or more multi-nucleated cells during culture. Multi-nucleation was first observed on day-2 in 12.5% of the embryos whereas only 5% of embryos had multi-nucleation first observed on day-3. This abnormality was associated with maternal age less than 31 years, low basal follicle stimulating hormone (FSH) levels, shorter than average ovarian stimulation, ICSI, severe male factor, and embryo culture in HTF supplemented with human serum albumin (HSA). Multi-nucleation did not occur with the same frequency during repeat cycles. Overall implantation and pregnancy rates were lower when all replaced embryos were affected by multi-nucleation (13.6% and 19.0%, respectively; n=110) than when none were affected (27.5% and 53.8%, respectively; n=5573). Exclusive replacement of bi-nucleated embryos (n=11) led to higher implantation and pregnancy rates (18.1% and 36.3%) than other types of multi-nucleation (n=25; 10% and 20%). Exclusive replacement of embryos with MNB observed on day-2 (n=63) led to a higher implantation rate (12.2%) than those in which MNB was first observed on day-3 (n=23; 3.5%). In fifteen cycles, embryos in which all cells were multi-nucleated on day-2 were replaced; four clinical pregnancies resulted; all have led to live birth.

1.3.4 Zona pellucida architecture

It is well established that the zona pellucida (ZP), the transparent glycoprotein shell that surrounds the mammalian egg plays an important role in normal mammalian embryo development (Wasserman and Mortillo, 1991). In the mouse, *in utero*, the zona is eventually shed by the embryo, and the timing of this event is well established (McLaren, 1967; 1968; 1970). In this species, the transcripts encoding constituent zona proteins are continuously translated during oocyte growth and their levels drop dramatically after ovulation (Reviewed by Zhao and Dean, 2002). Although

expression of zona pellucida proteins has been found to be oocyte-specific in the mouse (Epifano et al., 1995; El Mestrah et al., 2002), in other mammals such as the cow, rabbit, dog, and pig, follicle cells have also been found to express these proteins (Reviewed by Sinowatz et al., 2001). The latter is also true in cynomolgus (Martinez et al., 1996) and marmoset monkeys (Bogner et al., 2004). Moreover, FIGα, a transcription factor required for expression of zona proteins in the mouse, is also required for initiation of folliculogenesis in this species (Soyal et al., 2000). Together these data are at least suggestive that zona biosynthesis in most mammalian species is carried out by the oocyte in co-operation with its surrounding follicle cells.

The ZP can appear dark or pigmented, multilayered, distorted, too thick or too thin in human IVF embryos (Figure 1.6B). The exact origin of these characteristics is not known and in most cases, their relationship to embryo viability is difficult to assess. Zona pellucida thickness (ZPT) is an exception to this.

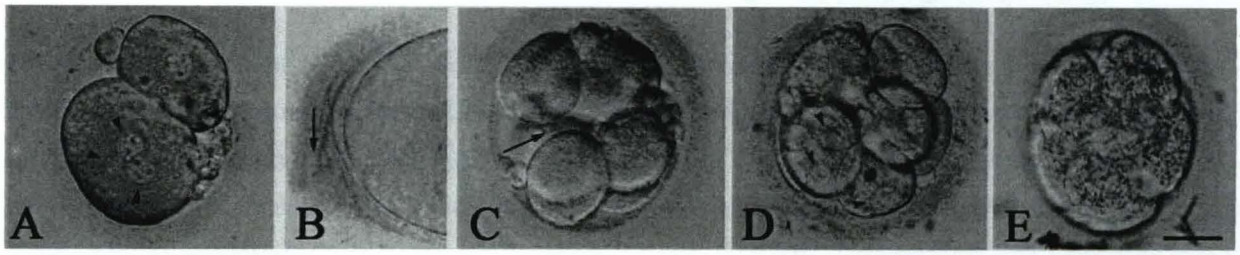


Figure 1.6 Human embryos on day-3 of development, showing various abnormal features. (A) multi-nucleation (arrowheads), (B) distorted and multi-layered zona pellucida (arrow), (C) poor cell adhesion (arrow), (D) vacuoles in the cytoplasm (arrowheads), and (E) cytoplasmic vesiculation or "pitting". Scale bar is approximately 50μm.

Cohen et al. (1988; 1990) discovered that ZPT and ZPT variation (ZPTV) were predictive of embryo hatching ability, hence potentially also predictive of viability following *in vitro* culture. Garside and colleagues (1997) studied the normal progression of ZP thinning during *in vitro* culture and the variables that correlate with ZP thickness. They confirmed earlier findings of Cohen (1993) that ZPT decreased during *in vitro* culture, and that these changes were more evident in embryos that resulted in successful pregnancies. They also found a significant correlation between ZPT and embryo quality (cell number and fragmentation) and maternal age. Increasing cell number as well as increasing maternal age (most notably >35 years) correlated with decreased ZPT, while increased fragmentation was associated with thicker ZP.

The preovulatory hormonal environment (Bertrand et al., 1996; Loret De Mola et al., 1997) has been linked to ZPT. Oestradiol levels at the time of hCG administration have been shown to have an inverse linear relationship and the dosage of hMG a direct linear relationship with ZPT. This

was interpreted to mean that ZP formation within the follicle is optimal in "good responders" who require fewer doses of gonadotrophins for adequate ovarian response (Bertrand et al., 1996). A direct correlation between ZPT and basal FSH levels was also established, but this was reversed by 48 hours of culture: embryos of patients with the highest FSH had thinner ZP at the time of transfer than embryos of other patients (Loret De Mola et al., 1997).

The variation of ZPT has been shown to be a strong predictor of pregnancy and therefore a useful parameter for embryo selection (Palmstierna et al., 1998; Gabrielsen et al., 2000). Zona pellucida thickness variation is obtained by taking the average value of 2-4 measurements of ZP thickness around the circumference of the embryo. A ZPTV value of less than 15% was a poor prognosis for pregnancy, while inclusion of one or more embryos with more than 20% variation led to very high pregnancy rates in a small group of patients (26/34 or 76.5%). Gabrielsen et al. (2001) showed that in embryos with cleavage abnormalities, the consideration of ZPTV during embryo selection led to a two-and-one-half fold increase in the chance of achieving a clinical pregnancy.

More recently, Shen et al. (2005) used orientation-independent polarization microscopy to evaluate ZP morphology quantitatively. They found that the magnitude of "light retardance" in the inner layer of the ZP is more than 30% higher in the oocytes of the conception cycles than the non-conception cycles. They concluded that this parameter may provide a non-invasive marker for oocyte development potential.

1.3.5 Cell-cell contact

Cell adhesion plays a pivotal role in morphogenesis (Johnson et al., 1986; Fleming and Johnson, 1988). The expression of cell adhesion molecules is partly dependent on cell-cell contact. For instance, aggregation of developmentally blocked cells from 2-cell mouse embryos with cells of a non-blocking genotype can restore the developmental capacity of the former, which resume expression of cell adhesion molecules on their surfaces (Neganova et al., 2000). The degree to which human embryos show intercellular contact is therefore likely to impact their development, including allocation of cells to the inner cell mass.

Consistent with this, disorganization and reduced cell adhesion after thawing was found to lower implantation rate of frozen-thawed cleavage stage embryos (Cohen et al., 1988). Fresh embryos can exhibit the same feature (Figure 1.6C). Cell-cell contact is also reduced by cytoplasmic fragmentation. It is more than likely that the loss of cell adhesion impacts a number of key events that precede differentiation, including cell polarization, compaction, and structural junction formation.

1.3.6 Cytoplasmic appearance

The presence of numerous intracytoplasmic vacuoles (Figure 1.6D) and cytoplasmic contraction in blastomeres signals possible degenerative processes (Sathananthan, 1993). Conversely, the appearance of vesicular (sometimes referred to as "pitted") cytoplasm, commonly observed in day-3 embryos (Figure 1.6E), may be a positive sign for genomic activation: fractional volumes of at least two cytoplasmic organelles, tubular endoplasmic reticulum and secondary lysosomes, were found to significantly increase with transcriptional activity (Tesarik et al., 1988). In one scoring system devised by Desai et al. (2000), vesiculation was found to correlate well with pregnancy and implantation; embryos were given positive points in the absence of vacuoles and the presence of a "pitted" cytoplasm. Other cytoplasmic inclusions, such as "pyknotic nuclei" or areas of intracellular necrosis, observed and studied in oocytes (Van Blerkom, 1991; Alikani et al., 1995), are often isolated in one or more blastomeres of the cleaving embryo. Their specific impact on development is undetermined but appears to be relatively insignificant.

1.3.7 Cytoplasmic fragmentation

During the early days of human embryo culture *in vitro*, Edwards (Edwards et al., 1970) observed a phenomenon he described as "cell division without nuclear division". This was noted in both developing and arrested embryos. That observation still holds true: partial or total loss of cellular integrity can affect a large percentage of cleavage stage embryos. Some examples of fragmented/fragmenting embryos are shown in Figure 1.7.

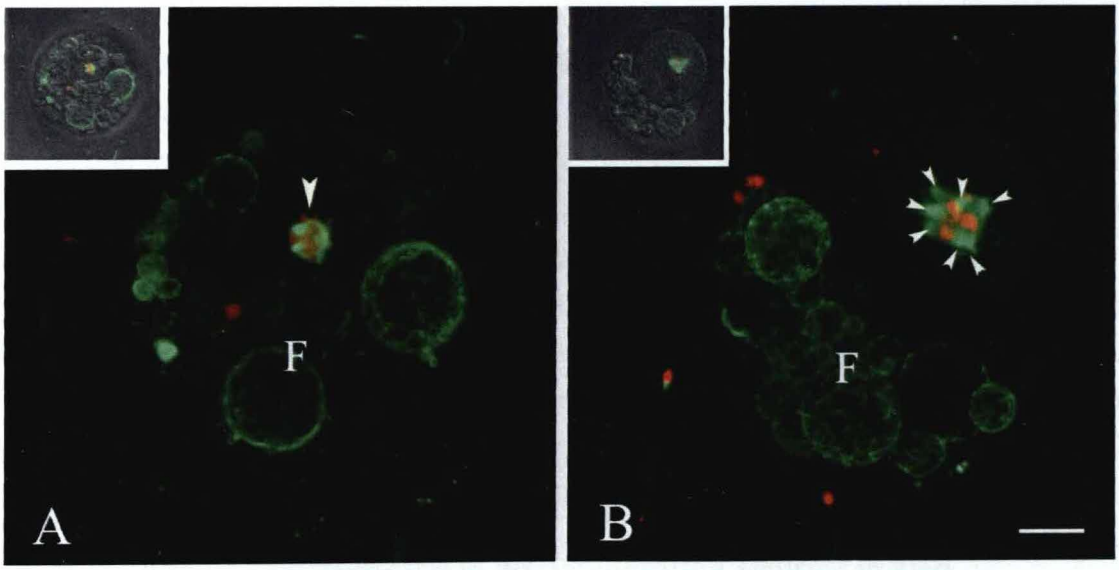


Figure 1.7 Human embryos with extensive fragmentation. Embryos have been stained for tubulin (green) and DNA (red). DIC images are single 3-4μm thick optical sections obtained on the laser scanning confocal microscope. Many fragments (F) and two abnormal spindles (arrowheads) are visible in both (A) and (B). Scale bar is 20μm.

Fragmentation before syngamy also occurs, albeit very rarely, and in many cases is associated with zygotic/embryonic arrest. In one study by Schmiady and Kentenich (1993), 38% of arrested zygotes exhibited fragmentation. Our own database reflects the rarity of this type of fragmentation (less than 2% of all zygotes) and shows that a great number of pronucleate embryos with fragmentation either arrest on day 1, arrest on day-2, or become excessively fragmented by day-3 and are neither suitable for transfer nor for cryopreservation. Antczak and Van Blerkom (1999) reported complete arrest either at the pronuclear or the syngamic stage, if fragmentation at the one-cell stage was pervasive and affected a significant portion of the oolema. Cytoplasmic fragmentation is the most common cleavage anomaly affecting human embryos developing in culture.

1.3.7.1 Cytoplasmic fragments

Fragments are cytoplasmic pieces that do not contain a nucleus but may contain chromatin. On the scanning electron microscope (SEM), cell fragments appear to have a surface structure very different from that of whole cells. The surface of whole cells is covered by microvilli that are organized with consistent density and length, whereas fragments have somewhat disorganized, small, rounded blebs covering their surfaces (Dale et al., 1995).

Transmission electron micrographs reveal a heterogeneous internal organization and content, that is normal, apart from the absence of a nucleus. Depending on their origin and position, fragments may contain a full complement of organelles, including mitochondria, (Sathananthan et al., 1993; Van Blerkom et al., 2001), or they may be virtually devoid of organelles (Van Blerkom et al., 2001).

Fragments may be reincorporated or they may lyse under some circumstances. Hardarson et al. (2002) reported an observation to this effect with the aid of time-lapse photography. Of three fragments that appeared on the surface of one blastomere of a two-cell embryo during the first cleavage, one was completely internalized within minutes, and its contents became continuous with the underlying blastomere. The basis of such reincorporation has been provided by transmission electron microscopy (TEM) showing cytoplasmic continuity between columns of fragments and the underlying cell (Van Blerkom et al., 2001). Evidence also points to lysis of small apical fragments while, according to Van Blerkom et al. (2001), larger fragments can swell, display a transparent cytoplasm and disappear in a "burst-like" fashion. It has been suggested that lysis of detached fragments may be due to absence of mitochondria and the associated depletion of ATP stores in these structures (Van Blerkom et al., 2001).

1.3.7.2 Cytoplasmic fragmentation in preimplantation embryos in vivo

Fragmentation is not exclusive to embryos developing *in vitro*. *In vivo*-produced cleavage stage embryos collected from a number of species, including the human, exhibit various degrees of cellular fragmentation. Embryos recovered after natural or induced ovulation in the baboon

(Hendrickx and Kraemer, 1968) and rhesus monkey (Batta et al., 1978; Hurst et al., 1980; Enders et al., 1982) contained "fragmenting blastomeres". Killeen and Moore (1971) reported a high incidence of "anucleate particles" in viable sheep embryos collected from fallopian tubes and portions of the uterine horn 60 hours following uterine insemination or natural mating.

In the human, it is difficult to know whether fragmentation is as common *in vivo* as it is *in vitro*. Information on morphological features of *in vivo* fertilized and developed human embryos is understandably limited. Three important studies, Hertig et al. (1954), Ortiz and Croxatto (1979), and Buster et al. (1985) provide some clues, however. Hertig and co-workers (1954) examined 4 ova that they judged to be abnormal. Apart from multi-nucleated blastomeres, the authors referred to "cellular degeneration or necrobiosis" in these ova which vaguely implies fragmentation, although this is not obvious from the thick sections through the embryos.

Ortiz and Croxatto (1979) associated fragmentation with aging of the unfertilized oocyte: 42% of unfertilized ova recovered 96 or more hours after the LH surge from women practicing sexual abstinence showed fragmentation. They also identified 25 abnormal ova recovered from the genital tract of women who had intercourse both outside and within the fertile period. Nine of the 25 ova had not cleaved and were presumed to be unfertilized. Thirteen ova exhibited no sign of cleavage or cytoplasmic fragments. Whether fragmentation resulted from aging of unfertilized ova or had occurred subsequent to fertilization could not be determined.

In the study of Buster et al. (1985), the four 1-cell ova recovered by uterine lavage at 100 or more hours post ovulation from fertile donors were found to be fully intact. These investigators collected 15 other ova in which development was arrested between the 2 and 16-cell stage. One 6-cell embryo had fully intact, but unevenly sized cells with contracted cytoplasm, while the intact cells of an unevenly divided 2-cell embryo were in close proximity to necrotic debris (possibly a lysed cell) in the perivitelline space (PVS). Another embryo evaluated as a 14-cell with "non-uniform" cells may have actually contained some fragments, but this is difficult to ascertain.

Overall, it may be safe to say that although fragmentation seems to be a feature of both *in vitro* and *in vivo* fertilized embryos, it is more prevalent among the former, at least when the scenarios represent comparable situations of infertility. Perhaps *in vitro* conditions and/or ovarian hyperstimulation are more conducive to the induction of embryonic fragmentation.

1.3.8 Causes and mechanisms of cytoplasmic fragmentation in blastomeres

The causes of fragmentation in human embryos remain largely unknown, but a number of factors may play a role.

1.3.8.1 Ovarian stimulation

Ovarian hyper-stimulation and oocyte maturation under adverse follicular conditions may contribute to fragmentation and related abnormalities. Poorly vascularized, and thus hypoxic follicles, have been reported to produce eggs with increased cytoplasmic and chromosomal abnormalities and to embryos with reduced development potential (Van Blerkom et al., 1997). However, fragmentation is clearly not limited to embryos generated following exogenous gonadotrophin administration; embryos from non-stimulated natural ovulatory cycles also display fragmentation in mild to severe forms (Monks et al., 1993) but of course the common denominator between the two situations is exposure of embryos to *in vitro* culture conditions.

1.3.8.2 Culture conditions

Quite apart from the influence of media composition, the conditions under which embryos are maintained *in vitro* have an impact on embryonic development. Extra-cellular pH (Squirrell et al., 2001), and temperature (Pickering et al., 1990), for instance, can impose their effects by altering the cytoskeleton. A decrease in the pH of the culture medium may lead to destabilization of cell membranes through inhibition of actin polymerization (Begg and Rebhun, 1979).

Similarly, high oxygen tension has been shown to lead to accumulation of reactive oxygen species in blastomeres, and said to jeopardize the integrity of their membranes, triggering apoptosis and fragmentation (Yang et al., 1998).

1.3.8.3 Chromosomal abnormalities

Numerical and structural chromosome abnormalities inherent in male and female gametes (Pellestor, 1991; Martin, 1987) may not preclude fertilization, but may produce embryos that subsequently fragment and fail to develop. Indeed, a very high percentage of embryos with extensive fragmentation has been shown to be chromosomally abnormal.

Pellestor et al. (1994) examined the karyotypes of 118 embryos with irregular blastomeres and extensive fragmentation. A normal diploid chromosome complement was found in only 12% of the embryos examined, the remainder showing a variety of abnormalities such as aneuploidy and mosaicism. Munné et al. (1995) applied fluorescent in situ hybridization (FISH) with probes for chromosomes X, Y, 18, and 13/21 to 154 slow and/or fragmented embryos. Chromosomal anomalies found in this group included extensive diploid mosaicism, aneuploidy, and polyploidy.

In further studies, all intact blastomeres of embryos with extensive fragmentataion were subjected to chromosome analysis. Sixty-six percent of embryos exhibiting more than 35% fragmentation were shown to be chromosomally abnormal (Munné et al., 2001). Though this was significantly

higher than the 47% abnormality rate among embryos with less than 35% fragmentation, the findings also imply that a fair number of fragmented embryos may be chromosomally normal.

1.3.8.4 Apoptosis

Body cell renewal takes place in a rapid process that also involves the degradation and disposal of damaged cells. This process is termed apoptosis or programmed cell death (PCD). The morphological signs of apoptosis are described as condensation of the nucleus and the cytoplasm, nuclear fragmentation, plasma membrane ruffling and blebbing, and ultimately production of cell fragments of varying sizes (Kerr et al., 1972). This is different from necrosis, the other form of cell death, which usually results from injury and affects entire groups of cells (Wyllie et al., 1980).

The morphological similarities between somatic cell apoptosis and embryo fragmentation, as well as evidence for programmed cell death in mammalian blastocysts (El-Shershaby and Hinchliffe, 1974; Mohr and Trounson, 1982; Enders et al., 1990) was noted by some investigators, and a proposal was put forth linking apoptosis and early embryo blastomere fragmentation (Jurisicova et al., 1995; 1996b).

This was based on evidence obtained by applying transferase-mediated dUTP nick-end labeling (TUNEL) to arrested fragmented embryos to identify condensation and degradation of chromatin (Jurisicova et al., 1996b). Subsequently, Levy et al. (1998) argued that TUNEL alone may fail to distinguish between apoptosis and necrosis; they used TUNEL in combination with annexin V labeling in order to detect cells in "early stages of apoptosis". Annexin V binds phosphatidylserine, a molecule that translocates from the inner to the outer cell membrane during apoptosis (Martin et al., 1995). All arrested or fragmented human embryos stained positively for annexin V, while cryopreserved embryos that continued to develop normally after being thawed did not show any staining (Levy et al., 1998).

However evidence disputing the role of apoptosis in blastomere fragmentation is accumulating. Van Blerkom et al. (2001) have convincingly argued that the TUNEL assay and annexin V labeling cannot reliably determine whether cell death has occurred, because normal active nuclei can be TUNEL positive, and, if cell death has occurred, annexin cannot reveal what type of death the cell has suffered since cells undergoing necrosis or lysis also stain positively with annexin V. Van Blerkom et al. (2001) therefore subjected fragmented embryos to the 'comet' assay in addition to TUNEL and annexin V in order to detect DNA damage. Positive TUNEL fluorescence and comets were evident only in second polar bodies, and in embryos with cell lysis, or where DNA cleavage was experimentally induced by DNase treatment. All fixed embryos displayed annexin V labeling. The latter demonstrated a major weakness of the study by Levy et al. (1998) in which fixed embryos displaying annexin V staining were thought to be undergoing apoptosis.

1.3.8.5 Other non-apoptotic pathways

Van Blerkom et al. (2001) offer an attractive alternative to apoptosis, in that they consider the role of the cytoplasm in fragmentation. They propose that an oncosis-like process, may be the underlying mechanism of fragmentation in human embryos. Oncosis has been described as a potentially reversible set of events that occur in oxygen-deprived somatic cells and can lead to cell death (Majno and Joris, 1995); oncosis is also characterized by membrane elaboration resembling fragmentation. Van Blerkom et al. (2001) argue that in blastomeres of early human embryos, transient and focal ATP deficiencies resulting from discrepant distribution of mitochondria in blastomeres may be the trigger for such a process.

This suggestion is based on the observation that large regions of cortical cytoplasm in zygotes as well as early blastomeres can be devoid of mitochondria (Van Blerkom et al., 2000). This deficiency in the inherited complement of these organelles, they propose, can lead to insufficient ATP concentrations for proper maintenance of cell membrane integrity, ultimately leading to fragmentation.

1.4 Aims of the study

The low efficiency of therapeutic IVF is a formidable problem in assisted reproduction. Analysis of data show that two in three eggs that are retrieved from superovulatory treatment become clinically unusable. They are judged to be unsuitable for transfer or cryopreservation (Figure 1.8) (e.g., 94,653 discarded / 133,956 eggs collected; M. Alikani and G. Tomkin, unpublished data) and are discarded at the end of the treatment cycle. The majority of these eggs are "lost" between fertilization and compaction, primarily to fragmentation, slow cleavage, and arrest.

It is the aim of this thesis to delineate the consequences of the most frequent cause of embryonic loss during *in vitro* culture—cytoplasmic fragmentation. This may be achieved through detailed analysis of embryology data obtained in the course of patient treatment cycles at the IVF Clinic (at Saint Barnabas Medical Center, NJ, USA).

Several elements in the laboratory of the Clinic have helped to create this opportunity. Culture of individual rather than groups of embryos allows the monitoring of their development from fertilization through the last day in culture. This meticulous follow-up of every egg collected is possible because of the availability of an extensive embryology database. The database was designed to allow the type of data collection and analysis integral to the present studies, and has been used for this purpose for over a decade. Once a question is formulated, specific queries can be written to obtain

the pertinent data, which can be studied and analyzed in-depth. The large numbers of embryos in the database enable higher levels of significance to be determined in multivariate analyses.

It is an aim of these studies to provide answers to several important questions in order to fill in the large gaps in current knowledge about the clinical significance of fragmentation: Is fragmentation morphologically indistinct (more or less uniform) or are there certain patterns? What is the potential of a fragmented embryo to continue development *in utero* and form a fetus? Can this potential be improved by opening the ZP (assisted hatching) and removing the fragments? What is the potential of a fragmented embryo to form a normal blastocyst *in vitro*? Is it preferable to

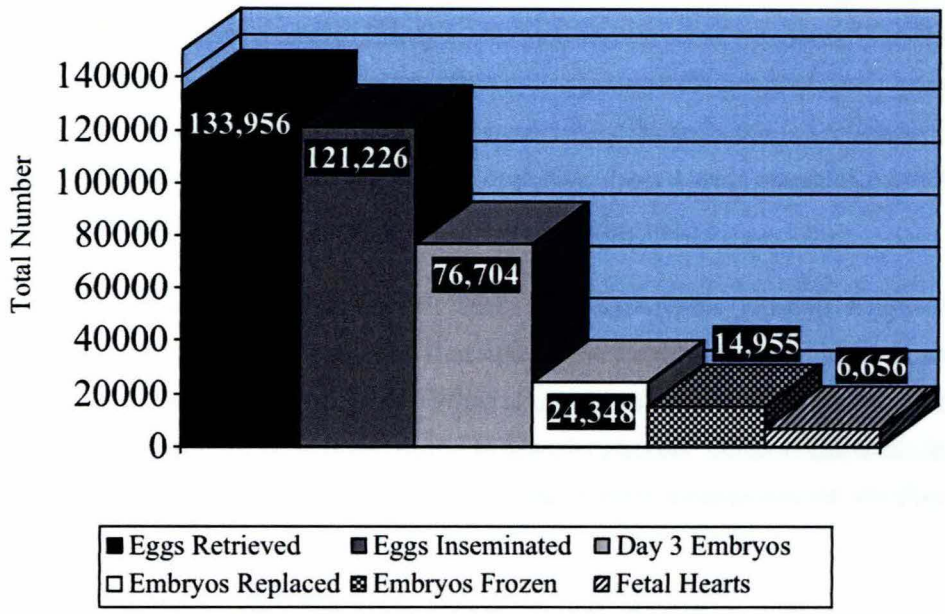


Figure 1.8 The low proportion of clinically usable eggs following super-ovulation. Roughly one third of the total number of eggs collected is frozen or transferred; the remaining eggs are discarded at various stages.

transfer fragmented embryos to the uterus as soon as possible or to leave them in culture to see whether they survive? When fragments are present, is survival *in vitro* more of a challenge than it is *in vivo*?

Another aim of these studies is to address a fundamental problem in the investigation of cytoplasmic fragmentation: the lack of an appropriate experimental model in which the phenomenon itself may be studied. Fragmentation has been observed in all species of mammals in which embryo transplantation has been implemented. However, when the phenomenon is encountered, following superovulation with exogenous gonadotrophins, or during the process of nuclear transplantation, it is attributed, respectively, to apoptosis and failed nuclear transfer. But unless fragmentation

can be reliably and easily induced, and studied in a readily available animal model, uncovering its origins and mechanisms would be very difficult. In the absence of such studies, fragmentation in human embryos has been viewed simply as a degenerative process and an unavoidable nuisance. The few studies in this area, have, perhaps understandably, viewed fragmentation from the perspective of cell death, attempting to link the phenomenon to apoptosis. There has been little effort to investigate fragmentation from other angles. This has placed an arbitrary limit on the scope of such investigations, ignoring some basic observations regarding fragmentation. For instance, unfertilized human eggs rarely ever show signs of fragmentation during *in vitro* culture (the same is true generally of unfertilized or unactivated eggs in other species as well); nor is fragmentation unequivocally associated with arrest of development in cleavage stage embryos, although arrested embryos are destined to die. If fragmentation were part of a process whereby unfertilized (or unfertilizable) eggs are eliminated *in vivo* through a genetically programmed process, why do they then not consistently fragment during culture after they fail to become fertilized? If fragmentation represents death, why do fragmented human embryos show a wide range of morphologies and why is limited fragmentation compatible with further development?

The studies that follow attempt to answer other basic questions: How do fragments interfere with development—is it their mere presence that affects the health of the embryo as a whole or do they signify other underlying abnormalities? What is fragmentation and does it occur randomly during the course of development or is it restricted to certain phases? What is the basis for the association of fragmentation with other abnormalities? Is this a mere association or are they mechanistically inter-related?

The undertaking of these studies is hoped to lead to a better understanding of early human development *in vitro*, and provide the basis for development of strategies to prevent the loss of large numbers of embryos during therapeutic IVF.

Monash University

Declaration for Thesis Chapter 2

In the case of Chapter 2, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	50	Human IVF laboratory protocols, research protocols, writing of manuscript
2. Tim Schimmel	25	Ordering and management of materials/equipment/animals for research
3. Elena Kissin	25	Ordering and management of materials for clinical IVF

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Tyho-Galileo Research Laboratories, Hudson, NY, USA

		Date
Signature 1	[redacted]	24 DECEMBER, 2005
Signature 2		1-3-2006
Signature 3		1.4.2006

Chapter 2

General materials and methods

2.1 Clinical data collection and analysis

Data from IVF treatment cycles were collected and analyzed using the EggCyte database (EggCyte, ART Institute of NY and NJ, Livingston, NJ, 1995-2005)(Tomkin and Cohen, 2001). A computer network with both paper and on-line database record-keeping was maintained in the clinical IVF laboratory. Data were recorded at the point of generation and afterwards confirmed with the paper records. Using MS-Access (Microsoft Corporation, Seattle, USA), a suite of tables, forms, program functions, and reports tailored to the requirements of clinical embryology for individual oocyte and embryo tracking were created. Like any relational database, the tables link the relevant records by indexed numeric fields common to each.

After retrieval, oocytes were numbered and tracked individually with separate pages or tables for each day of development, just as in a daily journal.

When embryos were transfered, separate records for each were again created and linked to the record of the transfer procedure. As pregnancy results became available, they were entered in a further table, always linked by procedure number. Data for the analyses in these studies were extracted by creating selection lists or queries from relevant tables in the database. EggCyte has over 40 data tables, all or some of which may be used in any of the analyses. Hundreds of queries of varying complexity with Structured Query Language (SQL) and Visual Basic programming functions (Microsoft Corporation, Seattle, USA) were used to generate the data presented in the following chapters.

2.2 Ovulation induction, embryo transfer, luteal phase support, and assessment of pregnancy

All patients undergoing IVF were placed on one of two protocols: 1) mid-luteal (cycle day-21) down-regulation with leuprolide acetate (0.5mg)(Lupron; Tap Pharmaceuticals Inc., Lake Forest, IL, USA) followed by stimulation with gonadotrophins on day-3 of the subsequent menses, or 2) a microdose leuprolide acetate (50μg twice a day) flare protocol utilizing leuprolide on day-2 of

an oral contraceptive withdrawal bleed followed by gonadotrophin stimulation beginning one day later (day-3 of menses). Patient diagnosis and previous response were used to tailor a clinically optimal protocol. Various urinary gonadotrophins (Metrodin; Serono Laboratories Inc., Randolph, MA, USA; Pergonal; Serono Laboratories Inc., Randolph MA, USA; Humagon; Organon Inc., West Orange, NJ, USA; Repronex; Ferring Pharmaceuticals Inc., Tarrytown, NY, USA), and recombinant gonadotrophins (Gonal F; Serono Laboratories Inc, Randolph, MA, USA; Follistim; Organon Inc., West Orange, NJ, USA) were employed at different times. The daily gonadotrophin dosage varied but was usually between 300 and 450 IU. Ovulation was triggered with either 5,000 IU or 10,000 IU of human chorionic gonadotropin (hCG; Profasi; Serono Laboratories Inc, Randolph, MA, USA; Pregnyl; Organon Inc., West Orange, NJ, USA; Novarel; Ferring Pharmaceuticals Inc., Tarrytown, NY, USA). Oocyte retrieval was performed 35 hours after hCG administration.

Embryo transfers were scheduled between noon and 14.00 hrs on day-3 or day-5 of embryo development (day 0 = day of insemination). This was done under ultrasound guidance, using a Wallace (Irvine Scientific, Irvine, California, USA) or Edwards-Cohen (IVF online) transfer catheter. From August 1995 until October 1999, the average numbers of embryos replaced in regular (average age 35.46) and oocyte donation cycles were, respectively, 3.31 and 2.72. In October 1999, a major organizational overhaul in the Saint Barnabas IVF program allowed implementation of a policy that reduced the number of embryos for replacement. This was despite a significant increase in the average age of the patients treated. The average numbers of embryos replaced in regular (average age 36.57) and oocyte donation cycles after that time were, respectively, 2.84 and 2.21.

Luteal phase support was by intramuscular progesterone (50mg) injection, first administered three days after oocyte retrieval and continued until ßhCG was assayed 16 days after hCG administration. Progesterone was continued in case of significant levels of ßhCG until the first ultrasound was performed at 5 weeks gestation. At this time, the number of gestational sacs, and if visible, yolk sacs, was noted. At 6 weeks gestation, fetal heartbeat (FHB) was assessed and crown-rump length (CRL) measurements were taken. At 7 weeks, in addition to measurement of FHB, interval growth was assessed with CRL. The final ultrasound prior to patient discharge for obstetrical care was performed at 8 weeks and again evaluated growth and presence of FHB within the normal developmental range.

2.3 Human gamete collection and culture

2.3.1 Oocyte collection

A list of all media used for culture and manipulation of human gametes and embryos is shown in Table 2.1.

Table 2.1 Culture and manipulation media in use in the clinical and research laboratories [1]

Medium	Source	Supplement/additives	Use
Acidified Tyrode's Medium (AT)	In-house	None (pH 2.36 - 2.4)	Zona drilling
Chatot Ziomek Bavister (CZB)	In-house	3% BSA	None
Calcium-magnesium-free CZB (CMF-CZB)	In-house	3% BSA	Dissociation of mouse embryos
Modified CZB (M-CZB)	In-house	3% BSA, 10µg/mL CCB [2]	Mouse egg/embryo enucleation
Human Tubal Fluid (HTF)	In-house	10% PS, 5% HSA, or 5% Plasmanate	Human embryo culture, semen preparation
HTF-HEPES	In-house	10% PS, 5% HSA, or 5% Plasmanate	Human egg collection and MM
CMF-HTF-HEPES	In-house	5% HSA, 0.05M sucrose	Human embryo biopsy
Gardner's growth medium, phase 1 (G1.2)	Scandinavian IVF	5% HSA	Human embryo culture (D1-D3)
Gardner's growth medium, phase 2 (G2.2)	Scandinavian IVF	5% HSA	Human embryo culture (D3-D6)
Global Medium (GLM)	LifeGlobal	10% HSA	Human embryo culture (D1-D6)
KSOM	1% BSA	None	Mouse embryo culture
KSOM [AA]	1% BSA	None	Mouse embryo culture

[1] Some media are no longer in use.

[2] On some occasions, 1µg/mL colcemid was also added.

BSA = Bovine serum albumin

CCB = Cytochalasin B

D1-D3 = Day 1 to day 3 of development

HSA = Human serum albumin

MM = Micromanipulation

PS = Patient's heat inactivated serum

Oocytes were collected in HEPES-HTF medium. After cumulus dissection (during which part of the cumulus was removed) and washing, oocytes were placed in 100-200mL drops of protein-supplemented HTF under mineral oil (Squibb, Princeton, New Jersey, USA).

Protein supplementation was provided either by 10% heat-inactivated maternal serum, 6% plasmanate (Miles Inc., Indiana, USA) or 6% Synthetic Serum Substitute (SSS; Irvine Scientific, Irvine, California, USA). [1] Oocytes and embryos were cultured at 37°C in an atmosphere of 5% CO_2 and 95% humidity in protein-supplemented HTF. Culture dishes containing oil (only) for each case were prepared and placed in the incubator one day before oocyte retrieval. Culture media were prepared on that day but placed at 4°C until 2-4 hours before oocyte retrieval. At that time, drops were made in the pre-equilibrated oil dishes and the dishes were placed in the incubator again.

[1] Serum and SSS are no longer in use.

2.3.2 Semen collection and preparation

The semen sample was collected by masturbation on the day of oocyte collection. The specimen was allowed to liquefy for 20 minutes at room temperature. Sperm concentration (count) was determined on a CELL-VU slide (Millenium Sciences Corporation, New York, NY, USA). Semen was diluted 1:2 with protein-supplemented HTF and spun in a centrifuge at 200g for five minutes. The cell pellet was resuspended in 1.0mL protein-supplemented HTF. Sperm were separated on a discontinuous density gradient (PureSperm 100; Nidacon, Gothenberg, Sweden). The Isotonic PureSperm was used to prepare the dilutions for the gradients. The gradients were made as follows: 1.0mL of 95% on the bottom, 70% in the middle, and 47.5% on the top. Once the sperm suspension was placed on the 47.5% layer, the gradient was spun in a centrifuge at 400g for 10 minutes.[1] After centrifugation, at least 0.5mL of the 95% layer was collected, washed twice in 3mL fresh medium, and resuspended in fresh medium, the volume depending on the total count of sperm. Sperm morphology was assessed using Kruger's strict criteria (Kruger et al., 1986). The sample was kept in suspension in protein-supplemented HTF until insemination (4-6 hours after oocyte collection). Insemination was either by standard methods by addition of approximately 50,000-200,000 sperm per mL to each medium drop containing eggs, or by intracytoplasmic sperm injection (ICSI) (Palermo et al., 1995). ICSI was applied primarily in case of male factor infertility.

2.3.3 Assessment of fertilization and zygote changeover

Fertilization was assessed roughly 24 hours from oocyte retrieval (16-20 hours from insemination). This was done by mechanical removal of corona-cumulus cells by repeated aspiration-expelling through finely pulled glass pipettes (200-600μm inner diameter). Oocytes in which a single pronucleus or two pronuclei were seen after standard insemination and those in which two pronuclei were seen after ICSI were separated and transfered individually into fresh drops of protein-supplemented HTF or 10% HSA-supplemented Global medium (GLM; Life Global, Guilford, Connecticut, USA), under mineral oil (Squibb, Princeton, NJ, USA or GenX International, Guilford, CT, USA). They remained in the same dishes until day-3 or day-6 of development, respectively. The dishes were prepared as for oocyte collection when HTF was used or they were prepared and equibrated in the incubator if G2.2 (Scandinavian IVF Science, Sweden) or GLM were used. For extended culture of embryos that were in HTF during days 1-3, the embryos were switched from HTF to G2.2 medium on day-3 of development. A second changeover to fresh G2.2 was done on the morning of day-5, after embryo evaluation and before embryo transfer.[2]

[1] Speed was adjusted based on sample quality. Oligospermic/azospermic samples were spun at 1800 rpm.
[2] G and S media are no longer in use in the clinical lab; they have been replaced with Global media (Life Global, CT, USA).

2.3.4 Embryo evaluation

On days 2, 3, 4, and 5 of development, embryos were evaluated on an Olympus IX70 inverted microscope (Olympus America, Melville, New York), equipped with Hoffman Modulation Optics (Narishige, Japan) at a total magnification of 600X. On the morning of days 2 and 3 of development, a number of parameters were recorded: uneven appearance of cells, including the presence of a dominant blastomere, the degree of fragmentation expressed as a percentage and defined as the embryonic volume occupied by anucleate cytoplasmic fragments, fragmentation patterns defined and based on spatial distribution and relative size of the fragments, the number of blastomeres with multiple nuclei (fully sized or micro-nuclei) and the number of nuclei in each multi-nucleate blastomere, reduced cell-cell adhesion/contact and generally disorganized appearance of the embryo, presence of cytoplasmic inclusions, including vacuoles, contraction of the cytoplasm, zona pellucida abnormalities and thickness (occasionally measured).

Assessment criteria for day-4, 5, and 6 embryos and blastocysts are described in Chapter 4.

2.4 Source of human embryos for experimentation

The embryos used in the experiments were obtained from consenting patients, undergoing infertility treatment by IVF/ET, and under a protocol approved by the Internal Review Board of Saint Barnabas Medical Center in 1995 and 1999, revisions of which were re-approved in 2000, 2002, and 2004. The protocol concerned the in-depth study of abnormal gametes and embryos generated during clinical IVF procedures and judged to be unsuitable for transfer or cryopreservation. A copy of the consent forms appear in appendices A and B.

Embryos with extensive abnormalities were collected upon completion of each treatment cycle, that is, after transfer and cryopreservation of viable embryos, and with the signed consent of the patient. Fresh or frozen/thawed embryos with one or more of the following characteristics were normally not considered for transfer: 1) fewer than two cells on day-2 of development, 2) fewer than five cells on day-3 of development, 3) no division in 24 hours of culture, 4) one or more highly uneven cleavage divisions, 5) loss of more than 35% of the total cytoplasmic volume to fragmentation or degeneration, 6) large fragments associated with few remaining blastomeres, 7) one or more multi-nucleated blastomeres appearing either on day-2 or day-3 of development, 8) less than 50% of the cells remaining intact after thawing of cryopreserved embryos.

2.5 Mouse oocyte and embryo collection and culture

A list of all media used for culture and manipulation of mouse eggs and embryos may be found in Table 2.1.

Eight to tweleve-week old F1 (C57BL/6J x BALB/c) females were injected intraperitoneally with 10 IU of pregnant mare's serum gonadotrophin (PMSG; Sigma-Aldrich Company, St Louis, Missouri, USA), followed 48-49 hours later by 10 IU hCG (Sigma-Aldrich) to induce ovulation. Ovulated oocytes were collected 13-18 hours later (depending on the experiment) by dissecting the ampullae of the oviducts in modified CZB medium (Chatot et al., 1989; Chatot et al., 1990). The modified medium (M-CZB) contained D-glucose, HEPES, and reduced bicarbonate (Kimura and Yanagimachi, 1995) and was supplemented with 0.1% hyaluronidase and 3% bovine serum albumin (BSA, fraction V; Sigma-Aldrich). Zygotes were recovered 18-20 hours post hCG injection also by dissecting the ampullae. All oocytes and zygotes were cultured in drops of KSOM (Lawitts and Biggers, 1993) or KSOM with amino acids (KSOM^{AA}; Ho et al., 1995; Biggers et al., 2000) (Specialty Media, Phillipsburg, NJ, USA) under mineral oil (Squibb), at 37°C in 5% CO_2 and 95% humidity.

2.6 Zona pellucida removal and disaggregation of embryos

To disaggregate embryos, the zona pellcida (ZP) was removed from embryos by 10-minute incubation in the protease, pronase (from *Streptomyces griseus*; Sigma-Aldrich) (Mintz, 1962). The protease solution was made in M-CZB and contained 0.5% pronase, 0.1% PVP, and 3% BSA. During this incubation period, the ZP began to loosen and dissolve. At this point, the ZP was completely removed by pipetting the embryo through a pulled glass pipette. Zona pellucida-free embryos were incubated in calcium-magnesium-free CZB (CMF-CZB) for 10 minutes. The cells were separated mechanically by pipetting through a pulled glass pipette. In the case of fragmented human embryos, incubation in CMF medium was often not necessary since the cells were held together loosely and disaggregated easily following removal of the ZP and pipetting.

2.7 Micromanipulation of human and mouse eggs and embryos

2.7.1 Microtools

Holding pipettes, ICSI needles, and blastomere biopsy needles were purchased (Humagen Fertility Diagnostics, Charlottesville, VA, USA). Holding pipettes had an inner diameter of 20μm, and an outer diameter of 65-100μm, depending on the type of egg/embryo under manipulation. Needles for ICSI had an outer diameter of about 7-8μm, an inner diameter of about 5μm, and a sharp spike of about 2μm. The biopsy needles had an inner diameter of 40μm. Assisted hatching needles were

prepared in-house and had an inner diameter of 10-12μm. Closed-tip ZP dissection needles were prepared in-house by pulling pre-pulled pipettes on a microforge (model MF-9; Narishige, Tokyo, Japan). For enucleation, a pipette with an inner diameter of roughly 30μm was manufactured in-house. The tip of the pipette was rounded using the heated filament of the microforge. All tools were bent at about 800μm from the tip at a 40° angle.

2.7.2 Equipment, media, and procedures

2.7.2.1 Human

For human egg and embryo manipulation, the micromanipulation medium was HEPES-buffered HTF (HEPES-HTF) supplemented with 10% human serum albumin (HSA; SAGE *in vitro* Fertilization, Inc., Trumbull, CT, USA). Embryo biopsy and assisted hatching procedures were performed in shallow culture dishes (Falcon 1006; Becton and Dickinson, Franklin Lakes, NJ, USA) with drops arranged under oil as shown in Figure 2.1.[1] Manipulations were done on a heated microscope stage (Thermo Plate, Tokai Hit, Shizuoka-ken, Japan), set to 37°C. The work surface of the laminar flow hood used for general handling of eggs and embryos was also set to 37°C. The procedures were performed at 40X magnification using an IX-70 inverted Olympus microscope (Olympus America, Melville, New York, USA), connected to a 14-inch monitor, and equipped with Hoffman Modulation Optics (Narishige, Tokyo, Japan) and hydraulic miromanipulators (Narishige, Tokyo, Japan).

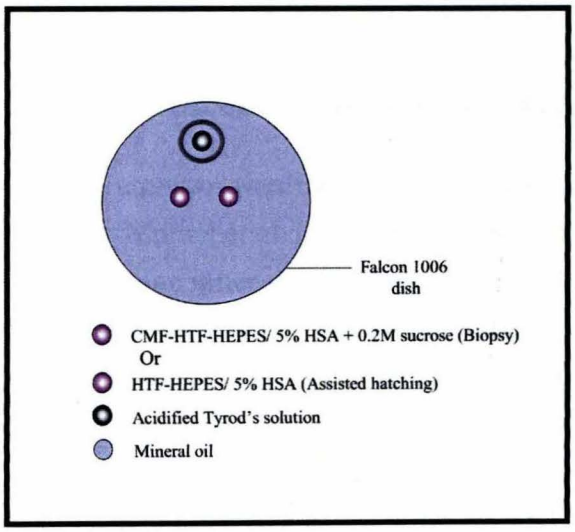


Figure 2.1 A schematic representation of a culture dish prepared with microdrops under oil for human embryo biopsy or assisted hatching micromanipulation procedures.

2.7.2.2 Mouse

All micromanipulations were performed in shallow Falcon culture dishes with medium drops arranged under oil as shown in Figure 2.2. For enucleation, 10μg/mL cytochalasin B (CCB; Sigma-Aldrich) was added to M-CZB, as specified in methods in the relevant chapter. Microscope and manipulators were the same as that used for human embryo work.[2] Some procedures were performed at room temperature while others were performed at 37°C, as specified in methods in each chapter.

[1] Figure adapted from The Embryology Laboratory Manual, IRMS at SBMC.
[2] The research laboratory is completely separated from the clinical IVF laboratory.

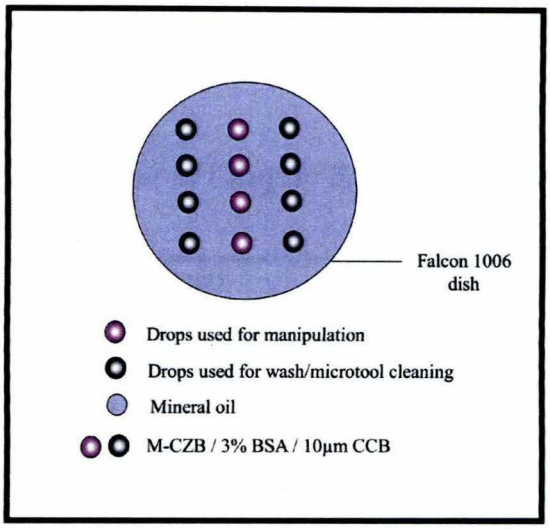


Figure 2.2 A schematic representation of a culture dish prepared with microdrops under oil for mouse egg and embryo micromanipulation procedures.

2.8 Preparation of host zonae pellucidae

Mouse, bovine, or human eggs or embryos were placed in a drop of M-CZB for micromanipulation. The ZP was either opened mechanically, using a ZP dissection microneedle, or by laser irradiation (Fertilase; ZMS, Vero Beach, FL, USA). The egg/embryo was then positioned so that the ZP incision was at the three o'clock position. An enucleation needle with an outer diameter of 30μm was inserted through the opening and the cytoplasm was aspirated until the ZP was empty.

2.9 Fixation of cells for cell counts and fluorescence *in situ* hybridization

Whole embryos were fixed on glass slides using a method first described by Tarkowski (1966), modified by Munné et al. (e.g., 1993; 1998) and described in detail by Velilla et al. (2002). A capillary tube with an inner-diameter of 0.16 mm was filled with a 1:3 solution of methanol: acetic acid. One embryo was picked up and placed on a clean glass slide on the stage of a stereoscope (Leica MZ 9.5; Leica Microsystems Bannockburn, IL, USA) along with a minimum volume of the fixative. While the ZP dissolved and the solution evaporated, humidity was increased by blowing on the slide until all cytoplasm was dissolved and the nuclei were fixed onto the glass slide. Nuclei were counted on a compound microscope (Olumpus BX 41, Olympus America, Inc., Melville, NY, USA).

[1] Figure adapted from The Embryology Laboratory Manual, IRMS at SBMC.
[2] The research laboratory is completely separated from the clinical IVF laboratory.

Monash University

Declaration for Thesis Chapter 3

In the case of Chapter 3, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	60	Concept, data collection and analysis, writing of manuscript
2. Jacques Cohen	15	Database design, data collection and analysis
3. John Garrisi	6	Embryology data collection
4. Giles Tomkin	15	Database design, data analysis
5. Caryn Mack	2	Endocrinology data collection
6. Richard Scott	2	Clinical Director of IVF

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, West Orange, NJ, USA

		Date
Signature 1	[redacted]	24 DECEMBER, 2005
Signature 2		12-24-2005
Signature 3		1/3/06
Signature 4		24 DEC., 2005
Signature 5		1/4/06
Signature 6		24 DEC., 2005

Chapter 3

Human embryo fragmentation *in vitro* and its implications for pregnancy and implantation

3.1 Summary

Although fragmentation is the most common feature of human embryos *in vitro*, its impact on pregnancy and implantation is only generally understood and the phenomenon itself and its origins remain largely undefined. Through non-invasive morphological assessments, we have defined five distinct patterns of fragmentation based on the size and position of fragments in relation to those of the nucleated cells. It is shown that these patterns along with the degree of fragmentation determine the developmental potential of fragmented human embryos. Exclusive transfer of embryos in which more than one third of the cytoplasmic volume was represented by fragments or those with large fragments led to very low implantation and pregnancy rates. The data also suggest that microsurgical removal of the fragments may be of benefit to some fragmented embryos.

3.2 Introduction

Fragmentation is a common feature of human embryos developing *in vitro* (Trounson and Sathananthan, 1984; Sathananthan et al., 1990). When exceeding 10% of the embryonic volume, it is thought to reflect a certain aberration in development. Although a study by Jurisicova et al. (1996b) suggested that programmed cell death or apoptosis is triggered in arrested fragmented human embryos, the exact manner in which fragments interfere with development of non-arrested embryos remains to be clarified.

The current protocols assess the quality of an embryo based largely on the degree to which it has fragmented. This system of embryo "grading" reflects early knowledge of human embryo morphology (Veeck, 1988). It is now inadequate, as it distinguishes fragmented embryos only at a rudimentary level and may underestimate the developmental potential of certain types (Erenus et al., 1991; Shulman et al., 1993).

The largest studies published to date on the impact of fragmentation and the outcome of *in vitro* fertilization and embryo transfer (IVF/ET) are those of Staessen et al. (1993), Giorgetti et al. (1995), and Ziebe et al. (1997). These investigators have reported very low implantation rates (approximately 5%) after transfer of embryos with 10-50% fragmentation on day two of development. These reports do not address the differences among embryos within this wide range, nor do they consider the size and distribution of the fragments.

Since August 1995, record keeping of embryonic development and transfer at the Saint Barnabas Clinic has included detailed tracking of individual embryos, making it possible to retrospectively analyze large series of cases. Here we have considered specific degrees and patterns of fragmentation and indicate their impact as well as the effects of fragment removal on the implanting potential of fragmented embryos.

3.3 Materials and Methods

3.3.1 Patients in the study

An institutional review board approval was not required for this study since the work is routine at the Saint Barnabas Clinic and was based on a previously approved protocol. This involved over 3000 patients without obvious side effects (Cornell University Medical College, 1989). The study population consisted of patient procedures for which pregnancy outcome was known (n=2410). Transfer cycles involving frozen-thawed embryos were excluded. Ovulation induction, luteal phase support, and pregnancy assessment protocols are described in Chapter 2 (section 2.2).

3.3.2 Homogeneous transfers

To eliminate or minimize correlative uncertainties when multiple embryos were transferred, only patient procedures with "homogeneous transfers" were selected for these analyses. A transfer was considered homogeneous when more than one half of the embryos replaced were in the same category. A pool of 2410 procedures was searched for homogeneous transfers. With respect to the degree of fragmentation, 1,727 procedures were found to be homogeneous ("W" groups). With respect to the pattern of fragmentation, 570 procedures were homogeneous ("T" groups). Procedures common to "W" and "T" groups were homogeneous with respect to both degree and pattern of fragmentation and were analyzed separately.

The degree of fragmentation in replaced embryos in groups W1 to W5 was 0-5%, 6-15%, 16-25%, 26-35%, and greater than 35%, respectively. The pattern of fragmentation in groups T1 to T5 was Types I through V, respectively, and followed the definitions below. Since only six patients were in group T5, this group was excluded from all analyses.

3.3.3 Definition of fragmentation patterns

Based on distribution and size of the fragments, five patterns of fragmentation were defined. Embryos with these patterns are presented in Figure 3.1, before and after fragment removal.

Type I fragmentation was minimal in volume and the fragments were typically associated with only one blastomere. Type II fragments were small and localized, forming a column associated with one or more cells. Type III fragments were small and scattered throughout the embryo. Type IV fragments are large, sometimes resembling whole blastomeres. These fragments were scattered throughout the embryo and were associated with uneven cells. Type V fragments appeared necrotic, and were associated with a characteristic granularity and cytoplasmic contraction in the intact blastomeres.

A designation of "no distinct pattern" implied that fragmentation did not fit in any of the above categories.

3.3.4 Assisted hatching and fragment removal

All embryos fragmented beyond 5% arriving to day-3 of development and selected for transfer were subjected to selective assisted hatching (Cohen et al., 1992), followed by microsurgical removal of some or all fragments (see Figure 3.1).

Fragment removal was attempted at high magnification and with continuous re-focusing and rotation of the embryo to avoid damaging intact blastomeres and to provide access to more fragments. The needle was moved towards the contra-lateral side of the aperture in the zona pellucida (ZP) or placed between blastomeres in order to remove fragments. Any blastomeres lysed accidentally during the procedure were removed completely.

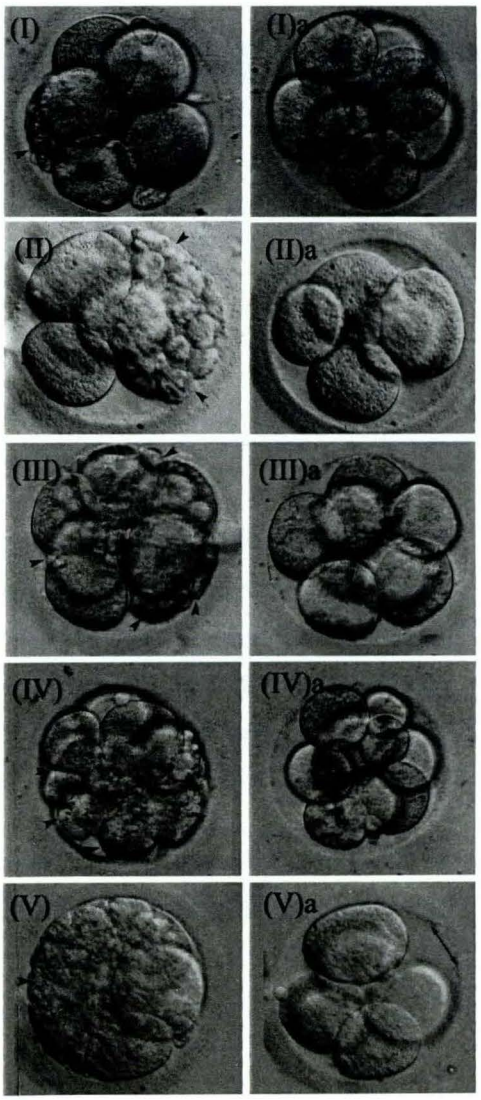


Figure 3.1 Day-3 fragmented human embryos with fragmentation patterns I, II, III, IV, and V. The left panel shows the embryos on day three of development before assisted hatching and fragment removal. Arrowheads point to the position of fragments. The right panel shows the same embryos after assisted hatching and fragment removal. Note differences in the organization and appearance of the remaining blastomeres after removal of different types of fragments. Arrow in (V)a shows the size of the ZP hole.

3.3.5 Statistical analysis

To find the relationship between fragmentation and pregnancy and implantation, variables represented as proportions (pregnancy and implantation rates) were analyzed by means of logistic regression. The Tukey multiple comparisons test was used to compare mean cell numbers among different fragmentation patterns.

3.4 Results

3.4.1 The degree of fragmentation and its impact on implantation and pregnancy

To measure the impact of fragmentation on implantation and pregnancy, we considered the degree and type of fragmentation as evaluated on the morning of developmental day-3. Data for the homogeneous transfer groups with respect to the degree of fragmentation ("W" groups, n=1727) are presented in Table 3.1.

Table 3.1 Pregnancy and implantation in five transfer groups according to the degree of fragmentation

Fragmentation Range (%)	Transfer Group	Total No. of Procedures	No. of Patients with Positive ßhCG	No. of Procedures with FHB>0	Clinical Pregnancy Rate (%)	No. of Embryos Replaced	No. of Sacs	No. of FHB	Implantation Rate (%)
0-5	W1	1,062	769	669	63.0	3,651	1,312	1,160	31.8
6-15	W2	533	365	308	57.8	1,845	584	521	28.2
16-25	W3	64	42	33	51.6	231	64	54	23.4
26-35	W4	41	22	19	46.3	111	32	26	23.4
>35	W5	27	9	4	14.8	78	6	5	6.4
Total		**1,727**	**1,207**	**1,033**	**59.8**	**5,916**	**1,998**	**1,766**	**29.9**

FHB= Fetal Heart Beat

During the 1727 procedures reviewed, a total of 30,772 oocytes were retrieved, 5916 embryos were transferred of which 2105 had fragment removal. The average fragmentation rate for all day-3 embryos and for those replaced on day-3 was 15.4% and 8.6%, respectively. The incidence of clinical pregnancy, defined as fetal heart beat (FHB) detected on ultrasound, was 59.8% (1033/1727). Implantation rate, defined as FHB per embryo replaced, was 29.9% (1766/5916). The average maternal age in this population was 35.7 ± 4.25 (S.D.) years.

There was a significant decrease in implantation and pregnancy as fragmentation increased in degree ($p<0.001$). This significance was largely (but not entirely) due to the drop in pregnancy and implantation beyond 35% fragmentation.

3.4.2 Microsurgical fragment removal

Following microsurgical fragment removal, none of the transfers in the study involved embryos with more than 25% fragmentation. The most fragments were removed from embryos with greater than 35% fragmentation (Figure 3.2).

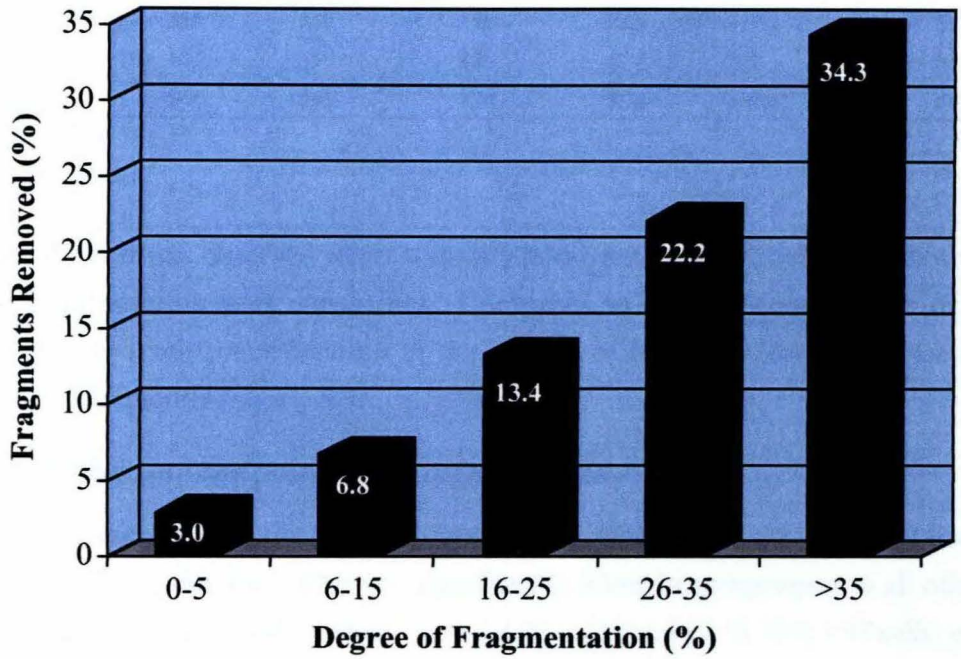


Figure 3.2 The percentage of fragments removed from fragmented embryos Vs. the degree of fragmentation. Nearly all fragments were removed from embryos with more than 35% fragmentation. In other groups, on average, roughly half the fragments were removed.

The damage rate was calculated based on the number of embryos in which one or more blastomeres lysed as a result of contact with the assisted hatching needle. The overall damage rate was 6.33% (255/3775) and it ranged from 1.7% (11/654) in embryos 0 to 5% fragmented, to 11.4% (22/193) in embryos with more than 35% fragments. The pattern of fragmentation also affected the degree to which cells were accidentally damaged. Embryos with no distinct pattern of fragmentation were those most frequently damaged (27/223; 12.1%), followed by Types V, IV, III, II, and I (4/190; 2.1%).

3.4.3 The pattern of fragmentation and its impact on implantation and pregnancy

Data for the "T" groups are presented in Table 3.2. There was a general decline in implantation and pregnancy in successive groups T1 to T4, with the largest decrease in group T4 ($p<0.001$).

Table 3.2 Pregnancy and implantation in four transfer groups according to the pattern of fragmentation

Transfer Group	Mean (%) Day-3 Fragmentation [a] (±SD)	Total No. of Procedures	No. of Patients with Positive ßhCG	No. of Procedures with FHB>0	Clinical Pregnancy Rate (%)	No. of Embryos Replaced	No. of Sacs	No. of FHB	Implantation Rate (%)
T1	5.9 (2.91)	63	48	41	65.1	206	87	78	37.9
T2	14.0 (6.77)	62	51	42	67.7	216	85	73	33.8
T3	14.4 (5.89)	335	239	199	59.4	1,089	378	332	30.5
T4	25.2 (10.43)	104	53	42	40.4	318	68	58	18.2
Total		**564**	**391**	**324**	**57.4**	**1,829**	**618**	**541**	**29.6**

FHB= Fetal Heart Beat

[a] Before fragment removal

A meaningful trend was observed when transfers homogeneous with respect to both degree and pattern of fragmentation were considered. Compared to the implantation rate of embryos in W2-W4 groups (without consideration of the pattern of fragmentation), embryos in group T4 implanted least frequently (Figure 3.3).

3.4.4 Cell number and the patterns of fragmentation

These data are presented in Table 3.3. Embryos with localized fragments (Type II) had an average of 3.22 ± 1.14 cells on day two. This was significantly lower in comparison to all other fragment types ($p<0.001$). On day-3, both types II (5.98 ± 1.99 cells) and IV (5.58 ± 1.97 cells) embryos had fewer cells compared to others ($p<0.001$).

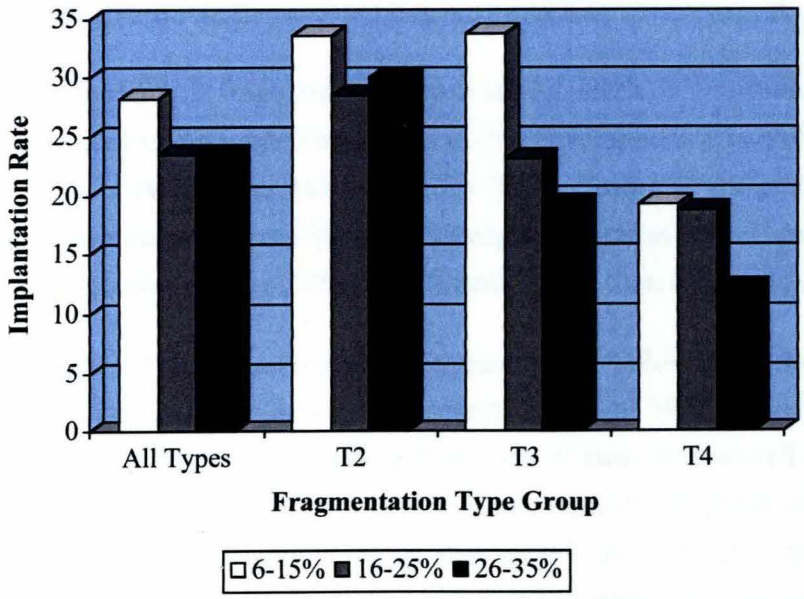


Figure 3.3 The incidence of implantation in transfer groups T2, T3, and T4 compared to implantation of embryos according to the degree of fragmentation and without consideration of the pattern of fragmentation. Homogeneous transfer of type IV fragmented embryos led to the lowest rate of implantation.

Table 3.3 Average cell number on days 2 and 3 of development according to the four patterns of fragmentation

Fragment Type	Day-2 Cell Number	Day-3 Cell Number
I	3.64	7.39
II	3.21 [a]	5.98 [a]
III	3.85	7.06
IV	3.65	5.58 [a]

[a] Significantly different from cell number of other types on that day ($p<0.05$)

3.5 Discussion

What is evident from years of recorded observations on fragmentation is that it does limit development of the human embryo *in vitro*. The specifics of this developmental limitation, however, have been far from clear. Comparisons among programs have been uncertain because of inadequate descriptions of morphology or indirect evaluations such as assignment of grades or scores. This has hampered the development of an acceptable embryo classification system that, for instance, would serve as a general aid in embryo selection for transfer.

The present study focuses on an important aspect of embryo morphology, namely fragmentation, from an angle other than that used traditionally. This work establishes a relationship between the degree and pattern of fragmentation on day three of development and the incidence of implantation and pregnancy following selective assisted hatching and fragment removal.

Our findings indicate that not all fragmentation, even at high levels, is detrimental to the embryo. The pattern of fragmentation has a profound effect on the developmental fate of the embryo, thus must be considered in its evaluation. Homogeneous (half or more of the embryos in the same category) transfer of embryos with large fragments, designated here as type IV pattern of fragmentation, produced an implantation rate (18%) significantly lower than that of other types.

There was also a significant decrease in clinical pregnancy rate following homogeneous transfer of such embryos (40% compared to an overall pregnancy rate of 58.5%). Electron microscopic evidence suggests that large fragments originate from cells of two- or four-cell embryos, as these fragments have mitochondria that are more electron-dense than in later stage cells (Sathananthan et al., 1993). The release of large fragments at an early stage may deplete the embryo of essential organelles such as mitochondria, or structures such as pinocytotic caveolae which are involved in uptake of exogenous protein. Moreover, the portion of the cell that retains the nucleus may

actually arrest following such a great loss. The minimum cytoplasmic volume required by a human blastomere at various stages in order to undergo further division is not known at present.

The average degree of fragmentation on day-3 was higher (25%) in group T4 (Type IV embryos replaced primarily), than all others. This high degree of fragmentation was probably one contributing factor to decreased implantation. However, to delineate the effect of fragmentation pattern apart from fragmentation degree, we considered implantation in transfers homogeneous for both degree and pattern of fragmentation. This analysis showed that transfer of Type IV fragmented embryos, with 6 to 35% fragmentation, led to implantation rates lower than their Type II or III counterparts, or the implantation rate of fragmented embryos regardless of the pattern of fragmentation. However, these differences were not significant.

It should be noted that implantation in group W3 with 16-25% fragmentation was in fact identical to that of group W2 with 6-15% fragmentation. It is thus more likely that the difference in implantation of Type II and Type IV embryos is at least influenced by fragmentation type.

In contrast to large fragments, small, scattered fragments (Type III) do not appreciably affect cell number during the three days in culture. Nor do they appear to pose a serious threat to further development. Based on the appearance of these fragments, it is likely that they are generated during successive divisions. They may not point to a systemic anomaly, but to "imperfect" cytokinesis.

The distribution of fragments is also of significance, as in the case of localized fragments (Type II), which appear to have resulted from the complete fragmentation of one or more cells. Despite lower cell numbers both on day-2 and day-3 of development, the implantation rate of embryos with Type II fragmentation is nearly 34%. Elimination of selected blastomeres by the embryo may reflect its effort in restoring or maintaining viability when anomalies affect particular blastomeres. For instance, limited diploid mosaicism is not uncommon among day-3 embryos (Munné et al., 1995). Early loss of such cells would prevent their later contribution to the inner cell mass or the trophectoderm of the blastocyst.

There was no correlation between the appearance of any pattern and maternal age, suggesting that ovarian response to exogenous gonadotrophins is not a significant factor in the evolution of fragmentation patterns. Although implantation is best when embryos are not fragmented, moderately fragmented embryos implant frequently. This is consistent with cytogenetic data indicating that about 60% of highly fragmented embryos (up to 40%) are chromosomally normal (Reviewed in Munné and Cohen, 1998). However, these data are in sharp contrast to previous reports which estimate the implantation rate of such embryos at 5% (Staessen et al., 1993; Ziebe et al., 1997).

In the current study, a very low implantation rate (6%; 3 delivered) occurred when replaced embryos had greater than 35% fragmentation before fragment removal. The relatively high implantation rate of embryos with 20-35% fragmentation may be attributable to microsurgical fragment removal, since it was applied routinely during the course of this study. The application of fragment removal is based on our previous finding of a 4% overall increase in implantation rate when assisted hatching and fragment removal were applied simultaneously; this was in comparison to embryos which were only zona-drilled (Cohen et al., 1994).

We propose two hypotheses, one or both of which may explain this effect. The first is that removal of extracellular fragments restores the spatial relationship of cells within the embryo. Fragments positioned between cells (as in fragmentation pattern III) may cause distortion of division planes or interfere with normal cell-cell contact, leading to abnormal compaction, cavitation, and blastocyst formation. Furthermore, fewer cells may be allocated to the inner cell mass, as this is dependent on contact between sister blastomeres (Edwards and Beard, 1997). A second explanation for the relatively high rate of implantation of moderately fragmented embryos following fragment removal may be the prevention of secondary degeneration. On the transmission electron microscope, blastomeres immediately adjacent to fragments show early signs of vacuolar degeneration (Sathananthan et al., 1990). Removal of fragments and cell degradation products may stop deterioration of the remaining cells. Perhaps a lesson is to be learned from lower organisms, such as *C. elegans*, in which at least eight genes are dedicated to the function of cell corpse recognition, engulfing and degradation by neighboring cells (Ellis et al., 1991). Since blastomeres of early human embryos do not appear to have such phagocytic activity, necrotic fragments and cell remains are not effectively removed from the developing embryo and may become problematic.

In addition to fragments and remains of cells, the perivitelline space in fragmented embryos often appears to be filled with other debris. Some of this debris is traced to the polar body or corona cell processes (Trounson and Sathananthan, 1984). However, it is possible that debris is also generated by lysed fragments.

During artificial removal, fragments, "cells" completely devoid of cytoplasm, as well as structures resembling extra-cellular vesicles occasionally break as they come in contact with the needle. The close proximity of any of these components to the surrounding cells can cause further damage leading to the demise of an otherwise viable embryo. This has been demonstrated in the mouse, where the presence of deliberately lysed cells in company of healthy cells led to reduced blastocyst expansion and hatching (Alikani et al., 1993).

This work is partly an effort to expand the focus of embryo morphology assessment to include the size and distribution of fragments. This is a different concept from the traditional view, indicated only by blastomere size and degree of fragmentation. These data also suggest a positive influence of fragment removal on the implanting potential of partially fragmented embryos.

Monash University

Declaration for Thesis Chapter 4

In the case of Chapter 4, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	60	Concept, data collection and analysis, writing of manuscript
2. Golria Calderon	10	Data collection and analysis
3. Giles Tomkin	10	Database design, data analysis
4. John Garrisi	5	Data collection
5. Magdalena Kokot	5	Data input and analysis
6. Jacques Cohen	10	Database design, data analysis

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, West Orange, NJ, USA

		Date
Signature 1	[redacted]	24 DECEMBER, 2005
Signature 2		24 Dec, 2005
Signature 3		24 Dec, 2005
Signature 4		1/3/06
Signature 5		1/4/06
Signature		12-24-2005

Chapter 4

Cleavage anomalies in early human embryos and survival after prolonged culture *in vitro*

4.1 Summary

This study examines the relationship between common morphological anomalies of cleaving embryos and their ability to form apparently normal blastocysts *in vitro*. The impact of cleavage rate, fragmentation, and multi-nucleation on compaction, cavitation, along with inner cell mass and trophectoderm formation has been assessed. The study population consisted of 102 patients who elected or were selected to have a day-5 embryo transfer. Clinical pregnancy and implantation rates were 66.7% and 49%, respectively. Slow and fast cleavage had a significant negative correlation with normal blastocyst formation. Only 13.8% (67/484) of embryos with fewer than 7 cells and 27.5% (25/91) of those with more than 9 cells on day-3 formed blastocysts with apparently normal morphology, compared to 41.9% (252/602) with 7-9 cells on day-3 ($p<0.001$). Fragmentation had a negative impact on normal blastocyst formation. Embryos with more than 15% fragmentation formed normal blastocysts significantly less frequently (46/279; 16.5%) than embryos with 0-15% fragmentation (311/935; 33.3%)($p<0.001$). Furthermore, the pattern of fragmentation correlated with blastocyst formation. Type IV fragmentation led to a significant reduction in blastocyst formation (25/170 or 14.7%), compared to types I, II, and III which performed much better (38.6%, 32.9%, and 32.4%, respectively). Only 15.9% (22/142) of embryos with one or more multi-nucleate cells on day-2 and/or 3 formed normal blastocysts compared to 31.9% (335/1051)($p<0.001$) of those without multi-nucleation. Collectively, the data suggest that cleavage anomalies, some of which do not preclude development after short-term culture, may reduce the developmental competence of embryos after prolonged culture.

4.2 Introduction

While 70% of eggs fertilized *in vitro* undergo the first three cleavage divisions during three days in culture, less than half advance to cavitation after five days (Gardner et al., 1998a, b; Jones et al., 1998; Behr et al., 1999), and about one third form morphologically optimal blastocysts with a well

defined inner cell mass (ICM), a cohesive trophectoderm (TE), and full expansion (Racowsky et al., 2000).

It has been proposed that prolonged culture allows development of "normal" embryos with implantation potential, while "abnormal" and non-viable embryos arrest before or shortly after the onset of genomic activation (Huisman et al., 1994; Dawson et al., 1995; Janny and Menezo, 1996; Gardner and Lane, 1997).

Indeed, a number of intrinsic factors have been shown to influence survival to the blastocyst stage in extended culture, among them are sperm quality (Menezo et al., 1992; Jones et al., 1998), the etiology of infertility (Menezo et al., 1992), and maternal age (Menezo et al., 1992; Schoolcraft et al., 1999).

Cleavage patterns have also been linked to blastocyst formation (Bolton et al., 1989; Wiemer et al., 1995; Balakier and Cadesky, 1997; Rijnders and Jansen, 1998). These observations, together with the link between chromosomal abnormalities and aberrant early embryo morphology (Pellestor et al., 1994; Munné et al., 1994; Munné et al., 1995; Sadowy et al., 1998), as well as some reports indicating the high viability of *in vitro* grown blastocysts (Gardner et al., 1998a, b; 2000; Schoolcraft et al., 1999) lend support to the "survival of the fittest" proposal.

However, the relationship between morphology, chromosomal integrity, embryogenesis, and viability is clearly more complex. Embryos with normal morphology may be chromosomally abnormal, but can reach the blastocyst stage; on the other hand, many chromosomally normal embryos with atypical or normal morphology fail to undergo differentiation in extended culture (Sandalinas et al., 2000). There is also some clinical evidence that suggests a loss of development potential with extended culture of compromised embryos. Racowsky et al. (2000) reported that patients with no 8-cell embryos on day-3 had a 33% pregnancy rate after day-3 transfers, but failed to achieve pregnancies after day-5 transfers.

Hence, extrinsic factors such as prolonged culture under sub-optimal conditions may also contribute to the loss of *in vitro* generated embryos over time in culture, but, it is unclear which embryos are affected and to what extent. In the present study, the impact of cleavage rate, fragmentation, and multi-nucleation on compaction, cavitation, along with ICM and TE formation after prolonged culture is assessed. Morphological abnormalities of blastulation are described. The relationship between blastocyst morphology and implantation after day-5 transfer was also evaluated. The findings may have important implications for the application of day-5 transfer after IVF.

4.3 Materials and Methods

4.3.1 Patients in the study

Patients were treated between October 1997 and March 1999 at the Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center. The Internal Review Board of Saint Barnabas Medical Center approved the review of these patient records. Ovulation induction protocols are described in Chapter 2 (section 2.2). The study population consisted of 102 patients (including 38 oocyte recipients) who elected or were selected to have embryos transferred on day-5 of development, mainly to avoid high order multiple pregnancy. The mean ages of regular patients and oocyte recipients were 33.89 ± 3.7 and 40.06 ± 4.21 years, respectively.

Procedures during which embryos were pooled in groups (n=11) were excluded from the study, as the embryos could not be individually tracked during culture.

4.3.2 Embryos in the study

Embryo culture methods are described in Chapter 2 (section 2.3). A total of 1,395 zygotes from 102 patients were cultured, of which 112 were cryopreserved on or before day-3. An additional 69 embryos were discarded before reaching day-5, as they were judged to have completely arrested. Thus the total number of embryos cultured to day-5 was 1,214. The number of embryos in the cleavage rate analyses differs slightly from this number, since the pertinent information was not available in the database for all the embryos in the study.

4.3.3 Embryo evaluation

Embryos were evaluated according to the protocol described in Chapter 2 (section 2.3). For the present analyses, cell numbers on days 2 and 3 were adjusted according to each embryo's individual time in culture after retrieval using the following formula: [(Cell number/Hours in culture) x Average hours in culture for all embryos in the database]. The average number of hours in culture from oocyte retrieval to day-2 and day-3 were 48.28 and 71.68, respectively.

Development on days 4 and 5 was recorded in detail during the study but was classified only during data analysis. At that time, embryos were classified according to the criteria listed in Table 4.1. As is the nature of all morphological classification systems, the one proposed here is subjective, but based on our own observations as well as those of others on morula and blastocyst morphology in the human (e.g., Cohen et al., 1985; Hardy et al., 1989; Dokras et al., 1991; 1993; Van Blerkom, 1993; Gardner and Schoolcraft, 1999; Menezo et al., 1999). Our analyses do not include day-6 development since the majority of the blastocysts in this study, as in other studies (Gardner et al., 1998a, b), formed on day-5, and embryos that did not undergo differentiation by day-5 rarely

formed normal blastocysts on day-6. This is not to say that day-6 blastocysts do not have an implantation potential, but, in the context of this study, timing was an important factor in definition of normal development.

Table 4.1 Criteria used to evaluate development of embryos on day-4 and day-5

Developmental Event	Day-4	Day-5
Compaction		
Complete	N	S
Incomplete	N	S
Regional	S	A
Fusion-like	S	A
None	S	A
Cavitation		
Single Cavity	*	N
Beginning	N	S
Large vacuoles	A	A
Multiple cavities	A	A
Inner Cell Mass formation		
Distinct/organized	*	N
Forming	*	S
Large cells	*	S
Small mass	*	S
None	*	A
Trophectoderm organization		
Cohesive	*	N
Large cells	*	S
Irregular	*	A

N = Normal; S = Sub-optimal; A = Abnormal

*This developemntal event is not expected on this day.

Compaction was expected to occur on day-4 (Nikas et al., 1996); a delay of one day in compaction was considered an indication of slow development and thus sub-optimal. Absence of compaction on day-5 was considered abnormal. Compaction on both days 4 and 5 was defined as complete, incomplete, regional, or fusion-like. Complete or incomplete compaction involved all cells within the embryo, with all cells appearing flattened and cell boundaries becoming vague (complete) or only some cells demonstrating this morphology and cell borders still recognizable

(incomplete). Regional compaction (Figure 4.1; panel A) marked exclusion of one or more cells from the compacted morula. A less common form of compaction was termed "fusion-like" and involved the complete disappearance of intercellular spaces not by flattening of cells, but by what appeared as "fusion" of cells (Figure 4.1; panel B). The extent to which these morphologies determine the potential of the morula for development needs further assessment; but for the purposes of this study and based on the likelihood of these embryos to form normal blastocysts on day-5, we have classified regional, fusion-like, and delayed compaction (one full day's delay) as sub-optimal.

It was not unusual to see the beginning of cavitation on day-4. However, a single defined cavity was never observed before day-5. If a cavity was beginning to form on day-5, this was sub-optimal, but included as normal. The persistence of large vacuoles, small and multiple cavities on day-5 was considered abnormal (Hardy et al., 1989; Dokras et al., 1991; 1993) (Figure 4.1; panels C, D, and E, respectively).

A distinct ICM, organized as a compacted mass of numerous cells, was considered normal. If the inner cells formed a small mass, or if the mass comprised loosely packed cells, the formation was sub-optimal but considered normal (Gardner and Schoolcraft, 1999)(Figure 4.1; panels F and G, respectively). The absence of an ICM was abnormal (Hardy et al., 1989) (Figure 4.1; panel H). A co-

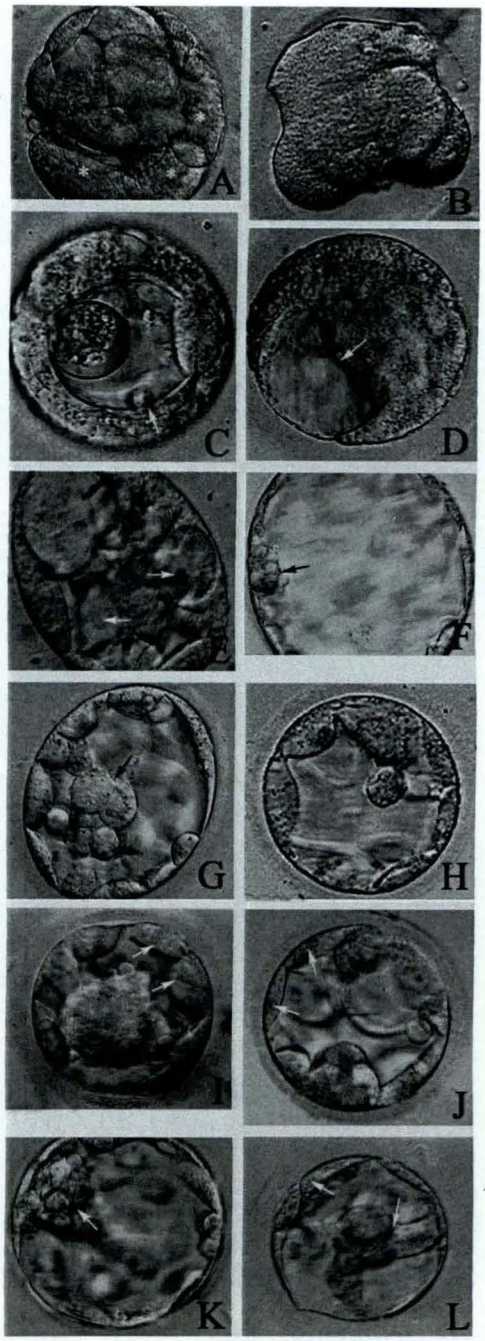


Figure 4.1 Various abnormal forms of compaction, cavitation, inner cell mass formation, and trophectoderm organization (A-L). Regional compaction occurred when several blastomeres (marked with asterisks) were excluded from the morula (Panel A); Complete disappearance of inter-blastomeric spaces was termed "fusion-like compaction" and was considered abnormal (Panel B); large vacuoles, sometimes with internalized excluded cells or fragments (Panel C, arrow), small cavities (Panel D, arrow), and multiple cavities (Panel E, arrows) were all considered abnormal forms of cavitation; A small mass of inner cells (Panel F, arrow) or few large and disorganized inner cells (Panel G, arrow) were sub-optimal formations, while the absence of an ICM (Panel H) was abnormal. Large TE cells, few in number (Panels I and J, arrows) were considered sub-optimal. Irregular forms of ICM and TE cells (Panels K and L, arrows) were considered abnormal.

hesive layer of numerous tightly packed cells in the TE was normal; large TE cells were sub-optimal but again considered normal (Figure 4.1; panels I and J). Highly irregularly arranged ICM and/or TE cells were considered abnormal (Figure 4.1; panels K and L). A blastocyst was considered morphologically normal if it contained either a normal or sub-optimal ICM in combination with either a normal or sub-optimal TE on day-5 (Table 4.1). The appearance of either an abnormal ICM or an abnormal TE, or a full day's delay in differentiation placed the day-5 embryo in the abnormal category. Ten representative blastocysts of normal appearance are shown in Figure 4.2; six of these have been delivered (shown in A, C, D, E or F, I, and J).

4.3.4 Statistical analysis

Differences in the number of embryos compacting, cavitating, and forming blastocysts in each fragmentation group as well as in each cleavage group were tested for significance using Chi square analysis. Differences between mean cell numbers were analyzed using ANOVA. P values <0.05 were considered significant.

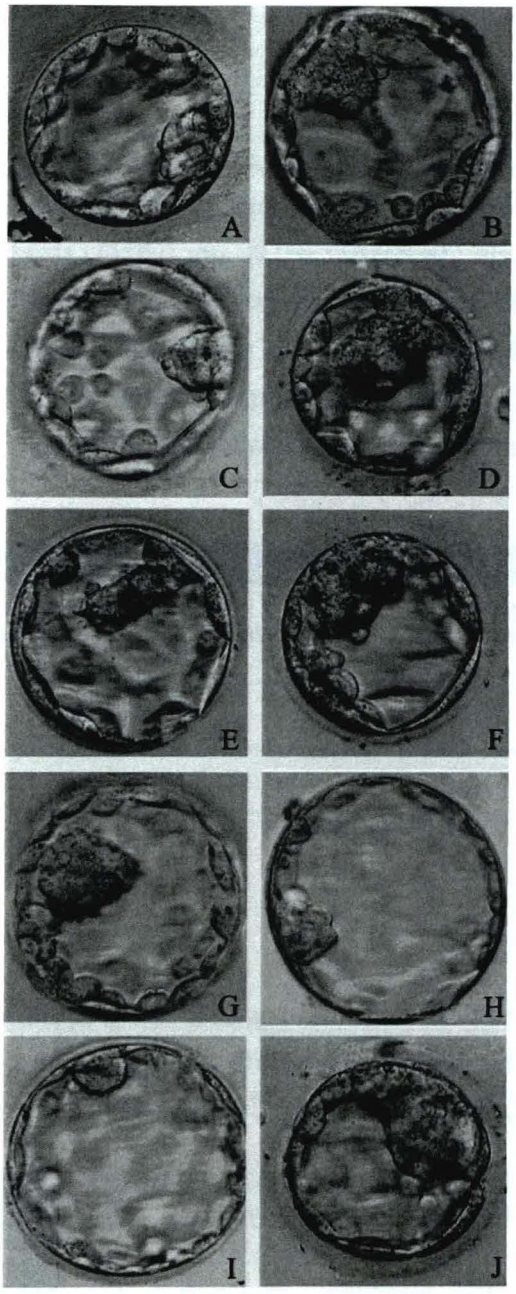


Figure 4.2 Representative day-5 embryos with normal morphology. A remarkable variation in size and shape of the ICM, as well as TE cells is visible. Embryo in panel **A** (delivered male) was an 8-cell without fragmentation on day-3. Embryo in panel **B** was an 8-cell with 15% fragmentation type III and did not establish a heartbeat. Embryo in panel **C** (delivered male) was an 8-cell with 5% fragmentation type I. Embryo in panel **D** (delivered male) was an 8-cell with 10% fragmentation type III. Embryos in panels **E** and **F** were 9-cell with 20% fragmentation type III and 8-cell with 15% fragmentation type III; they both implanted, but only a single female was delivered. Embryo in panel **G** was a 14-cell without fragmentation and did not implant. Embryo in panel **H** was an 8-cell with 15% type III fragmentation and did not implant. Embryo in panel **I** (delivered female) was an 11-cell without fragmentation. Embryo in panel **J** (delivered female) was an 8-cell without fragmentation.

4.4 Results

4.4.1 Overall clinical results

Table 4.2 is a summary of clinical outcome for the 102 cases reported here. Clinical pregnancy rate with confirmed fetal heart activity on ultrasound was 66.7% (68/102). Implantation rate (percentage of transferred embryos represented by fetal heartbeats (FHBs) was 49.0% (102/208). The average number of embryos transferred per patient was 2.06. Five patients received one embryo, 88 received 2 embryos, and 9 patients received 3 embryos. Overall blastulation rate was 44.9%; and normal blastocyst formation rate was 30.6% (371/1214) (some were sub-optimal; see Table 4.1, Figure 4.2, and materials and methods section). The majority of the blastocysts were obtained on day-5. All transfers were performed on day-5.

Table 4.2 Overall clinical outcome for day-5 transfers

Total No. of Procedures	No. of Procedures with FHB >0	Clinical Pregnancy Rate (%)	No. Delivered*	No. Lost	No. of Eggs Retrieved (Average)	No. of 2 PN	No. of Blastocysts (%)	No. of Embryos Replaced	No. of FHBs	Implantation Rate (%)
102	68	66.7	55	12	2146 (21.0)	1,395	576 (44.9)**	208	102	49

FHB = Fetal heart beat

*Delivery information was not available for one patient.

**Blastocyst formation rate reflects 114 embryos that were cryopreserved before or on day-3.

4.4.2 Development rate and blastocyst formation

Figure 4.3 is a summary of the data for a total of 1,177 embryos in the cleavage rate analysis. The rate of development was an important determinant of normal blastulation. Only 7.4% (15/204) of the embryos with fewer than 5 cells and 14.3% (17/119) of those with 5 to 6 cells on day-3 formed a

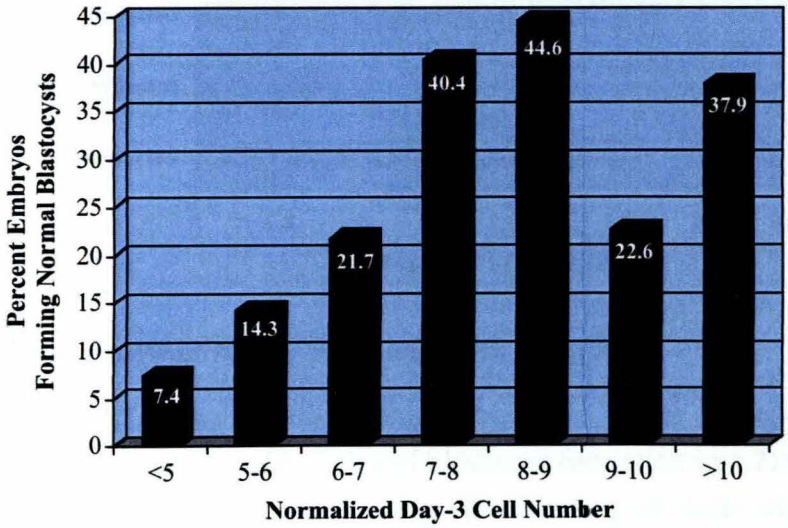


Figure 4.3 Normalized cell numbers on day-3 of development and the incidence of normal blastocyst formation. Embryos with fewer than 7 cells or more than 9 cells formed normal appearing blastocysts significantly less frequently than those with 7-9 cells ($p<0.001$).

morphologically normal blastocyst on day-5. The proportion of embryos developing into normal-appearing blastocysts increased significantly among embryos with 6-7 cells (35/161; 21.7%), 7-8 cells (157/389; 40.4%), 8-9 cells (95/213; 44.6%), 9-10 cells (14/62; 22.6%), and >10 cells (11/29; 37.9%) ($p<0.001$).

When divided into three groups, embryos with <7 cells, 7-9 cells, and >9 cells on day-3, the proportion of normal-appearing blastocysts were 13.8% (67/484), 41.9% (252/602), and 27.5% (25/91), respectively. The proportion of normal-appearing blastocysts was significantly higher among embryos with 7-9 cells compared to embryos with <7 cells ($p<0.001$) and >9 cells ($p<0.01$). The average (± Standard Deviation) cell numbers on day-2 and day-3 for normally-compacted day-4 embryos were 3.92 ± 0.75 and 7.65 ± 1.48, respectively. The day-2 and day-3 averages for embryos that formed a cavity on day-5 were 3.95 ± 0.71 and 7.70 ± 1.45, respectively. If a normal ICM had formed, average cell numbers for days 2 and 3 were 3.96 ± 0.49 and 7.97 ± 1.3, respectively. By contrast, cell numbers on both day 2 and day-3 were significantly lower in non-compacted embryos (3.52 ± 1.21 and 6.03 ± 2.15, respectively) and embryos that did not cavitate (3.56 ± 1.30 and 6.08 ± 2.24, respectively) ($p<0.001$). Embryos that did not form a normal ICM had significantly fewer cells on day-3 (7.42 ± 1.50) ($p<0.001$). Day-2 cell numbers did not differ between embryos forming a normal ICM verses an abnormal one (3.96 ± 0.49 Vs. 3.91 ± 0.78). The data for day-3 cell numbers are presented in Figure 4.4.

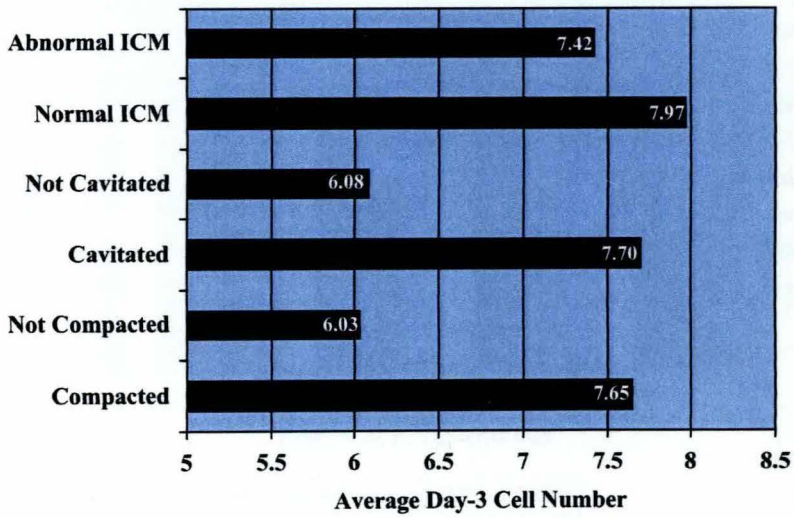


Figure 4.4 Average cell number of day-3 embryos and blastulation. Embryos that successfully underwent normal compaction, cavitation, and inner cell mass formation had significantly higher cell numbers than those embryos that failed to complete these developmental transitions ($P<0.001$).

4.4.3 Fragmentation and blastocyst formation

Figure 4.5 shows the relationship between the degree of fragmentation and the incidence of normal compaction, cavitation, and blastocyst formation in 1,214 embryos available for this analysis. Normal blastocyst formation rate decreased significantly with increasing fragmentation: 33.3%

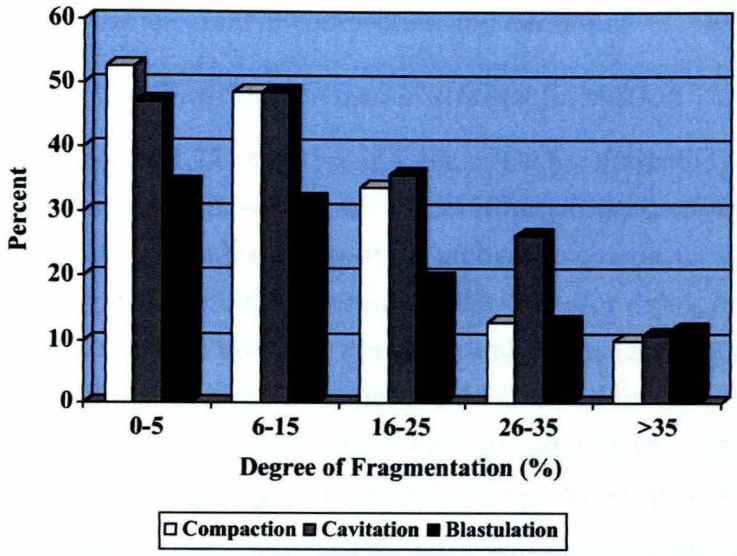


Figure 4.5 The relationship between fragmentation degree and normal compaction, cavitation, and blastocyst formation. When fragmentation exceeds 15%, the rates of normal compaction, cavitation and blastocyst formation are significantly reduced ($p<0.001$).

(311/935) among embryos with 0-15% fragmentation was significantly higher than 16.5% (46/279) among embryos with more than 15% fragmentation ($p<0.001$). The difference was apparent at compaction (51.1% vs. 24.6%, respectively) and cavitation (47.9% vs. 29.3%, respectively). When fragmentation exceeded 35%, all processes were severely compromised, and normal compaction (5/50; 10%), cavitation (5/47; 10.6%) and blastocyst formation (6/51; 11.8%) were low.

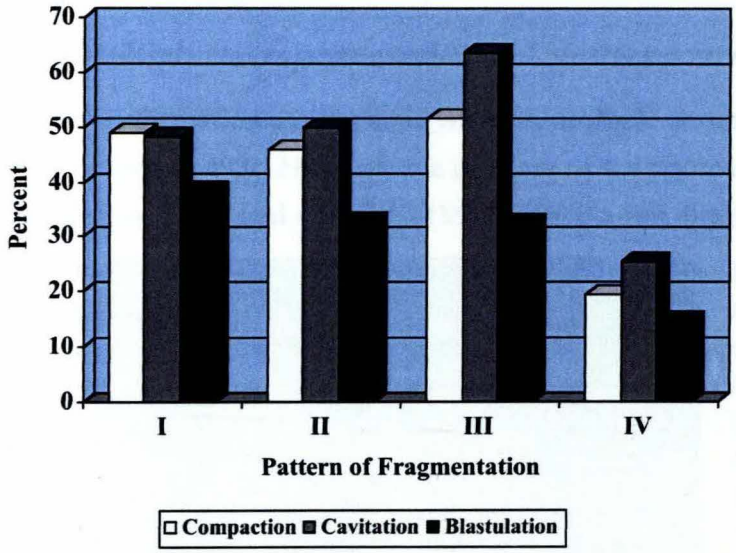


Figure 4.6 The relationship between pattern of fragmentation and normal compaction, cavitation, and blastocyst formation. Embryos with type IV fragmentation have significantly lower rates of compaction, cavitation and blastocyst formation than those with fragmentation types I, II, and III ($p<0.001$).

The pattern of fragmentation also correlated with blastulation; this is presented in Figure 4.6. Blastocyst formation rates were not different among embryos with fragmentation type I (54/140, 38.6%), type II (27/82, 32.9%), or type III (143/442, 32.4%). However, type IV fragmentation led to a significant reduction in normal blastocyst formation (25/170, 14.7%) ($p<0.001$). The same

trend was apparent at compaction and cavitation.

4.4.4 Multi-nucleation and blastocyst formation

The data for 1,214 embryos in this analysis are presented in Table 4.3. The presence of one or more blastomeres with two or more nuclei either on day-2 or day-3 of development had a strong negative correlation with the ability of the embryo to compact, cavitate, and form a blastocyst. Among the embryos with multi-nucleation on day-2 and/or day-3, only 30.6% (44/144) compacted normally, 27.5% (38/138) cavitated normally, and 15.9% (22/138) formed normal blastocysts. The rates of normal compaction, cavitation, and blastocyst formation for embryos without multi-nucleation were 47% (498/1060), 45.9% (482/1051), and 31.9% (335/1052), respectively. The differences between the two groups were statistically significant ($p<0.001$).

Table 4.3 The effect of multi-nucleation on day-2 and/or day-3 on development beyond day-3

Day-2/Day-3 Embryo Morphology	Normal Compaction Rate (%)	Percent Not Compacted	Normal Cavitation Rate (%)	Blastocyst Formation Rate (%)	Average Day-2 Cell Number [a] (±SD)	Average Day-3 Cell Number [a] (±SD)
Without MNB	47.0	38.4	59.9	32.6	3.96 (0.7)	7.76 (1.4)
With MNB	30.6	50.7	38.4	16.7	3.53 (1.0)	6.65 (1.96)
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MNB = Multi-nucleate blastomeres

[a] For normally compacted embryos

4.4.5 Multiple cleavage anomalies and blastocyst formation

Blastocyst formation among embryos with multiple anomalies was evaluated. Forty-one percent of all embryos (493/1214) had one or more of the anomalies described here, that is, on day-3 of development, they had <5 cells, >15% fragmentation, type IV fragmentation, and/or multi-nucle-

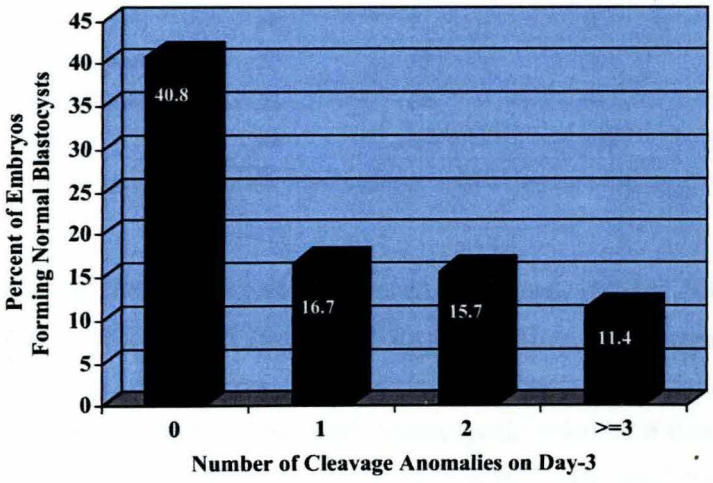


Figure 4.7 Blastocyst formation rate among embryos with 0, 1, 2, or >=3 cleavage abnormalities on day-3 of culture. The highest rate of blastocyst formation is among embryos without abnormalities. The appearance of a single anomaly led to a significant reduction in normal blastocyst formation ($p<0.001$); this was further reduced with multiple anomalies but not significantly.

ation. Figure 4.7 depicts blastocyst formation among embryos with 0, 1, 2, or >=3 abnormalities. A significantly higher proportion (294/721 or 40.8%) of embryos without abnormalities formed blastocysts than those with one anomaly (45/270 or 16.7%), two anomalies (24/153 or 15.7%), or three or more anomalies (8/70 or 11.4%) ($p < 0.001$). The rate of blastocyst formation did not change with increasing number of abnormalities.

4.4.6 Implantation and cleavage profile/morphology of transferred blastocysts

The morphology of the transferred blastocysts was analyzed in four patient groups with different pregnancy outcomes (Table 4.4): (i) 23 patients with no implantation, (ii) 11 patients with a biochemical or an anembryonic pregnancy, (iii) 36 patients in whom at least one of the transferred embryos gave rise to a fetal heart, and (iv) 32 patients in whom all the transferred embryos resulted in fetal hearts. The proportion of non-blastocysts and blastocysts with abnormal morphology in groups (iii) and (iv) with one or more fetal hearts was 15.8% (22/139). This was significantly lower than the proportion in groups (i) and (ii) with complete implantation failure (negative pregnancy result) or with implantation but development failure (biochemical or anembryonic pregnancy) (22/71; 31%)($p<0.01$). Moreover, blastocyst abnormalities in groups (iii) and (iv) were limited to the trophectoderm and some slow embryos, but abnormalities in the failed implantation/development groups (i) and (ii), included the ICM and many slow embryos (non-blastocysts).

Table 4.4 The incidence of morphological anomalies among transferred blastocysts in four groups of patients with different implantation outcomes

Implantation Outcome (Group)	No. of Patients	No. of Blastocysts Transferred	No. of FHB	No. Abnormal	No. Not Blastocysts	Percent Abnormal
Failed (i)	23	49	0	4	10	28.6
Anembryonic (ii)	11	22	0	8	0	36.4
Total	**34**	**71**	**0**	**12**	**10**	**31.0** [a]
Some implanted (iii)	36	75	38	7	5	16
All implanted (iv)	32	62	64	7	3	15.6
Total	**68**	**137**	**102**	**14**	**8**	**15.8** [a]

[a] $p<0.01$ (proportion of abnormal embryos in failed implantation groups compared to the groups with fetal heart activity)

Of the 208 blastocysts that were transferred, 182 (87.5%) were embryos with <=15% fragmentation on day-3, and 26 (12.5%) had more than 15% fragmentation. Six embryos (2.9%) had multinucleate blastomeres either on day 2 or day-3. In patients of group (iv), all transferred blastocysts implanted (64/62; 2 pairs of monozygotic twins). Of these blastocysts, only 8/62 (12.9%) had more than 15% fragmentation and 3/62 (4.8%) exhibited fragmentation pattern IV on day-3. In patients of group (iii), with implantation of 38/75 replaced embryos, day-3 fragmentation in excess

of 15% was only found in 8/75 (10.7%), and Type IV fragments in 5/75 (6.7%). In groups (i) and (ii) together, where none of the 71 transferred embryos resulted in a fetal heart, the proportion of embryos with more than 15% fragmentation or type IV fragmentation was similarly low and not different from that found in groups (iii) and (iv). This reflected the low percentage of such embryos reaching the blastocyst stage and the poor morphology of the ones that did since they were rarely selected for transfer.

4.4.7 Implantation potential of embryos with cleavage anomalies after short-term culture

The EggCyte database was searched for homogeneous day-3 transfers involving embryos with either no abnormality or one or more of the anomalies described here. Homogeneous transfers were those where all the embryos transferred belonged in the same morphological category. The resulting implantation rates were compared to blastocyst formation rate in extended culture and the theoretical implantation rate after day-5 transfer (Table 4.5). A similar concept was previously introduced by Edwards and Beard (1999).

Table 4.5 Implantation rate of embryos with specific anomalies after homogeneous day-3 transfer compared to theoretical implantation rate for similar embryos after 5 days in culture

Day-3 Cleavage Anomaly	Implantation Rate (%) (Day-3 Transfer)	Day-5 Blastocyst Formation Rate (%)	Theoretical Implantation Rate (%) (Day-5 Transfer)
>15% frag.	17.7	16.5	8.2
Type IV fragmentation	16.5	16.4	7.8
MNB	13	14.1	7.0

MNB = Multi-nucleate blastomere

Embryos with none of the abnormalities discussed here had an implantation rate of 40.1% (332/828) on day-3. Embryos with cleavage rate anomaly (<5 cells) had an implantation rate of 22.0% (78/354) after day-3 transfer.[1] It must be noted that 1-,2-, or 3-cell embryos were never transferred on day-3, but they were included in the blastocyst formation data. The implantation rate of embryos with more than 15% fragmentation was 17.7% (40/226). Exclusive transfer of embryos with type IV fragmentation on day-3 resulted in an implantation rate of 16.5% (22/133). Embryos with MNB on days 2 and/or 3 resulted in a low implantation rate of 13.0% (14/108).

The theoretical implantation rate of embryos in prolonged culture was calculated based on the following formula: [(Number of normal appearing blastocysts/Total number of embryos cultured) x 100] x Overall implantation rate of blastocysts. Overall, the theoretical implantation rate after day-5 transfer appeared to be lower than actual implantation rate after day-3 transfer, since many embryos with cleavage abnormalities were lost during extended culture (Table 4.5).

[1] Later analyses showed that this figure was exaggerated. The actual implantation rate of slow embryos is about 10%.

4.5 Discussion

This study demonstrates the relationship between early embryo morphology and blastocyst formation . Slow development (fewer than 5 cells on day-3), fragmentation (in excess of 15% and/or type IV), and multi-nucleation (on day 2 and/or 3) all interfered with the formation of apparently normal blastocysts.

The culture system used during this study, included culture in HTF on days 0 to 3, then in G2.2 or S2 on days 3.5 to 6. This is a variation on the currently advocated sequential systems, hence the possibility that the observed effects were a direct result of deviation from the established sequence. Although this possibility cannot be excluded, the culture system reported here produced an overall blastocyst formation rate similar to that reported by others (e.g., Gardner et al., 1998a, b). Moreover, a 49% implantation rate reflects the ability of this system to support development of viable blastocysts.

The reduced ability to form blastocysts of normal appearance was in many instances already obvious at compaction, which occurs on day-4 of development in the human (Nikas et al., 1996). Regional compaction with exclusion of a number of cells and fragments from the morula occurred among fragmented embryos (Figure 4.1). Blastocyst formation rate among normally compacted day-4 embryos was 46.8%, but this rate was reduced to 28.6% among regionally compacted embryos and 10.3% among embryos that did not show compaction on day-4 (data not shown). These data suggest that 1) the absence of compaction on day-4 is highly prognostic for normal blastocyst formation, and 2) while regional compaction by itself does not preclude blastocyst formation, the extent to which cell exclusion occurs may determine development potential of the embryo; we did not note the number of excluded cells when regional compaction occurred. Exclusion of cells in the blastocoelic cavity was also observed. This anomaly is associated with reduced embryo viability in sheep and cattle (Steen Willadsen, personal communication), but its impact on human embryo viability is uncertain.

Other abnormal features in human blastocysts described in this and other studies have been correlated to reduced total cell count, low or absent hCG secretion (Dokras et al., 1993), and reduced implantation after intrauterine transfer (Jones et al., 1998; Gardner et al., 2000). Furthermore, total blastocyst cell count has been correlated to hatching ability (Van Blerkom, 1993).

The occurence of a single cleavage anomaly on day-2 and/or 3 of development led to a significant reduction in normal blastocyst formation on day-5. Only 17% of embryos with >15% fragmentation formed a morphologically normal blastocyst. Such embryos have been shown to develop into fetuses at a higher rate after assisted hatching, fragment removal, and intrauterine transfer on day-3

(Chapter 3). A further analysis for this study of 118 homogeneous transfers (all embryos transferred in one morphological category) involving embryos with >15% fragmentation revealed that about 18% implanted; on the other hand, the loss of some 80% of such embryos between day-3 and day-5 *in vitro* yields a theoretical day-5 implantation rate of about 8%. This suggests that the developmental potential of some of these embryos may be reduced in extended culture.

The 8% figure seems to be in agreement with the approximately 5% implantation rate for fragmented embryos reported by a number of investigators (Staessen et al., 1993; Giorgetti et al., 1995; Ziebe et al., 1997). However, fluorescence *in-situ* hybridization (FISH) analysis of embryos with <= 35% fragmentation shows that about one half are normal for the number of chromosomes tested, and aneuploidy is not a major abnormality among such embryos (Munné et al, 1995; Marquez et al., 2000). Instead, these embryos display different forms of mosaicism, some of which are compatible with normal development (Reviewed by Munné and Cohen, 1998). Together, these findings suggest that fragmentation *per se* is not an abnormality.

Moreover, with the exception of extreme cases where more than a third of the embryo has been lost to fragmentation and chromosomal abnormality is almost certain, the potential of moderately fragmented embryos for implantation may be determined by the distribution and size of the fragments (Chapter 3; Antczak and Van Blerkom, 1999). Here, reduced rates of normal compaction, cavitation, and blastocyst formation were observed in embryos with type IV fragments (large scattered fragments associated with several cells) but not types I, II, or III. If fragmentation results in the depletion of cortically positioned regulatory proteins essential to the embryo, as suggested by Antczak and Van Blerkom (1999), then type IV fragmented embryos may be specially affected, since the fragments are much larger than those in other types. These embryos had the lowest implantation rate after transfer on day-3 (Chapter 3) but extended culture may reduce their potential even further and less than a quarter may survive.

In Chapter 3, it was suggested that fragment removal may, at least in part, explain the relatively high implantation rates for 6-35%, non-type IV fragmented embryos. Whether fragment removal on day-2 or 3 can lead to better blastocyst formation remains to be shown, but there is some indication that both cleavage between day-2 and 3 (Zaninovic et al., 1999) and compaction between day-3 and day-4 (Alikani, 2001) are promoted if fragments are microsurgically removed.

Embryos that successfully underwent normal compaction, cavitation, and inner cell mass formation had significantly higher mean cell numbers on days 2 and 3 compared to those that failed to complete these developmental stages. Somewhat surprisingly, fast cleaving embryos, particularly those with 9-10 cells on day-3, showed a reduced capacity to form normal blastocysts. Unusually

fast embryos may exhibit high levels of chromosomal aberrations (Magli et al., 1998), some of which are due to polyspermic fertilization (Harper et al., 1994).

In a large study by Huisman et al. (1994), slow embryos showed lower implantation potential after 2, 3, or 4 days in culture. In the present study, prolonged culture of such embryos often led to abnormal patterns of compaction, specifically "fusion-like" compaction, and abnormal cavitation.

The reduced capacity of embryos with MNB to form blastocysts was demonstrated by Balakier and Cadesky (1997). The majority of the embryos in that study arrested at 2-15 cells and only 14% formed morphologically normal blastocysts. It has also been shown that multi-nucleation leads to low implantation rates after day-3 transfer (Jackson et al., 1998; Pelinck et al., 1998), as was observed in this study. Multi-nucleation, therefore, appears to have a significant negative correlation to the development potential of embryos regardless of the duration of culture.

Many of the embryos with MNB failed to show signs of compaction. When they did compact, their attempt at cavitation often ended with the persistence of what should be only transitory structures (e.g., intracellular vacuoles) involved in the formation of the blastocoel (Gualtieri et al., 1992). Embryos with MNB had a lower cell number (on average 6.5) on day-3, and formed blastocysts at a rate of 14.1%; this figure is not different for the rate at which all embryos with fewer than 7 cells formed blastocysts (13.8%). So, it is possibly both chromosomal anomalies associated with multi-nucleation (Kligman et al., 1995; Laverge et al., 1997; Staaesen and Van Steirteghem, 1998) as well as their lower cell number that contribute to reduced blastocyst formation and reduced implantation in this group.

Under the conditions of this study, extended culture led to a reduction in viability of embryos with cleavage abnormalities. On this basis, and until further refinement of culture media to accommodate compromised embryos, this study suggests that extended culture should be limited to those embryos with optimal development during the first three days in culture.

Monash University

Declaration for Thesis Chapter 5

In the case of Chapter 5, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	70	Concept, experiment design/execution, writing of manuscript
2. Tim Schimmel	30	Experiment preparations/execution

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Tyho-Galileo Research Laboratories, Hudson, NY, USA

		Date
Signature 1	[redacted]	24 DECEMBER, 2005
Signature 2	[redacted]	1-3-2006

Chapter 5

The developmental capacity of isolated mouse blastomeres following exposure to heterologous cytoplasmic fragments

5.1 Summary

The impact of heterologous cytoplasmic fragments on isolated, intact mouse blastomeres was evaluated. Two-cell stage mouse embryos were disaggregated and each blastomere (1/2 blastomere) was placed in an emptied mouse or bovine zona pellucida. One 1/2 blastomere was aggregated in the host zona pellucida with heterologous cytoplasmic fragments of different origins while the other was left alone and served as control. The volume of fragments inserted was either roughly equal to the missing 1/2 blastomere or far exceeded it. The frequency of blastocyst formation was 81% (64/79) among experimental and control half embryo constucts; average cell count on day-5 was 13.5 cells for both groups, considerably lower, as would be expected, than the average cell number of fully intact embryos. The volume of fragments did not have a measurable impact on blastocyst formation, however, it did somewhat influence the morphology of the blastocysts that developed. The source of the fragments was of no consequence; egg fragments from non-mated and mated mice, and human embryo fragments produced the same results. We conclude that, at least in the absence of a fully intact zona pellucida, mouse blastomeres are neither overtly sensitive to close physical association with heterologous (often degenerative) cytoplasmic fragments nor responsive to the intra-zonal microenvironment created by them. This suggests that the generally negative impact of fragmentation on embryo viability is unlikely to be a result of the presence of fragments *per se*. Although fragment interference in the spatial organization of the embryo and communication among cells can not be ruled out, fragmentation most likely represents already established atypical cellular conditions that affect the overall performance of the embryo.

5.2 Introduction

In the human, the fate of embryos with blastomere fragmentation is degree and type dependent, as was shown in Chapter 3 as well as in other reports (Puissant et al., 1987; Warner et al, 1998; Antczak and Van Blerkom, 1999; Van Blerkom et al., 2001). The loss of up to 15% of the volume of the embryo

to fragmentation is largely tolerated during short-term culture and transfer to the uterus and during extended culture to the blastocyst stage. However, if fragmentation exceeds 15%, or if large fragments form, development is seriously compromised (Chapter 3; Racowsky et al., 2003; Stone et al., 2003).

Clearly, the non-viability of some fragmented embryos is a result of extensive chromosomal mosaicism, polyploidy, and haploidy that often characterize such embryos (Munné and Cohen, 1998; Munné et al., 2001). In other embryos, the drastic reduction in total cytoplasmic volume or the obvious loss of internal resources in the fragments may lead to arrest (Antczak and Van Blerkom, 1999; Van Blerkom et al., 2001). But, many fragmented embryos with adequate cytoplasmic volume and limited or no chromosomal abnormality still fail for reasons that are not well understood.

The laboratory mouse has been used as a model to study potential causes and mechanisms of cytoplasmic fragmentation (Hawes et al., 2001; Liu et al., 2002). But, so far, the mouse has shown limited potential as a model to study the impact of fragmentation on embryo viability (Dozortsev et al., 1998). To some extent, this is consistent with the observation that fragmentation in the mouse seems to have an 'all or none' effect. Division subsequent to normal fertilization and under optimal culture conditions is rarely if ever accompanied by fragmentation in this species, but ovulated oocytes (from mated and non-mated animals) found to be fragmented at the time of isolation have no chance of further development (Perez et al., 1999).

The present work evaluates the prospect of mouse embryos as a model system for human embryo fragmentation, focusing specifically on the influence of superfluous, presumably degenerating cytoplasm on healthy cells. The experiments were based on the proposal that cell fragments may create a 'hostile' microenvironment within the embryo causing deterioration and eventual arrest of the vital blastomeres, much the same as has been suggested for degenerate cells in amphibian (Denker, 1976), and mouse embryos (Alikani et al., 1993).

Taking advantage of totipotency in mouse early blastomeres (Tarkowski, 1959), two-cell embryos were dissociated (generating 1/2 blastomeres); in each case, one 1/2 blastomere was placed in a host zonae pellucida (ZP) and aggregated with cytoplasmic fragments while the sibling 1/2 blastomere was placed in the ZP alone. The development of the half embryo-fragment aggregates and the control half embryos was evaluated over the course of the following three to four days in culture.

5.3 Materials and Methods

5.3.1 Source of cytoplasmic fragments

Cytoplasmic fragments of both mouse and human origin were used. For mouse fragments, ovu-

lated eggs were collected 16-18 hours post hCG administration according to the protocol described in Chapter 2 (section 2.5). Eggs showing fragmentation at the time of isolation were selected for the experiments. For human fragments, non-viable, discarded human embryos on days 3-6 of development with extensive fragmentation were used.

5.3.2 Preparation of mouse 1/2 blastomeres

Mouse, bovine, and human eggs or embryos were placed in a drop of modified CZB medium (M-CZB)(Chapter 2, section 2.5). To obtain mouse 1/2 blastomeres, the ZP was removed from 2-cell embryos according to the procedure described in Chapter 2 (Section 2.6). Zona pellucida-free embryos were incubated in calcium-magnesium-free CZB (CMF-CZB) for 10 minutes. The cells were separated mechanically by pipetting through a pulled glass pipette, keeping the two blastomeres of each embryo together and identifiable.

Mouse eggs and embryos containing fragments were pronase-treated as before. Human eggs and embryos required only a brief exposure to pronase for ZP removal. After removal of the ZP, eggs and embryos were placed in CMF-CZB (mouse) and pipetted through a finely drawn glass pipette to dissociate them.

5.3.3 Preparation of mouse half embryos and half embryo-fragment aggregates

Micromanipulation procedures were carried out in M-CZB under mineral oil, at room temperature. A micromanipulation dish with up to 10 drops of M-CZB was prepared. One empty ZP, several fragments, and one dissociated 2-cell embryo were placed in each drop of medium. One ZP was held by the holding pipette while a blastomere was picked up by a micropipette (roughly 50μm outside diameter) and was inserted into the empty ZP alone (control blastomere). The other blastomere was placed in the host ZP along with several fragments (experimental blastomere)(Figure 5.1). Once all the half embryos and half embryo-fragment aggregates were constructed, they were washed and placed in KSOM^{AA}. The development of these embryos was monitored during the next three days in culture.

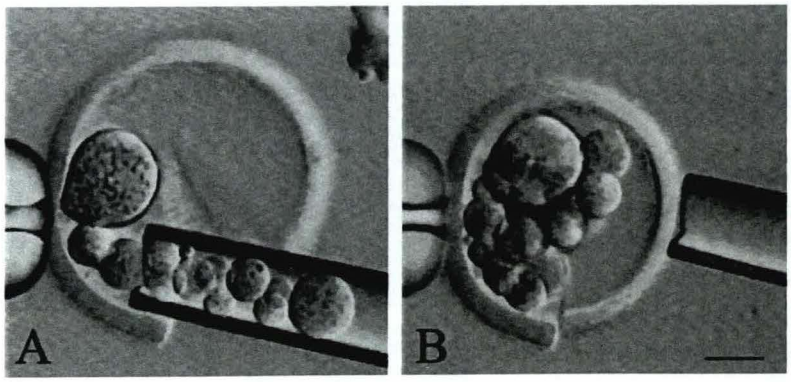


Figure 5.1 Aggregation of 1/2 blastomeres from 2-cell mouse embryos with cytoplasmic fragments from mouse eggs. Several fragments were picked up in a pipette and inserted into a partially dissected empty zona pellcida while it was held in place by a holding pipette (A). The zona pellucida slit was forced to close to prevent loss of inserted fragments (B). Scale bar is approximately 20μm.

5.4 Results

5.4.1 Development of half embryos in mouse host zonae pellucidae

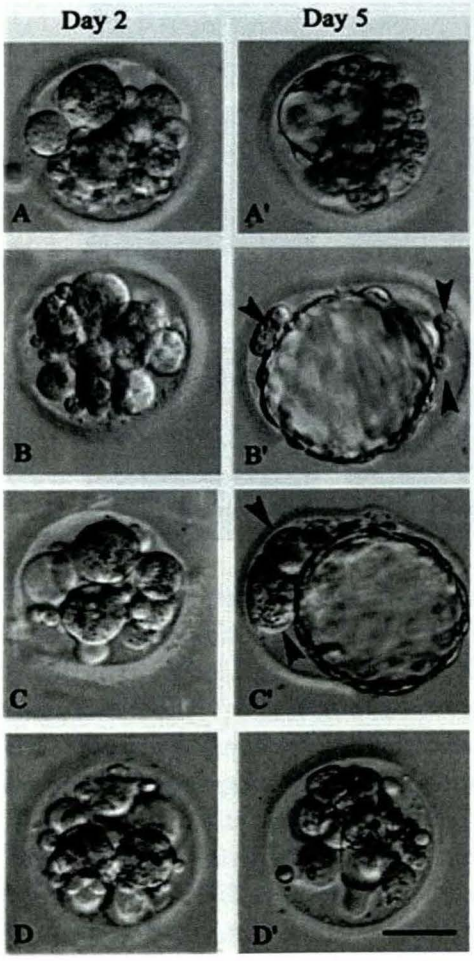
These results are summarized in Table 5.1. Representative embryos on days 2 to 5 are shown in Figure 5.2 (next page). Mouse ZP were used for both the experimental and the control blastomeres, limiting the volume of inserted fragments to less than or roughly equivalent to the missing sister blastomere.

Table 5.1 Development of half embryos with or without heterologous cytoplasmic fragments on day-5 or 6

Half Embryos	Total No.	Small Blastocyst	Expanded Blastocyst	All Blastocysts (%)	Abnormal Cavitation	Degenerated or Arrested
With Fragments [a]	79	19	45	64 (81)	9	6
Without Fragments	79	23	41	64 (81)	9	6

[a] Volume of fragments in mouse host zonae pellucidae was equivalent to or less than that of the missing blastomere.

Seventy nine embryos were dissociated, generating 158 blastomeres. Among the 79 half embryo-fragment aggregates, 19 developed into small blastocysts (24%), and 45 (57%) developed to the expanded blastocyst stage, producing an overall blastocyst formation rate of 81% (64/79). Abnormal cavitation, including multiple cavities and delayed cavity formation was not frequent (9/79 or 11%) neither was degeneration and complete arrest before compaction (6/79 or 7.5%).



The control half embryos developed identically to the experimental half-embryo-fragment aggregates, as 23/79 (29%) made small blastocysts and 41/79 (52%) formed expanded blastocysts for an overall blastocyst formation rate of 81% (64/79). Abnormal cavitation and degeneration was observed in 11% and 7.5% of the control half-embryos.

Figure 5.3 Mouse 2-cell embryos dissociated and re-aggregated within human zonae pellucidae with several fragments from human fragmented embryos. The aggregates are shown on day-2 and on day-5 of development. Blastomeres are marked with asterisks. While the aggregates in A and D have formed abnormal morula/early blastocysts, the aggregates in B and C have blastulated apparently normally and have excluded some of the cytoplasmic fragments (arrowheads in B' and C'). Scale bar is approximately 50μm.

A few 2-cell embryos were dissociated and both cells were then placed in human ZP along with fragments from human embryos (Figure 5.3). Half of these developed to full blastocysts.

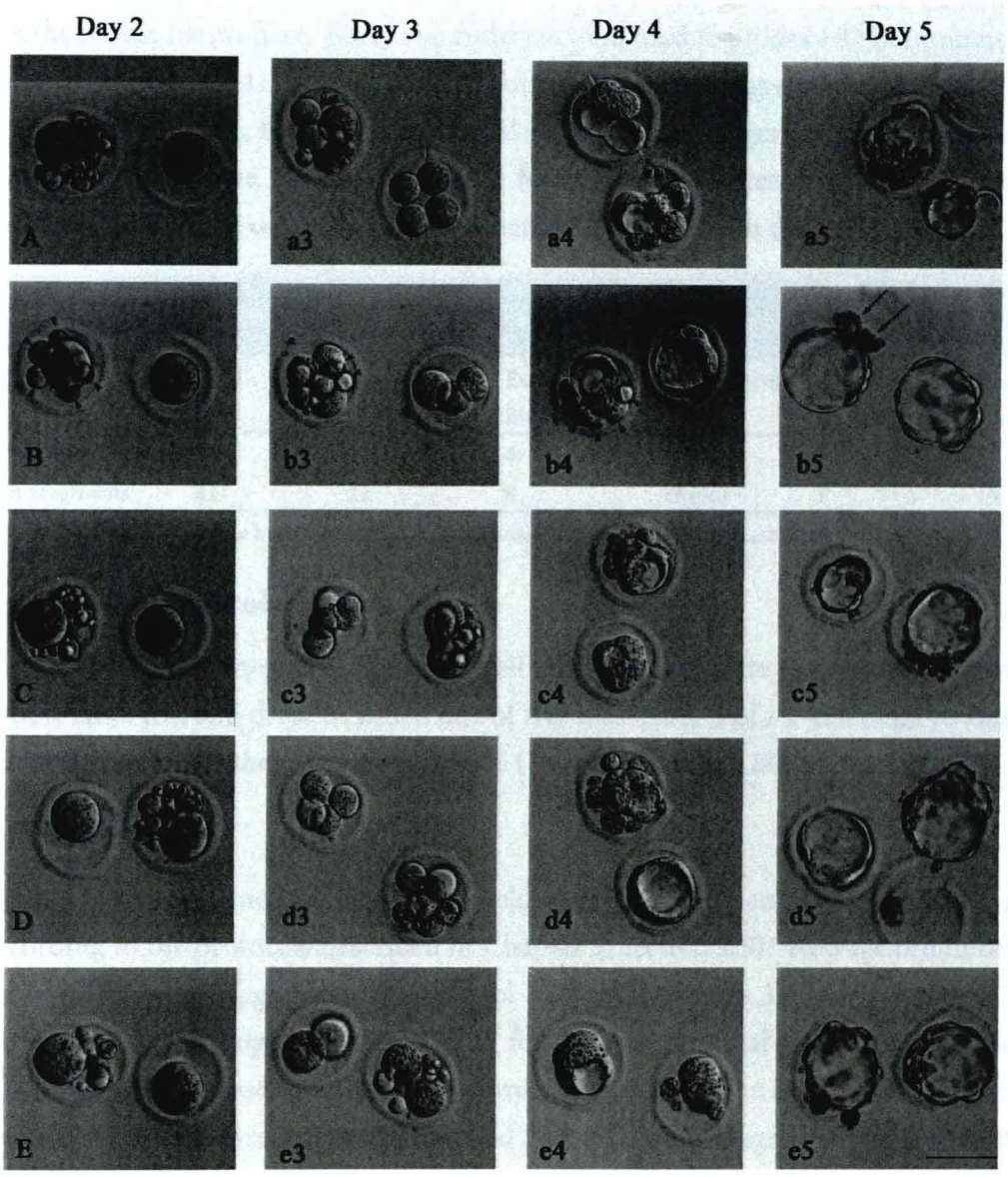


Figure 5.2 The development of experimental and control half embryos from day-2 through day-5 in culture. In panel B (second row from top), on day-2 of development, five large fragments (arrowheads) aggregated with one 1/2 blastomere are visible, while the sister 1/2 blastomere from the same 2-cell embryo is seen in a host zona pellucida alone. One day later, on day-3 of development (b3), the five fragments can be seen surrounding three blastomeres (1x1/4 and 2x1/8). The control half embryo has exactly the same number of cells (1x1/4 and 2x1/8). On day-4 (b4), the fragments in the experimental half embryo have moved to one side of a compacted mass that has begun to cavitate. The control half embryo has also compacted and cavitated but appears better organized. By day-5, both half embryos have formed blastocysts. The fragments (arrows in b5), now degenerated, are loosely attached to the experimental half embryo. Scale bar is approximately 50μm.

5.4.2 Development of half embryos in bovine or human host zonae pellucidae

These results are summarized in Table 5.2. The large size of the human and bovine ZP (about 150-160μm) allowed a greater number of fragments to be inserted without imposing physical restriction on the mouse blastomere. Forty one embryos were used to produce 82 blastomeres. Blastocyst formation was 41% (17/41) among the half-embryo-fragment aggregates and 46% (19/41) among the control half embryos; the rate of abnormal cavitation and degeneration or arrest was 41% and 37% in respective groups. Overall blastocyst formation was lower than that in the first series of experiments, though the control and experimental groups did not differ significantly.

Table 5.2 Development of half embryos with or without heterologous cytoplasmic fragments on day-5 or 6

Half Embryos	Total No.	Small Blastocyst	Expanded Blastocyst	All Blastocysts (%)	Abnormal Cavitation	Degenerated or Arrested
With Fragments [a]	41	9	8	17 (41)	7	17
Without Fragments	41	11	8	19 (46)	7	15

[a] Volume of fragments in bovine host zonae pellucidae exceeded that of the missing blastomere.

5.4.3 Additional control embryos

Additional control embryos consisted of 2-cell embryos that were dissociated then reconstituted into whole embryos, and those in which one of two blastomeres of a 2-cell embryo was enucleated. Blastocyst formation in these groups was 83% (19/23) and 97% (28/29), respectively.

5.4.4 Cell counts

Cell counts were performed on day-5 of development for those embryos that had a distinct cavity (according to the protocols described in Chapter 2, section 2.9). Average cell number for both half-embryo-fragment aggregates and control half embryos was 13.5 cells (total of 38 embryos counted); total count ranged from two to 27 for the experimental group and from four to 29 in the control group. Reconstituted control embryos had 26.6 cells on average (22 embryos counted). Control embryos with one enucleated cell had 23.8 cells on average. Fully intact, unmanipulated embryos had 49.7 cells on average.

5.5 Discussion

The results of these experiments show that exposure of intact blastomeres of the mouse to mouse and human cell fragments does not measurably reduce their capacity to develop to the blastocyst stage. The model presented here takes advantage of totipotency of mouse blastomeres at first cleavage. Isolated cells from 2-cell mouse embryos are equivalent in their ability to form viable blastocysts (Tarkowski, 1959; Smith and McLaren, 1977). It is therefore possible to measure the impact of

various phenomena on development potential in strictly controlled experiments where one sister blastomere serves as an internal control for the other.

It should be noted that the method of fragment insertion described here required a rather large opening in the ZP. This model was problematic in two ways. First, it was not possible to evaluate any possible differences in the microenvironment within an intact and a partially dissected ZP. Secondly, and more importantly, some of the fragments were often found to have been expelled through the slit in the ZP, obviously reducing any potential negative impact on development.

Sibling 1/2 blastomeres used to construct half embryos with or without heterologous cytoplasmic fragments showed the same capacity to divide, compact, and cavitate. The fragments were not generated by the embryos being examined, so the experiments measured the impact of fragments or the micro-environment created by their presence on division of the original 1/2 blastomere and its descendant cells. Interestingly, the destruction of one of two blastomeres of a 2-cell embryo appears to impact development since hatching ability is compromised (Alikani et al., 1993). On the other hand, the present experiments suggest that enucealtion of one blastomere has no impact on development of the sister blastomere.

Timing of cleavage divisions following dissociation was not altered by the fragments. One day after micromanipulation, on day-3 of development, both the experimental and control half embryos had undergone one to two divisions to generate 2-4 cells (representing 1/8 and 1/16 blastomeres). By day-4, compaction had occurred and many had started to cavitate. On day-5, blastocysts formed. Judging by the reduced cell number of dissociated-reconstituted whole embryos, treatment with pronase followed by dissociation seemed to impact the rate of division since these embryos formed blastocysts with fewer cells than the intact controls. However, the effects of pronase treatment and dissociation can not be independently evaluated under the present conditions.

The source of the fragments was of no consequence to the half embryos. Potentially "apoptotic" fragments from unfertilized mouse eggs (Perez et al., 1999) and those from non-viable human embryos produced the same results. The only potential negative factor was that some fragments appeared to become internalized, thus they may have ultimately affected the viability of the blastocysts had they been transferred.

Based on the observations here, the presence of fragments *per se* does not have a significant impact on development. Fragments could, however, interfere in the spatial organization of the embryo and communication among cells. In the present experiments, compaction was disrupted in some of the 2-cell embryos that were dissociated and re-aggregated along with numerous fragments.

Nonetheless, the general suggestion of the experiments is that fragmentation represents already established atypical cellular conditions that affect the overall performance of the embryo. The validity of the latter should be further investigated.

Monash University

Declaration for Thesis Chapter 6

In the case of Chapter 6, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	70	Concept, experiment design/execution, writing of manuscript
2. Steen Willadsen	30	Experiment design/execution

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, West Orange, NJ, USA Tyho-Galileo Research Laboratories, Hudson, NY, USA

		Date
Signature 1	[redacted]	24 DECEMBER, 2005
Signature 2	[redacted]	23 December, 2005

Chapter 6

The developmental capacity of blastomeres from abnormally cleaving human embryos in isolation and following artificial aggregation

6.1 Summary

This study examined the developmental capacity of isolated mono-nucleated cells from human embryos with fragmentation and other abnormalities. Two approaches were used. The cells were either cultured individually or aggregated in a host zona pellucida in a chimaeric form and monitored over time in culture. Fifteen of the 37 (40.5%) mono-nucleated blastomeres arrested in development, but the remaining 22 (59.4%) divided during the course of culture in isolation. Of those that divided, 13 (59.1%) went on to form a cavity and blastulate albeit with fewer than four cells, hence atypically. In total, 235 usable cells from 116 non-viable (fresh or frozen-thawed) embryos were combined into 36 aggregates, ranging from 4-12 cells. Of these, 15 (42%) formed blastocyst-like structures with distinct inner cell masses. These results clearly demonstrate that some blastomeres from non-viable embryos do indeed maintain their developmental potential and regulatory capacity to the extent of being able continue limited development in isolation and to contribute to a normally organized blastocyst in aggregation.

6.2 Introduction

Clinical evidence accumulated over more than two decades of human *in vitro* fertilization and embryo transfer (IVF/ET; Steptoe and Edwards, 1978) shows that between one quarter and one half of the total cytoplasmic volume of an early embryo must be represented in normal cells as a condition for full embryo viability. However, a substantial proportion of embryos produced in the course of routine IVF show abnormal cleavage and lose cells and cytoplasmic volume through cell fragmentation, degeneration, mitotic arrest, and multinucleation (Reviewed in Chapter 1). Extensive cytogenetic studies have demonstrated a high frequency of numerical chromosomal anomalies in such embryos (Reviewed by Munné and Cohen, 1998). As a consequence, roughly 60% of

human IVF embryos do not meet basic viability criteria and in most clinics they are simply discarded at the end of treatment cycles.

Although non-viable in the sense that they are unlikely to develop into normally organized blastocysts (Chapter 4) or give rise to a clinical pregnancy (Chapter 3), such embryos often do contain one or more surviving, apparently normal blastomeres. Experimental animal studies have long demonstrated that single blastomeres from normally developing early cleavage stage embryos of a number of species are fully capable of development to advanced stages (Tarkowski, 1959; Moore et al., 1968; Rossant, 1976; Willadsen, 1979; 1981). However, the developmental capacity of the surviving blastomeres of abnormally cleaving embryos is not known; such cells have not been grown in isolation and most embryos with multiple abnormalities deteriorate significantly after day-4 of development making evaluation of the status of individual cells impossible.

Experimental chimaerism induced by cell aggregation has been used in other mammalian species to study the developmental potential and regulatory capacity of whole embryos or isolated blastomeres (Reviewed by Willadsen, 1989; Tarkowski, 1998). So far, however, this highly flexible approach seems to have been completely overlooked in the study of human embryos.

The present work is part of a wider investigation of the implications of abnormal cleavage and degenerative changes in early human embryos. It attempts to discover whether individual cells isolated from fragmenting non-viable embryos are developmentally competent, by examining their development in isolation and after artificial aggregation.

6.3 Materials and Methods

6.3.1 Blastomere isolation by biopsy

Micromanipulation equipment, media, and microtools were described in Chapter 2 (section 2.7). Non-viable embryos were placed (one at a time) in a drop of biopsy medium consisting of HEPES-buffered calcium-magnesium-free HTF (CMF-HTF) with 0.05M sucrose, under mineral oil. A micropipette with an outer diameter of 12μm was used to pick up an acidified Tyrode's (AT) solution and limited amounts of this were then expelled against the ZP in a localized area until a hole of approximately 35-45μm was made. The AT micropipette was replaced with a larger biopsy needle which had a 45μm outer diameter. Cells were biopsied one at a time and placed in the drop until all cells had been biopsied. All isolated cells and large fragments were then washed free of biopsy medium and placed in culture in individual drops. They were checked daily until day-5 (or in some cases, day-7) of development.

Seventeen discarded embryos with 1-10 cells and 20-75% fragmentation, mostly of type IV (Chapter 3; large scattered fragments) were used in these experiments. Forty-two 1/4-1/8 nucleated blastomeres and fifteen anucleate blastomere-size fragments were isolated and cultured individually.

6.3.2 Preparation of aggregates

Source embryos, obtained according to the protocol described in Chapter 2 (section 2.4) were disaggregated as described in Chapter 2 (section 2.6).

The mixture of cells and fragments, obtained by disaggregation of source embryos, was examined on the inverted microscope, and cells with a single nucleus and smooth cytoplasm (without vacuoles or contraction) were separated and transferred to a fresh medium drop along with several empty ZP (preparation described in Chapter 2, section 2.8). The dish was then placed on the warm microscope stage for micromanipulation. A holding pipette, attached to the left micromanipulator, was used to hold the ZP in place, with the opening between the 3 and 5 o'clock positions. The biopsy needle, attached to the right micromanipulator, was used to pick up the cells. Four to 12 nucleated cells were inserted, one cell at a time, into the ZP through the opening. The ZP-encapsulated cell aggregate was then released from the holding pipette, transferred to Global culture medium and cultured at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Culture was continued (typically for two days) until a blastocyst developed or the aggregate arrested in development.

In total, 116 discarded embryos were used in the experiments from which 235 usable mononucleated cells were isolated intact (average 2.03 cells per embryo). Roughly 10% of the cells were damaged during manipulation and could not be used.

6.3.3 Fluorescence in situ *hybridization*

Blastomeres and blastocysts were fixed according the protocol described in Chapter 2 (section 2.9). They were analyzed by fluorescence *in situ* hybridization (FISH) using probes for chromosomes X, Y, 13, 16, 18, 21, 22, and in some cases, 15 and 17. To denature nuclear DNA, the sample together with the first probe set, specific for chromosomes 13, 16, 18, 21, and 22 were held at 73°C for five minutes. They were then hybridized for three hours at 37°C. Slides were then washed and analyzed before a second hybridization round was performed. In the second round, the slide was co-denatured with the second probe set, specific for chromosomes X, Y, 15, and 17, at 73°C for five minutes, then hybridized for 1 hour at 37°C, washed and analyzed.[1]

Previously described criteria distinguishing mosaics from FISH errors were used without modification (Munné et al., 1994; Munné and Cohen, 1998). Cells and embryos were classified as normal, aneuploid, polyploid, haploid and/or mosaic according to established guidelines (Munné et al., 1995; Munné and Cohen, 1998).

[1] This work was done by Mireia Sandalinas, a member of the PGD team at IRMS-SBMC.

6.4 Results

6.4.1 Isolated blastomeres in individual culture

The outcome of culture for isolated 1/4-1/8 mono-nucleated (n=37) or multi-nucleated blastomeres (n=5), or anucleate blastomere-size fragments (n=15) in individual culture is summarized in Table 6.1. Representative blastomeres and their division products on days 3, 4, and 5 of development are shown in Figure 6.1. Fifteen of the 37 (40.5%) mono-nucleated blastomeres from fragmenting embryos arrested in development, but the remaining 22 (59.4%) divided during the course of culture in isolation. Of those that divided, 13 (59.1%) went on to form a cavity and blastulate albeit with fewer than four cells, hence atypically. Of the five multi-nucleated cells, four arrested, while one underwent a single division on the fifth day of development.

In 15 blastomere-size fragments, no nucleus was visible on the day of isolation. Ten of the 15 showed complete arrest in subsequent culture, indicating that they were either arrested in mitosis or were fragments of cells. Of those remaining, two fragmented, and three divided, the latter indicating that they were in mitosis at the time of isolation.

Table 6.1 A summary of development of 1/4-1/8 nucleated cells and anucleate fragments isolated from fragmented embryos

Source Embryo Cell Number on Day-3	Source Embryo Fragmentation Degree (%); Pattern	Nuclei in Isolated Cells	Number of Cells on Day-4	Final Status
5	50; IV	1	2	Divided; fragmented
		0	1	Arrested
		0	1	Fragmented
8	25, IV	1	1	Arrested in mitosis
		1	1	Arrested in mitosis
		1	1	Arrested in interphase
		0	1	Arrested
		0	uk	Fragmented
10	40; IV	1	2	Divided; blastulated
		1	2	Divided; compacted
		1	2	Divided; blastulated
		1	2	Divided; blastulated
		1	2	Divided; blastulated
		1	1	Arrested in interphase
6	30; IV	1	2	Divided; blastulated
		1	2	Divided; blastulated
		1	2	Divided
		2	1	Arrested; Multiple nuclei
		0	1	Arrested
5	25; II	>2	1	Divided on day 5

9	20; IV	1	2	Divided; blastulated
		1	2	Divided; blastulated
		>2	1	Arrested in interphase
		0	uk	Incomplete furrow
6	40; IV	1	2	Divided; blastulated
		1	2	Divided; degenerated
		1	1	Fragmented; degenerated
		2	uk	Fragmented; degenerated
		0	2	Divided; blastulated
		0	1	Arrested
		0	2	Divided; blastulated
5	30; IV	1	1	Divided; fragmented
		1	2	Divided; blastulated
		1	uk	Divided; blastulated
5	25; IV	1	1	Arrested in mitosis
		0	1	Arrested
		0	1	Arrested
1	80	2	1	Arrested in interphase
		0	1	Arrested
		0	1	Arrested
6	20; III	1	1	Arrested in mitosis
		1	1	Arrested in interphase
		1	1	Arrested in interphase
		1	1	Arrested in mitosis
7	25; IV	1	1	Arrested
		1	2	Divided
		1	1	Fragmented
		1	2	Divided; fragmented
		1	2	Divided; fragmented
		1	2	Divided
		1	2	Divided
7	30; IV	1	1	Arrested in interphase
		1	1	Arrested in interphase
		1	1	Arrested in mitosis
		0	1	Arrested in interphase
		0	1	Arrested; Multiple nuclei

uk = unknown

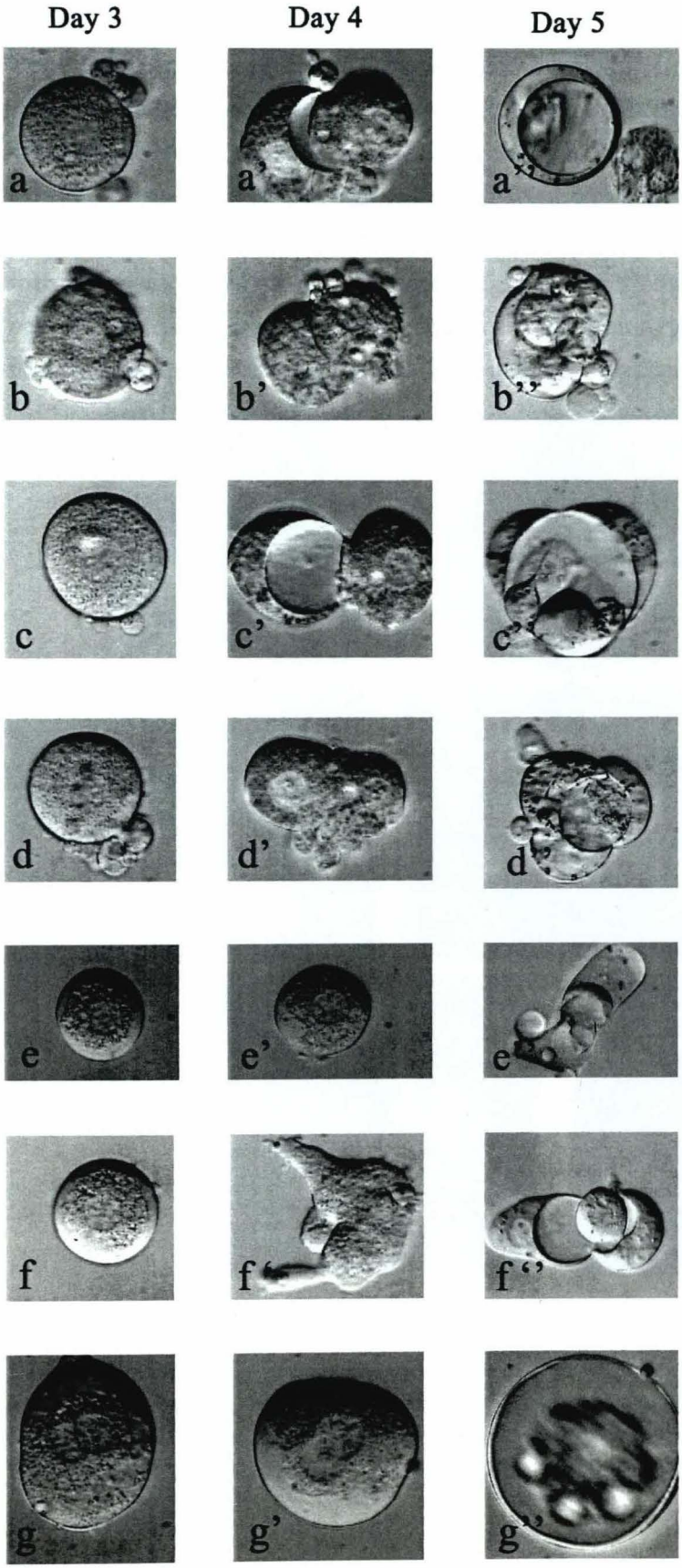


Figure 6.1 The outcome of culture of cells isolated from highly fragmented human embryos. Many of the mononucleated cells divided within 24 hours and formed a cavity, albeit atypically, within 48 hours (see a-a", c-c", and d-d") or compacted without forming a cavity (see b-b"). Other cells arrested (see g-g").

6.4.2 Aggregation chimaeras

The details of the 25 aggregation experiments are shown in Table 6.2. In total, 235 usable cells from 116 non-viable (fresh or frozen-thawed) embryos were combined into 36 aggregates, ranging from 4-12 cells or about one half to about one and a half times the normal cell volume. Of these, nine arrested completely; twelve others showed some development, but did not blastulate or did so in a disorganized way. The remaining 15 (42%), ranging in aggregate cell number from 6 to 12 cells, formed blastocyst-like structures with distinct inner cell masses. In most instances, some aggregate cells did not participate in compaction and were completely excluded during blastulation; therefore the blastocysts were on the whole smaller than ordinary blastocysts (Figure 6.2).

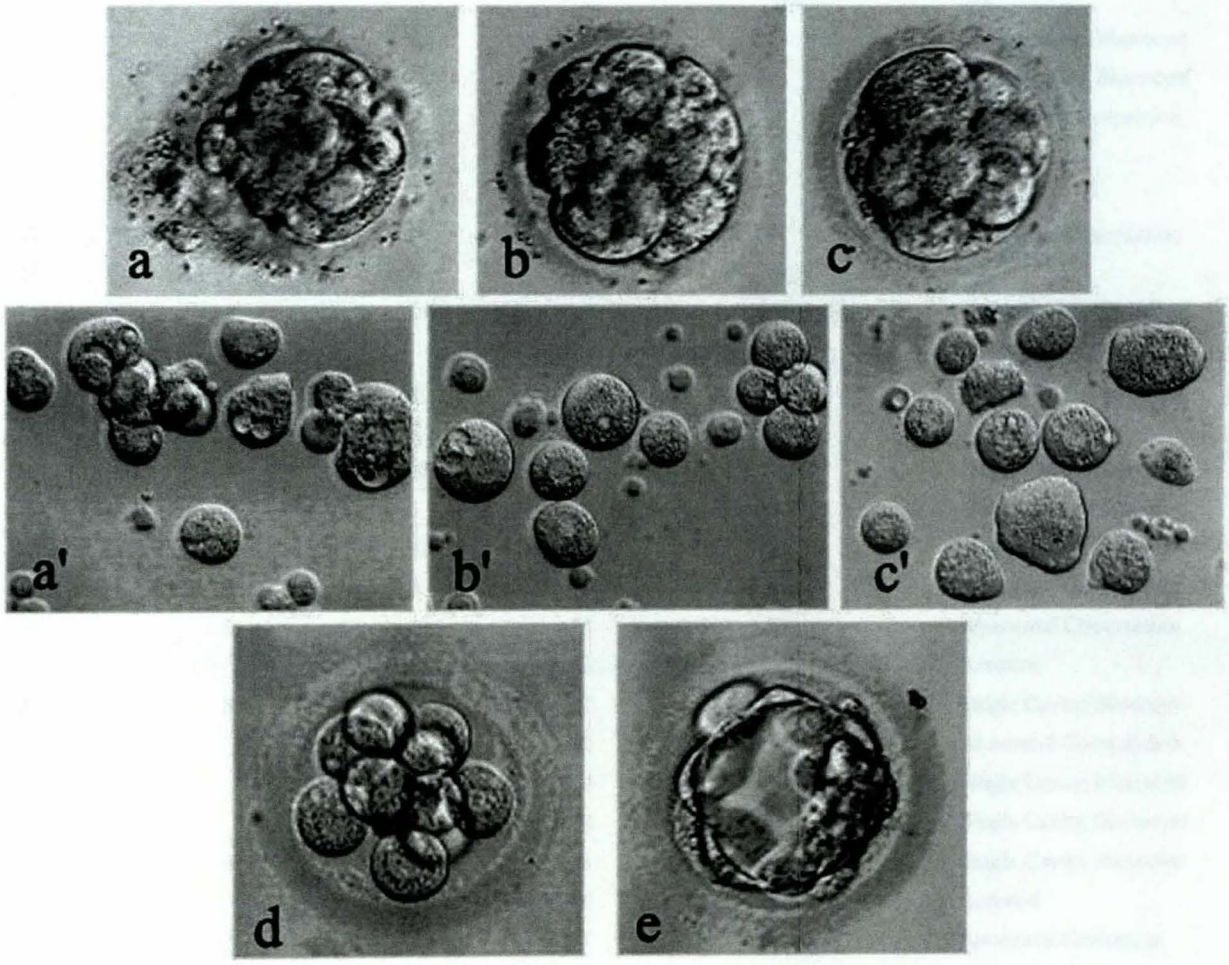


Figure 6.2 Development of a human aggregate. (a-c) Three discarded non-viable embryos on day-3 of development. (a'-c') Dissociated cells of the respective non-viable day-3 human embryos in a-c, comprising mononucleated, multinucleated, and anucleate blastomeres/fragments. (d) Eleven of the mononucleated cells from the three embryos were used on day-3 to construct an aggregate. (e) Following two days of culture, the aggregate formed a blastocyst.

Table 6.2 Summary of 25 aggregation experiments

Experiment Number	Number of Non-viable Embryos	Usable Cells	Aggregate Number	Number of Cells Aggregated	Final Developmental Status
1	5	5	1	5	Arrested
2	6	14	1	9	Single Cavity, Blastocyst
			2	5	Abnormal Compaction
3	6	0	0	n/a	n/a
4	3	5	1	5	Abnormal Compaction
5	6	13	1	8	No Cavity
			2	5	Abnormal Compaction
6	4	5	1	5	Single Cavity, Blastocyst
7	2	0	0	n/a	n/a
8	4	15	1	9	Single Cavity, Blastocyst
			2	6	Single Cavity, Blastocyst
9	2	5	1	5	Abnormal Compaction
10	5	14	1	9	Arrested
			2	5	Arrested
11	3	5	1	5	Abnormal Compaction
12	2	0	0	n/a	n/a
13	8	13	1	9	Single Cavity, Blastocyst
			2	4	Single Cavity, Blastocyst
14	2	6	1	6	Abnormal Compaction
15	5	6	1	6	Arrested
16	4	16	1	10	Single Cavity, Blastocyst
			2	6	Abnormal Cavitation
17	2	13	1	11	Single Cavity, Blastocyst
18	3	6	1	6	Abnormal Compaction
19	5	11	1	8	Abnormal Compaction
			2	3	Arrested
20	8	37	1	9	Single Cavity, Blastocyst
			2	9	Abnormal Compaction
			3	7	Single Cavity, Blastocyst
			4	12	Single Cavity, Blastocyst
21	9	15	1	11	Single Cavity, Blastocyst
			2	4	Arrested
22	7	22	1	8	Abnormal Cavitation
			2	8	Single Cavity, Blastocyst
			3	6	Arrested
23	2	8	1	8	Arrested
24	7	2	1	2	Arrested
25	6	19	1	12	Single Cavity, Blastocyst
			2	7	Single Cavity, Blastocyst

n/a = not applicable

Fluorescence *in situ* hybridization was carried out on seven blastocysts from four aggregation experiments (Figure 6.3) as well as 20 excluded cells from four regionally compacted aggregates. The overall genetic diagnosis of the blastocysts is given in Table 6.3. Fifty-two to 90% of the cells in the blastocysts, containing 31 to 56 cells, were diploid. Chaotic mosaicism was the most common abnormality found in these embryos. Sex chimaerism was seen in four of the seven blastocysts.

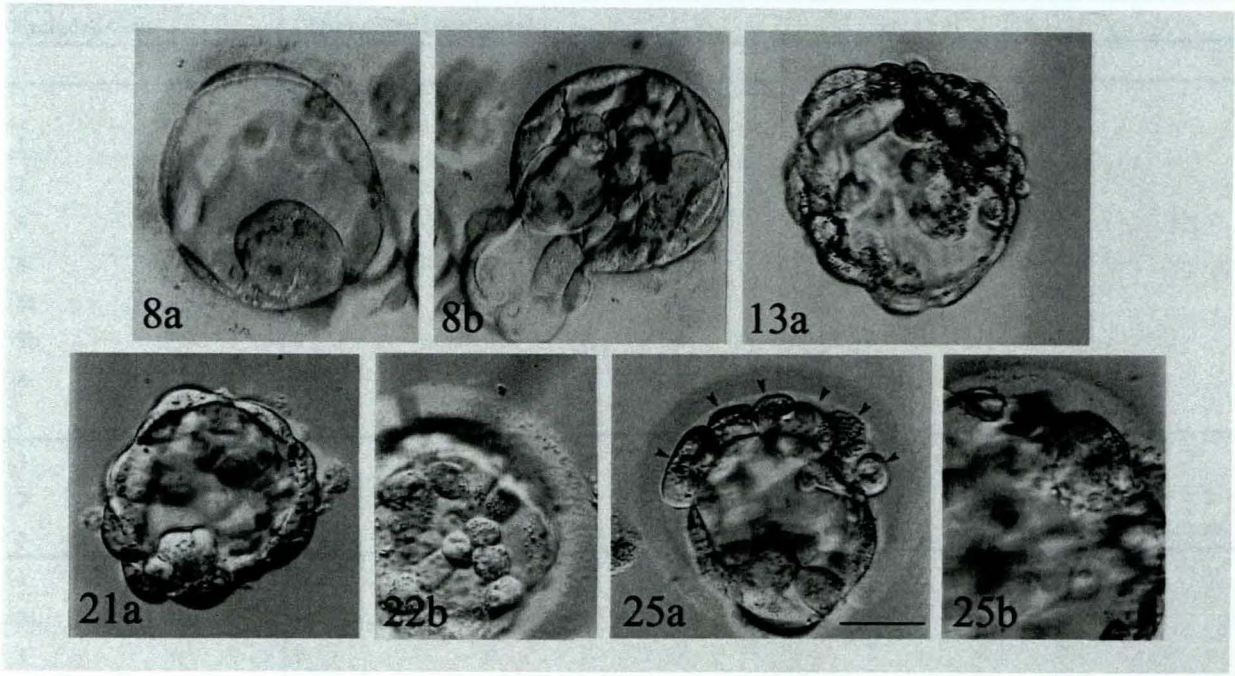


Figure 6.3 Seven blastocysts that developed from chimaeric aggregates and were analyzed by FISH. Blastocysts are overall smaller and have fewer cells than ordinary blastocysts. In 25a, several cells have been excluded (arrowheads). Scale bar is approximately 50μm.

Table 6.3 Chromosomal constitution of seven blastocysts that developed from chimaeric aggregates

Experiment Number	Aggregate Cell Number	Number Of Excluded Cells At Blastulation [a]	Blastocyst Cell Number [b]	% Diploid Cells	Diagnosis for Abnormal Cells [c]	Sex Chimaerism
8a	9	2	31	90	Polyploid	Yes
8b	6	0	56	89	Polyploid	Yes
13a	9	2	39	54	Polyplod/Chaotic	Yes
21a	11	5	31	58	Chaotic	No
22b	8	0	38	89	Chaotic	No
25a	12	6	27	0	Polyplod/Chaotic	No
25b	7	3	41	52	Poly/Chao/Aneuploid	Yes

[a] Excluded cells are those cells not participating in compaction and blastulation.

[b] All blastocysts were analyzed on day-5 of development, except 13a and 25a which were analyzed on day-6.

[c] Poly = polyploid; Chao = chaotic

Whether the blastocyst was normal or abnormal, when examined, the excluded cells associated with it were found to be abnormal, showing haploidy, polyploidy, and complex abnormalities that in nine cases affected all seven of the chromosomes examined. These results are presented in Table 6.4.

Table 6.4 Chromosome constitution of cells excluded from four aggregates showing regional compaction

Excluded Cells	Chromosomes Examined				
	13	16	18	21	22
1	3	3	3	3	0
2	2	0	2	6	0
3	2	2	2	1	2
4	9	nr	2	7	3
5a	3	2	2	2	3
5b	3	2	2	2	3
6	2	2	3	2	3
7	2	2	3	3	3
1	3	3	5	4	5
2	1	1	1	1	1
3	4	2	3	4	2
1	0	3	1	1	3
2	0	3	0	1	0
3	0	3	1	1	3
4	3	1	1	2	1
1	2	1	1	0	1
2	2	1	1	0	1
3	1	3	2	1	1
4	1	2	1	1	1
5	1	0	2	1	1

nr = no result

6.5 Discussion

The results of these experiments demonstrate that some surviving 1/4 and 1/8 blastomeres of extensively fragmented, clinically unusable human embryos are developmentally competent. Isolated from cell fragments and other abnormal cells and placed in individual culture, many mononucleated cells from such embryos continued to divide, and often proceeded to cavitate at the expected time, with three or four cells in total. A similar observation has been made when blastomeres were isolated from normally cleaving embryos and cultured for several days (Geber et al., 1995). This is generally consistent with the observation that morphogenetic events during mammalian embryo-

genesis are developmental age rather than cell number related (Johnson et al., 1984; Kidder et al., 1987; Petzoldt and Muggleton-Harris, 1987).

The aggregation of mono-nucleated blastomeres obtained from several non-viable embryos led, in about 40% of the aggregates, to the formation of normally organized blastocysts, demonstrating the capacity of some blastomeres to regulate development given the appropriate conditions. While the number of chromosomally abnormal cells within the aggregate blastocysts remained high, a proportion of the blastomeres and their descendent cells was chromosomally normal.

Pluripotency or, in some cases, totipotency of 1/4 to 1/8 blastomeres isolated from normal diploid embryos has been demonstrated in several mammalian species, including the rabbit (Moore et al., 1968), the sheep and the cow (Willadsen, 1981; Willadsen and Polge, 1981) and the mouse (Tarkowski et al., 2001).

In the human, reports of birth following transfer of embryos containing one blastomere as a result of freeze-thaw damage remain anecdotal and pertain only to quarter blastomeres (Veiga et al., 1987). The lowest number of 1/8 blastomeres that has produced a live birth, on the other hand, is probably three (M. Alikani, unpublished). However, during clinical human IVF, the concept of totipotency is rarely used to justify selection for intrauterine transfer of human embryos with numerous cleavage abnormalities but few surviving cells. This is partly because the occurrence of most morphological abnormalities, particularly when compounded, is associated with a very significant loss in development potential. For instance, cytoplasmic fragmentation in excess of 15% of the total volume of the embryo is associated with lower pregnancy and implantation (Chapter 3; Racowsky et al., 2003), regardless of chromosomal status of the embryo as a whole. Multinucleation is another abnormality that is associated with not only significantly lowered implantation rates but also failure to form normal blastocysts in extended culture *in vitro* (Chapter 4).

The isolation of component blastomeres from several fragmented/multi-nucleated embryos and their aggregation into a "new" chimaeric embryo therefore provides the means to restore the developmental ability of normal cells from abnormal embryos. This potential would otherwise have been lost.

In this study, embryos resulting from normally fertilized eggs were the primary source of cells for the aggregates. In the mouse, aggregation or injection chimaeras produced from diploid and parthenogenetic or androgenetic cells (Thomson and Solter, 1989), diploid and tetraploid embryos (Everett and West, 1996), and diploid blastomeres supported with tetraploid blastomeres (Tarkowski et al., 2001) are viable. Parthenogenetic/diploid chimaeras have also been found to be developmentally competent in the cow (Boediono et al., 1999). So it is possible that in the human,

the potential of surviving cells from normally fertilized but non-viable embryos can also be realized if they are aggregated with aneuploid cells (such as those from digynic embryos). Our initial efforts in obtaining blastocysts from such aggregates were not successful (data not shown) but the subject needs further evaluation, as was suggested by Edwards and Pool (2002).

The use of aneuploid cells in aggregation chimaeras may prove advantageous if indeed ploidy can influence the relative position of cells in the chimaeric blastocyst. This has been shown in mouse diploid-tetraploid chimaeras where within 30 hours of aggregating cleavage stage embryos, tetraploid cells were found to be non-randomly distributed among different tissues of the early blastocyst, having preferentially been allocated to mural trophectoderm (Everett and West, 1996). It was later discovered that the loss of tetraploid cells was a result of both selection against these cells throughout the embryo and their preferential allocation to the mural trophectoderm (Everett and West, 1998).

Although in our experiments, we often used blastomeres at different stages of development (from different embryos), we did not trace these cells to see whether they were preferentially allocated in the blastocyst. Nevertheless, developmental age has been shown to play a role in cell allocation in aggregation chimaeras. Fehilly et al. (1984) showed that lambs produced from chimaeric embryos made from asynchronous blastomeres (1/8 and 1/4) were predominantly non-chimaeric and of the type corresponding to the older blastomere. This led to the conclusion that the more advanced cells within the chimaera were more likely to form the inner cell mass of the blastocyst. More recently, Tang et al. (2000) also suggested that when aggregated mouse blastomeres differed in size but not ploidy, the derivatives of the larger cells contributed significantly more to the mural and polar trophectoderm than the ICM.

Chimaeric human aggregates generated from discarded embryos may be useful as an alternative source for human embryonic stem (ES) cells. The production of embryonic and trophectodermal stem cells has so far required the use of viable embryos and their maintenance in culture until the development of a blastocyst containing an inner cell mass and a trophectoderm. The source of viable human embryos has been either unwanted frozen embryos donated to research by couples who have already undergone an IVF treatment cycle (Thomson et al., 1998) or embryos created specifically for the purpose of stem cell production using gamete donors (Lanzendorf et al., 2001). But, both these options have generated considerable debate and criticism with major implications for the future of ES cell research, particularly in the United States. The use of the aggregation method discussed here may alleviate concerns regarding the destruction of viable embryos during the course of ES cell production.

Monash University

Declaration for Thesis Chapter 7

In the case of Chapter 7, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	100	Concept, data collection and analysis, writing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, West Orange, NJ, USA Tyho-Galileo Research Laboratories, Hudson, NY, USA

	Date
Signature 1	[redacted] 24 December, 2005

Chapter 7

Epithelial cadherin distribution in abnormal human preimplantation embryos

7.1 Summary

E-cadherin is a vital cell adhesion protein that plays a critical role in morphogenesis. Previous studies of E-cadherin distribution in human embryos have yielded equivocal results. In this study, immunocytochemistry in conjunction with laser scanning confocal microscopy was used to detect E-cadherin in 97 human cleavage stage embryos and 35 blastocysts from normal and abnormal fertilization. An antibody against human placental E-cadherin was used to locate the protein. In blastomeres of cleaving embryos, on the second and third days following insemination, E-cadherin was located in the cytoplasm—mostly concentrated in the cell margins. On the fourth day of development, the protein was relocated in compacting embryos to membranes in areas of cell-cell contact. In other abnormally compacted or non-compacted embryos with extensive cytoplasmic fragmentation, cell arrest, or blastomere multi-nucleation, E-cadherin relocalization was either absent or erratic. In apparently normal blastocysts, E-cadherin in the inner cells was diffuse and cytoplasmic while properly organized trophectoderm cells were surrounded by a band of membrane E-cadherin. Disorganization of trophectoderm was associated with disruption of the regular E-cadherin banding pattern. It is concluded that, as in other mammalian species examined, E-cadherin distribution in human embryos is stage-dependent. Disturbances in the distribution of E-cadherin occur in embryos with cleavage abnormalities and suggest one path to abortive or abnormal blastulation and loss of embryonic viability. The implications of similar changes in the blastocyst are well worth investigating since they could threaten blastocyst integrity.

7.2 Introduction

Cadherins are calcium-dependent transmembrane glycoproteins that play a critical role in regulation of morphogenesis through their involvement in junctional and non-junctional cell adhesion, cell polarity, and cell signaling (Reviewed by Fleming et al., 2001). The intracellular domain of each cadherin dimer is linked to the actin cytoskeleton by anchoring proteins (catenins), while the extracellular domain — which contains calcium binding sites — extends from the surface and can bind to another cadherin dimer on a neighboring cell.

E(epithelial)-cadherin (also known as uvomorulin) is the first cadherin to be expressed during mammalian development. In the mouse, detergent-resistant surface expression of uvomorulin has been detected 6-11 hours post activation (Clayton et al., 1993), but its de-novo synthesis apparently occurs at the late 2-cell stage (Vestweber et al., 1987), i.e. after the embryonic genome is normally activated (Flach et al., 1982). During subsequent stages and until compaction, uvomorulin remains uniformly distributed on free surfaces of blastomeres but it is more concentrated at regions of cell-cell contact. During compaction, the flattening of the outer embryonic cells coincides with a marked increase in intercellular adhesion and uvomorulin redistribution to areas of apposing cell membranes (Johnson et al., 1979; 1986; Vestweber et al., 1987; Becker et al., 1992). At the same time, free surface protein is reduced and becomes relatively more susceptible to detergent extraction (Clayton et al., 1993). This distribution pattern is maintained in outer blastomeres of the mouse morula and in the blastocyst, while the inner cells continue to show diffuse cytoplasmic distribution of the protein (Vestweber et al., 1987).

The function of E-cadherin is thought to be regulated through protein phosphorylation, a post translational modification (Sefton et al., 1996), and its "trafficking" to and from the cell surface (Reviewed by Bryant and Stow, 2004).

In the human, the E-cadherin gene (CDH1) is approximately 100 kb and is located on chromosome 16q22 (Berx et al., 1995). E-cadherin has been discovered on plasma membranes of human spermatozoa and (inseminated but undivided) oocytes (Rufas et al., 2000).

In human embryos presumed to be normal, E-cadherin mRNA has been found throughout the preimplantation stages, including in pronuclear eggs, cleavage stages, and blastocysts (Bloor et al., 2002). However, in apparent contradiction to observations in the mouse (described above), as well as in the pig (Reima et al., 1993) and the cow (Barcroft et al., 1998), E-cadherin expression in the limited number of human embryos examined so far, using antibodies primarily against E-cadherin from mouse carcinoma cells, has been defined as "cytoplasmic, punctate, extremely weak" (Bloor et al., 2002) and "evident in isolated regions of cell contact in trophectoderm or [inner cell mass]" (Ghassemifar et al, 2003).

In this study, immunocytochemistry in conjunction with laser scanning confocal microscopy (LSM) was used to examine the distribution of E-cadherin in some detail and in a large number of human embryos produced by different techniques during assisted reproduction. These embryos were of different genomic constitutions and showed a wide variety of morphologies. Reactivity of early embryonic E-cadherin with an antibody raised against human E-cadherin (of placental origin) was utilized.

All the embryos examined in the study had been judged to be unsuitable for clinical use based on several criteria, as described in Chapter 2 (section 2.4). However, at the time of fixation, some of the cleavage stage embryos did contain the number of cells appropriate for the day of development as well as cells that were mitotically active; some of these and some of the blastocysts appeared morphologically normal.

7.3 Materials and Methods

7.3.1 Embryos in the study

The abnormal embryos used in these experiments were obtained according to the protocols described in Chapter 2 (section 2.4).

In total, 92 embryos from normally fertilized eggs (intracytoplasmic sperm injection or ICSI and standard insemination) were examined. A total of 38 embryos were fixed on day-3 of development, while the remaining 54 were fixed on or after day-4 (up to day-7) of development. The latter group included 29 blastocysts. In addition, 40 abnormally fertilized embryos were examined on days 2 through 6 of development; these included tri-pronucleate IVF and ICSI embryos, single pronucleate ICSI embryos, and embryos that developed from inseminated eggs in which pronuclei were never seen. Six blastocysts were among the 40 abnormally fertilized embryos examined.

7.3.2 Fixation and confocal microscopy

Embryos were fixed and permeabilized for 30-60 minutes at room temperature in 2% formaldehyde and 0.5% Triton X-100 in PIPES buffer. Following fixation, they were washed and kept overnight (or until processed) at 4°C in PBS containing 3% bovine serum albumin (BSA) in order to minimize non-specific binding.

The primary antibody was a monoclonal mouse antibody (IgG2a isotype; Zymed Laboratories, Inc., San Francisco, Ca, USA) against E-cadherin from human placenta (diluted 1:500). Embryos

were incubated with primary antibody for 60 minutes at 37°C; this was followed by two 10-minute washes in 0.2% Tween 20 (Sigma-Aldrich, Inc., Saint Louis, MO, USA) and a minimum of seven washes in PBS supplemented with 3% BSA (PBS/BSA).

The secondary antibody was an FITC-conjugated goat anti-mouse IgG, whole molecule (Sigma-Aldrich, Inc., Saint Louis, MO, USA), diluted 1:200. Following 60 minutes incubation at 37°C with secondary antibody, the specimen was washed through a minimum of seven drops of PBS/BSA, with 0.2% sodium azide (PBS/BSA/Az). To counter-stain the nuclei/DNA, 20 minutes of incubation in 0.06mg/mL propidium iodide followed. Embryos incubated with secondary antibody only and stained with propidium iodide served as negative controls.

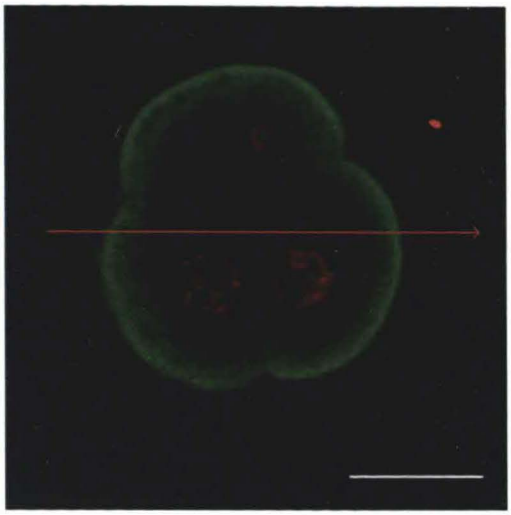
For examination, eggs and embryos were placed in 2µl drops of PBS/BSA/Az covered with mineral oil on a glass cover-slip set in a steel chamber (Attofluor cell chamber, Molecular Probes, Eugene, Oregon, USA).

Laser scanning confocal microscopy was carried out either with an Olympus FluoView laser scanning confocal microscope (Olympus America, Inc., Melville, NY, USA), consisting of an IX70 fluorescence-Nomarski DIC microscope, equipped with an argon laser (emitting at wavelength 488nm), a krypton laser (emitting at wavelengths of 568nm and 647nm), and a transmitted light detector, or with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc, Thornwood, NY, USA) equipped with an argon laser, an HeNe laser (emitting at wavelength 543nm). The images are presented either as single 3-5µm thick optical sections, or projections reconstructed from a series of sections.

7.4 Results

7.4.1 Days 2 and 3 of development

Embryos treated with secondary antibody only (negative controls) did not show any fluorescence (not shown). On days 2 and 3 of development, stage appropriate as well as slow growing embryos, whether resulting from normal or abnormal fertilization, showed diffuse cytoplasmic staining that was most pronounced in the cell margins, as was indicated visually and by the fluorescence profile of single optical sections of embryos (Figure 7.1). In fragmented embryos, blastomeres, anucleate, and some small nucleated fragments associated with them showed diffuse cytoplasmic staining (Figure 7.2).



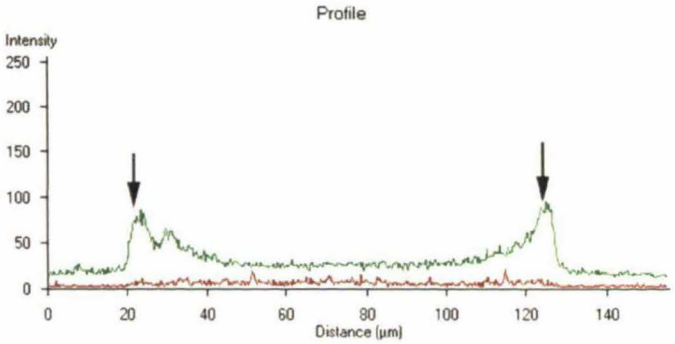


Figure 7.1 Representative day-3 embryo stained for E-cadherin (green) and nuclear DNA (red). The image is a 4µm thick single optical slice. Fluorescence intensity is graphed for the distance represented by the red arrow drawn across the embryo. E-cadherin fluorescence (green line in the graph) is concentrated in the cell margins, as shown by the graph peaks (marked by arrows). The intensity of DNA fluorescence (red line in the graph) is negligible in the same distance (i.e., no nuclei present). Scale bar is 50µm.

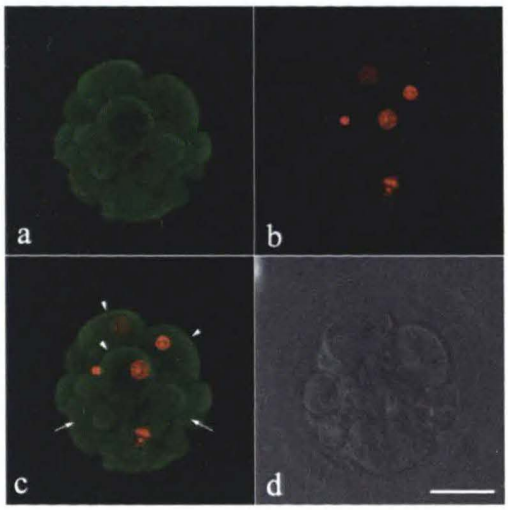


Figure 7.2 A representative day-3 embryo with extensive cytoplasmic fragmentation, stained for E-cadherin (green) and nuclear DNA (red). The image is a projection of multiple 3-4µm thick optical slices. E-cadherin localization is shown in panel (a), the nuclei in panel (b), and both in panel (c). E-cadherin appears diffuse in the three mono-nucleated cells (arrowheads in c) and in fragments (arrows in c). The DIC image in panel (d) is a single mid-section optical slice. Scale bar is 50µm.

7.4.2 Day-4 of development

Representative day-4 embryos are shown in Figure 7.3. Changes in fluorescence pattern were first seen on this day, albeit neither uniformly nor universally. Some embryos with multiple compacted cells showed staining in areas of cell-cell contact along with cytoplasmic staining (Figure 7.3A-D). Other embryos appeared compacted, but their component cells either had very limited membrane staining or lacked it altogether (Figure 7.3E). Embryos with extensive cytoplasmic fragmentation mostly exhibited diffuse cytoplasmic staining as they did during day-3 of development; however, erratic membrane staining was also present in a small proportion of the remaining nucleated cells (Figure 7.3F).

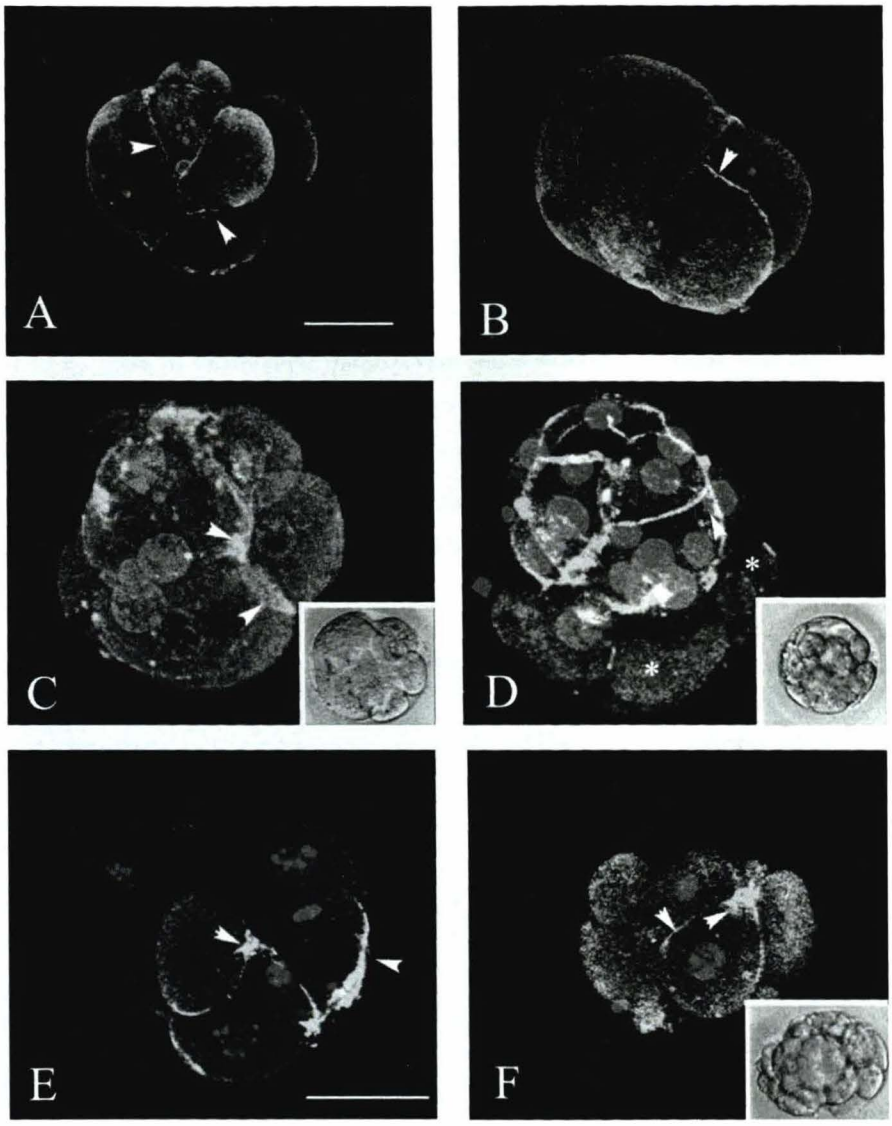


Figure 7.3 Representative day-4 embryos stained for E-cadherin and nuclear DNA. (A) Compacting 8-cell embryo showing relocation of E-cadherin to areas of cell-cell contact, although fluorescence is weak (arrowheads); this embryo had only 4 cells on day-3 of development. (B) Compacting 5-Cell embryo, showing membrane localization of E-cadherin, again weakly (arrowheads); compaction appears atypical; this embryo had 5 cells, 10% fragmentation, and one binucleate cell on day-3 of development. (C) Compacting 7-cell embryo, showing erratic membrane localization of E-cadherin (arrowheads). This embryo had five cells on day-3 of development. (D) Compacted embryo, beginning to cavitate late on day-4 of development; it shows extensive membrane-localized E-cadherin; at least two cells and one large fragment are excluded from the embryo proper (asterisks); staining is primarily cytoplasmic in these excluded cells. This embryo was an uneven and disorganized 11-cell with one or two large fragments on day-3 of development. (E) A partially compacted embryo with several multi-nucleated blastomeres and erratic membrane-located E-cadherin (arrowheads). (F) An extensively fragmented embryo, partly reconstructed from multiple optical sections, showing minimal membrane-localized E-cadherin (arrowhead). Insets show embryos just before fixation. Scale bar is 50μm.

7.4.3 Days 5-7 of development

The morphology of embryos on days 5-7 of development varied greatly, ranging from few vacuolized cells to fully differentiated and expanded blastocysts. The pattern of staining also varied with different morphologies.

The overall staining pattern of normal-appearing blastocysts was consistent: inner cell mass (ICM) cells displayed cytoplasmic but not membrane staining. Properly organized and uniformly sized trophectoderm (TE) cells in expanded blastocysts with normal or near normal morphology were surrounded by a strong band of fluorescence (Figure 7.4A-C). Differences in staining were not seen between mural (cells surrounding the blastocoel) and polar (cells overlaying the ICM) TE. Several hatching blastocysts were examined in which details of the embryo's escape from the zona pellucida could be visualized. Hatching was observed to have started either in the area of the ICM or away from it (Figure 7.5).

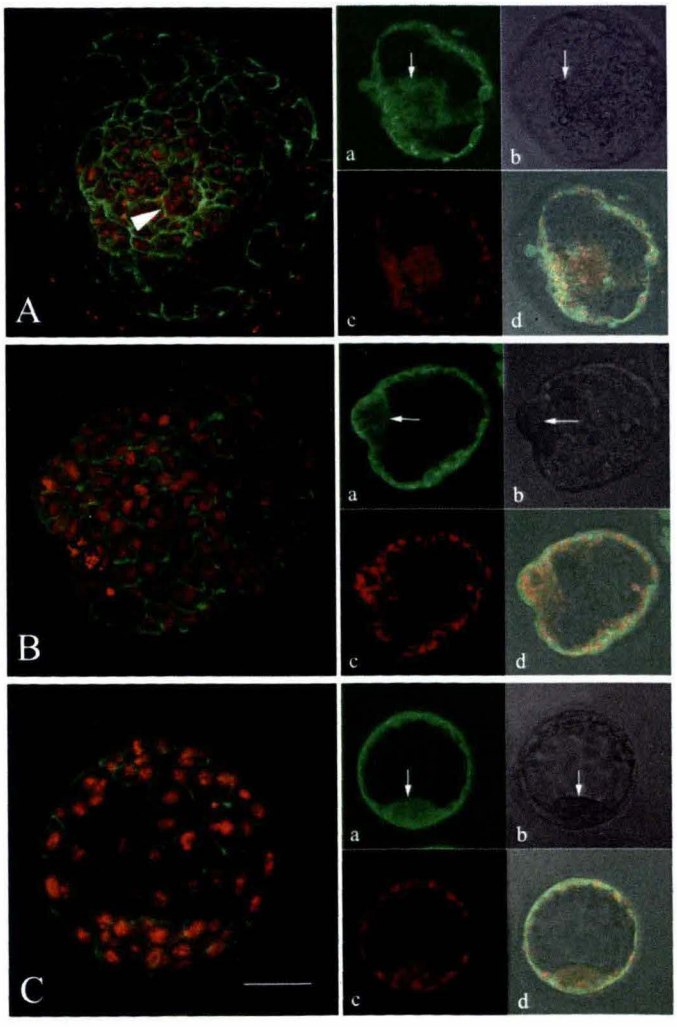


Figure 7.4 Apparently normal day-5-7 blastocysts stained for E-cadherin (green) and DNA (red). Images in A, B, and C are projections of multiple 5μm optical sections. Images in panels (a-d) are single optical sections and show (a) E-cadherin, (b) DIC, (c) DNA, and (d) the combination. (A) Day-7 expanded blastocyst that developed from an egg in which fertilization was not confirmed. The cells of the trophectoderm (both polar and mural) show an intense "belt" of fluorescence indicating localization of E-cadherin in the membranes. Cells within the ICM (arrows in panels a and b) show diffuse cytoplasmic E-cadherin, but no membrane localization of the protein. Arrowhead points to a multi-nucleate cell within the polar TE overlying the ICM. (B) Hatched day-7 blastocyst from a normally fertilized egg showing the same pattern as seen in A. The area of the ICM is indicated by arrows in panels a and b. (C) A day-5 expanded blastocyst that developed from an egg in which three pronuclei were seen 19 hours following ICSI. The pattern of staining is similar to that seen in A and B. The ICM is marked by arrows in panels a and b. Scale bar is 50μm.

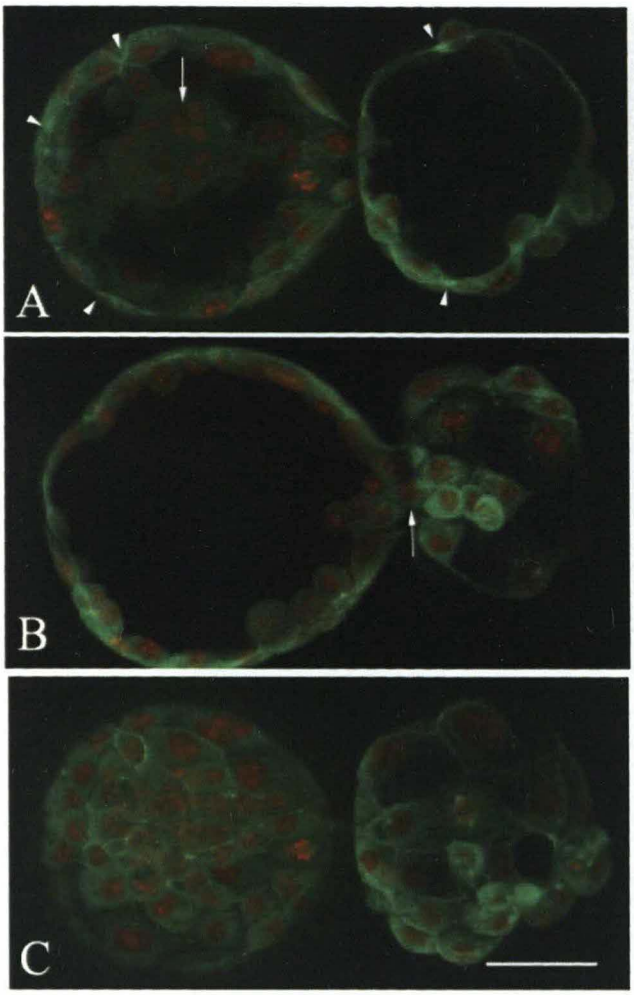


Figure 7.5 Apparently normal day-5 hatching blastocyst stained for E-cadherin (green) and DNA (red). This blastocyst developed from a normally fertilized egg that was biopsied on day-3 of development and was diagnosed as a trisomy 13 aneuploid embryo. (A) Although membrane localization of E-cadherin is visible in some TE cell junctions (arrowheads), in other areas it appears to be mostly cytoplasmic. The ICM (arrow) shows typical cytoplasmic staining. (B) The embryo is hatching through an artificial biopsy hole in the zona pellucida (arrow). (C) Partial reconstruction of the blastocyst shows its overall morphology. Scale bar is 50μm.

Representative embryos with abnormal blastulation are shown in Figure 7.6. The abnormalities included an absent ICM, a disorganized TE or one composed of abnormally large cells, and a perivitelline space (PVS) filled with fragments and excluded cells. Atypically large cells within disorganized TE often did not show any membrane staining (Figure 7.6A,C). Excluded arrested cells and fragments in the PVS did not show any cytoplasmic or other staining (Figure 7.6B). Morulae that formed on day-5 rather than day-4 of development (no cavity present) still showed cell-cell contact area staining, albeit not extensively.

Embryos that developed from presumed digynic (ICSI 3PN) fertilization were often found to be compacted on day-4 of development and they showed various degrees of cell-cell contact area staining on (or after) that day; some digynic embryos blastulated apparently normally by day-5 of development (Figure 7.5C). Presumably polyspermic embryos (IVF 3PN) also showed occasional compaction and erratic membrane staining on day-4, but fewer were found to blastulate on (or after) day-5 of development.

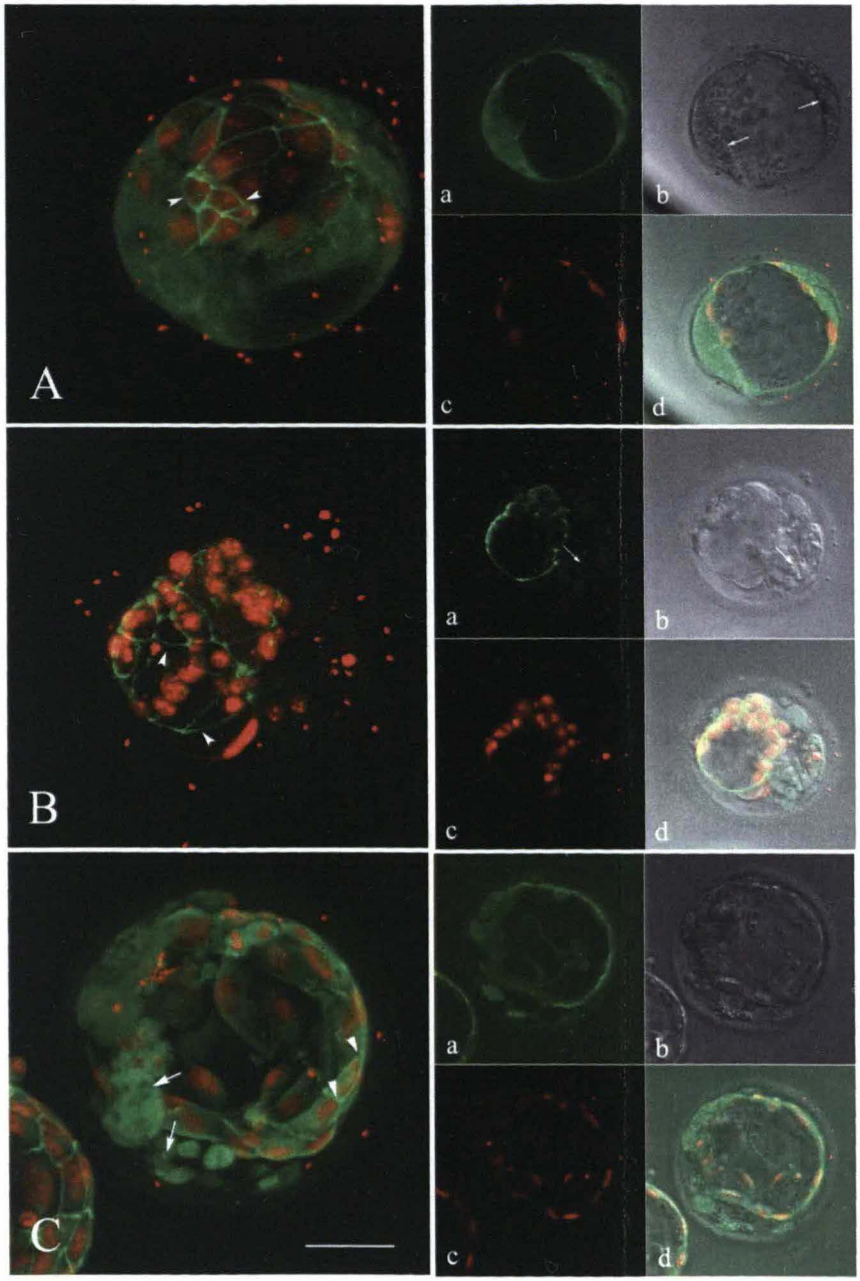


Figure 7.6 Abnormal blastulation on Day-5 of development; E-cadherin (green) and DNA (red) staining are shown. Images in A, B, and C are projections of multiple 4-5μm optical sections. Images in panels (a-d) are single optical sections and show (a) E-cadherin, (b) DIC, (c) DNA, and (d) the combination. (A) Day-5 embryo with very few TE cells, some showing the typical belt pattern of E-cadherin localization (arrowheads). The TE is generally disorganized (arrows in panel b) and there is no visible ICM. (B) An embryo showing abnormal blastulation and "belt" staining of TE cell membranes (arrowheads); many excluded cells and fragments which do not show any staining are visible (arrows in panels a and b). (C) A blastocyst with many large TE cells and limited localization of E-cadherin in TE cell junctions (arrowheads). Note the excluded fragments (arrows). Scale bar is 50μm.

7.5 Discussion

In the present study, the distribution of the vital cell adhesion protein E-cadherin was assessed in human embryos by immunocytochemistry and confocal microscopy. The images obtained suggest that E-cadherin distribution in human embryos is stage-dependent. During the first three days in culture, in embryos with apparently normal morphology, the protein appears to be cytoplasmic and mainly concentrated in the non-contact regions of cells. On day 4 of development, it is relocated to areas of cell-cell contact. Following differentiation, E-cadherin appears to be cytoplasmic in the ICM cells while it appears as a band delineating the borders between individual TE cells, as is characteristic for epithelial cells in general.

In a study by Bloor et al. (2002), only 2 of 8 "normal" blastocysts showed "localization of E-cadherin to TE cell junctions", while the remaining blastocysts showed "weak and punctate" staining. Many factors may have contributed to this and other differences between our two studies. The most conspicuous is the diversity of the embryos examined (and of IVF human embryos in general). Weak staining and complete absence of membrane staining was also observed in the present study in some compacted day-4 embryos and in some blastocysts that were apparently normal. The choice of primary antibody may be another contributing factor. In the present study, a monoclonal antibody, produced in the mouse, against E-cadherin from the human placenta was used. Bloor et al. (2002) utilized an antibody against mouse E-cadherin. The homology between mouse and human E-cadherin molecules is between 69 and 75%, as suggested by BLAST 2 sequences (Tatusova and Madden, 1999; http://www.ncbi.nlm.nih.gov/blast/bl2seg/wblast2.cgi). However, monoclonal antibodies are generally specific for a 4-5 amino acid sequence and are usually directed against the amino or carboxy terminal of a given protein (Pieczenik, 2004); the mouse and human E-cadherin differ at their amino end. It is therefore possible that the antibodies in the study of Bloor et al. (2002) did not fully recognize E-cadherin in human embryos, thus producing inconsistent results.

The timely expression and distribution of E-cadherin is essential for normal compaction and blastulation. In all species of mammals examined, compaction begins after a certain number of cleavage divisions. The actual number depends on the species; in the human, compaction takes place between the eight and the sixteen cell stages, i.e. between the third and the fourth cleavage divisions (Nikas et al., 1996). The images of cleavage stage embryos with divisional asynchronies and related abnormalities as well as those of abnormal blastocysts collected for this study suggest that E-cadherin distribution is perturbed in these embryos. Specifically, redistribution of the protein to cell-cell contact areas was found to be absent or erratic. This apparent failure of abnormal embryos to properly relocate E-cadherin may be the immediate cause of their frequent failure to compact (and subsequently blastulate) normally.

The failure to relocate E-cadherin may be a consequence of developmental asynchrony or loss of physical contact between blastomeres (for instance through fragmentation or arrest of one or more cells). In the mouse, reverse translocation of E-cadherin from cell-cell contact areas to the cytoplasm has been shown to occur in decompacted embryos (Clayton et al., 1993; Pey et al., 1998). Conversely, in the human, disaggregation of fragmented embryos into their component cells and fragments and reaggregation of the cells in a "chimaeric" form can, in some cases, overcome the barriers to normal compaction and lead to blastulation (Chapter 6).

At a more basic level, a possible reason for failure of embryos to fully engage E-cadherin in compaction is failure of genomic activation. Experimental evidence indicates that activation of the embryonic genome is a prerequisite for proper compaction. E-cadherin-null homozygous mutant mouse embryos which use residual maternal E-cadherin rather than zygotic E-cadherin to initiate compaction are unable to maintain the compacted state and fail to form blastocysts (Larue et al., 1994; Riethmacher et al., 1995).

Maintenance of compaction is dependent also on development of junctional complexes between apposing cells (Reviewed by Fleming et al., 2001). These complexes include gap junctions, tight junctions (zonula occludens), adherens junctions, and desmosomes; E-cadherin plays an integral part in the latter two.

In the human, Dale et al. (1991) and Gualtieri et al. (1992) have shown that TE and ICM cells are in communication through gap and tight junctions as well as desmosome-like structures. However, other evidence suggests that in some embryos, particularly those with morphological abnormalities, both the expression and the localization of junctional proteins are altered (Hardy et al., 1996; Ghassemifar et al., 2003).

The pattern of staining in abnormal blastocysts examined here was of particular interest. Some blastocysts did not show the expected localization of E-cadherin. On the other hand, TE cells in some abnormal blastocysts did not exhibit the typical epithelial cell type of E-cadherin banding; in the same blastocysts, the cells of the ICM, if present, showed the diffuse cytoplasmic staining just as was seen in blastocysts with normal morphology.

Clearly, the analysis of the images alone can not provide an explanation for these observations. Nonetheless, the absence of E-cadherin banding in some TE cells is reminiscent of epithelial-to-mesenchymal cell transformation, during which E-cadherin is internalized, facilitating the dissolution of adherens junctions between epithelial cells, thereby giving them mobility (Palacios et al., 2005). Notwithstanding a role for epithelial-to-mesenchymal transformation in interactions between uterine cells and the trophoblast during implantation (e.g., Thie et al., 1996), under the

circumstances of *in vitro* culture, a similar mechanism could lead to separation of TE cells from their neighboring cells and localized or general disruption of the trophectoderm. The accidental formation of trophoblastic vesicles during hatching of human blastocysts through narrow slits left after partial zona dissection lends support to this suggestion (Cohen et al., 1990). The implications of abnormal regulation and distribution of E-cadherin in human blastocysts are therefore well worth investigating, especially in view of the current trend toward universal application of blastocyst culture and transfer for treatment of infertility, and the twinning complications associated with this technology (Milki et al., 2000).

Monash University

Declaration for Thesis Chapter 8

In the case of Chapter 8, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	40	Concept, experiment design/execution, data collection and analysis, writing of manuscript
2. Tim Schimmel	10	Experiment preparations/execution
3. Steen Willadsen	50	Concept, experiment design/execution, data collection/analysis

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Tyho-Galileo Research Laboratories, Hudson, New York, USA

		Date
Signature 1	[redacted]	24 December, 2005
Signature 2		1-3-2006
Signature 3		29 December, 2005

Chapter 8

Cytoplasmic fragmentation in activated eggs occurs in the cytokinetic phase of the cell cycle, *in lieu* of normal cytokinesis, and in response to cytoskeletal disorder

8.1 Summary

The timing of cytoplasmic fragmentation in relation to the cell cycle was studied in mature oocytes and early cleavage stages using mouse eggs and embryos as experimental models. The central approach was to remove the nuclear apparatus, in whole or in part, from non-activated and activated eggs and early embryos and follow their response during subsequent culture *in vitro*. Eggs arrested in metaphase of the second meiotic division did not fragment following complete removal of the meiotic apparatus, provided they were not subsequently activated. Exposure of spindle-chromosome-complex-depleted eggs to activation conditions immediately after enucleation led to fragmentation, although not until control embryos entered first mitosis. Delaying activation until 24 hours post enucleation led to earlier fragmentation. Enucleation of normally fertilized or artificially activated eggs after emission of the second polar body also led to fragmentation coinciding with the first mitosis in nucleated control embryos. However, if artificially activated eggs were prevented from completing second meiosis, by exposure to cytochalasin, and then enucleated, this universal wave of fragmentation was preceded in some cytoplasts by limited fragmentation after just a few hours in culture, and coinciding with completion of meiosis II in nucleated eggs. Fragmentation also occurred in the second mitotic cell cycle, but it was limited to blastomeres of fertilized eggs that were enucleated in late interphase, or after the zygotic genome normally is activated. These results indicate that fragmentation in eggs and early embryos, though seemingly uncoordinated, is a precisely timed event that occurs only in mitotically active cells, during the cytokinetic phase of the cell cycle, *in lieu* of normal cytokinesis, and in response to altered cytoskletal organization.

8.2 Introduction

Both *in vitro* and *in vivo*, cleavage stage embryos of practically all mammalian species examined so far have been found often to comprise not only nucleated cells, but also non-nucleated cells, or "fragments", of varying size and number (e.g., Killeen and Moore, 1971; Enders et al., 1982; Edwards et al., 1970; Buster et al., 1985). The fragments do not contribute directly to the development of the embryo proper, and therefore the most obvious and immediate result of fragmentation is that the embryo has a smaller than normal cytoplasmic volume and is deprived of whatever endogenous resources are contained in the fragments. This in itself could reduce the developmental potential of the embryo to a point of non-viability, though fragmented embryos generally have a lower viability than can be attributed to the loss of cytoplasm alone.

In human embryos, extensive cytoplasmic fragmentation is almost always accompanied by other cytoplasmic and nuclear anomalies, for instance, blastomere multi-nucleation and chromosomal mosaicism (Pellestor et al., 1994; Kligman et al., 1996; Laverge et al., 1997; Marquez et al., 2000). Collectively, these abnormalities can explain the abnormal pre- and post-implantation development often seen in fragmented embryos (Chapters 3 and 4; Jackson et al., 1998; Van Royen et al., 2003; Racowsky et al., 2003; Hardy et al., 2003), though it is important to keep in mind that such embryos are not necessarily non-viable, and that even if non-viable, they may still contain viable cells (Chapter 6).

It is as yet unclear what causes fragmentation, and what mechanisms are involved. In the mouse, following super-ovulation, fragmentation of unfertilized mature eggs has been viewed as a manifestation of apoptosis, or programmed cell death (Takase et al., 1995, Perez et al., 1999; Morita and Tilly, 1999; Gordo et al., 2002), and it has been suggested that its occurrence in cleaved embryos is under the control of more than one genetic locus and affected by both parental genotypes (Hawes et al., 2001).

Waksmundzka et al. (1984), Ciemerych (1995), and Ciemerych et al. (1998) noted cortical deformations in anucleate halves of bisected (or enucleated) activated mouse eggs. They pointed out that this "autonomous cortical activity" preceded entry of the nucleated cells into first mitosis but did not identify its cause. Fragmentation of activated eggs with absent or damaged DNA led Liu et al. (2002) to conclude that this phenomenon represented apoptosis and resulted from activation of a unique checkpoint at first mitosis.

In fertilized human eggs and cleavage stage embryos, cytoplasmic fragmentation is considered the final manifestation of a degenerative process, whether apoptotic (Jurisicova et al., 1996b; 1998; 2003; Levy et al., 1998; Yang et al., 1998) or triggered otherwise (Van Blerkom et al., 2001). It is unclear, however, how fragmentation as a consequence of cell death could lead to, e.g., a wide range

of morphologies and developmental potentials (indeed, viability) as seen in fragmented human embryos (Chapter 3; Warner et al., 1998; Antczak and Van Blerkom, 1999).

While it is probable that cytoplasmic fragmentation in mammalian eggs and embryos has a number of different primary causes, the phenomenon itself, its occurrence and dynamics deserve to be studied in more detail.

In the context of *in vitro* fertilization in humans and animals, and nuclear transplantation in animals, fragmentation seems to take place mainly during the first one or two cell cycles. Major fragments have not been widely observed to occur in freshly ovulated or freshly matured unfertilized eggs or in 1-cell embryos prior to the first mitotic division; indeed, it is doubtful whether fragmentation occurs at all in non-activated mature eggs.

Fragmentation is reminiscent of cell division, at least in the sense that one original cell is partitioned. Normal cell division involves radical reorganizations of the cytoskeleton, particularly in the microtubules and their relationship with the cortical microfilaments. It is reasonable to expect, as suggested by early experiments in the sea urchin (described by Rappaport, 1996), that somewhat similar reorganizations are required for fragmentation to take place. This in turn raises the question whether fragmentation takes place at any time during the cell cycle or is restricted to certain phases. The present experiments were undertaken to find an answer to this question, but also, and more importantly, to uncover the causes and nature of the phenomenon and, eventually, explain its association with other abnormalities.

Drawing on experience gained in nuclear transplantation, enucleation was chosen as a way of making experimental eggs and blastomeres "fragmentation prone". The nuclear apparatus was removed from eggs and embryos during different phases of the meiotic or mitotic cell cycle, and the response of the cells to these manipulations was followed under different culture conditions by light microscopy. The mouse was chosen as the experimental species because, in the mouse, the timing of the second meiotic division and cleavage is well defined and relatively easy to control. Also, in the mouse, the nuclear structures relevant to the particular experimental approach chosen are relatively easy to see in fresh specimens. Laser scanning confocal microscopy was employed to visualize nuclear DNA and the occurrence and distribution of microtubules. In the course of the experiments, cytochalasin B, a destabilizer of microfilaments, and/or colcemid, a microtubule depolymerizer, were used to 1) minimize damage during cell manipulations, and 2) establish culture conditions that temporarily prevented cytokinesis and/or mitosis (e.g., Smith and McLaren, 1977; Surani et al., 1980; Schatten et al., 1985).

8.3 Materials and Methods

8.3.1 Oocyte collection

Oocytes were collected according to the general protocols described in Chapter 2 (section 2.5). Ovulated oocytes were collected at 18 hours after hCG injection in all experiments but experiment I.1 (parts a and b). In that experiment, the eggs were collected at 16 hours post hCG, to minimize the risk of spontaneous activation (Xu et al., 1997; Abbott et al., 1998).

8.3.2 Ethanol activation of mature oocytes

Within 30 minutes of collection (or enucleation), eggs to be activated were exposed to 7% ethanol in PBS for 4.5 minutes (Kaufman, 1982), washed thoroughly in M-CZB, and placed in culture. When intact eggs were ethanol-treated, only activated eggs showing one polar body and one emerging female pronucleus within 5 hours (generally above 85% of those treated) were selected for the experiments. Activation of enucleated eggs, on the other hand, could not be immediately confirmed.

In certain experiments, CCB (10μg/mL) was included in the culture medium to prevent cytokinesis. In certain other experiments, colcemid (0.02μg/mL) was included in the culture medium to prevent progression of mitosis beyond metaphase. In these two sets of experiments, experimental material was cultured for 5 to 24 hours, depending on the experiment, in medium containing either CCB or colcemid.

8.3.3 Enucleation procedures

To minimize damage during micromanipulation, eggs and embryos were incubated at 37°C in 10μg/mL CCB in M-CZB with 3% BSA for 20 minutes before the start of procedures. Micromanipulation was performed in the same medium. To keep the position of the meiotic spindle well defined, CCB was not used during manipulations in experiment I. Following completion of micromanipulation, the eggs and embryos were washed through 5-7 drops of culture medium, placed in culture for 10 minutes and then moved again to a fresh medium drop for further culture.

Micromanipulation equipment and tools are described in Chapter 2 (section 2.7). For enucleation, a pipette with an inner diameter of 25-30μm was used. When required, the zona pellucida was opened according to the protocol described in Chapter 2 (section 2.6).

Enucleation of activated eggs is illustrated in Figure 8.1 and for 2-cell embryos, in Figure 8.2.

Ten to 20 eggs or embryos were manipulated at a time and micromanipulation procedures took less than an hour to complete. Enucleations were timed to coincide with different phases of the

cell cycle, hence varied in different experiments, as detailed below (cell cycle timing in the mouse has been described in detail in Hogan et al., 1994). To slow down heat loss and minimize the risk of microtubule depolymerization while eggs and embryos were out of the incubator, micromanipulations and examinations were carried out on a heated microscope stage, set to 37°C; general handling of eggs and embryos was done on the work surface of the laminar flow hood which was also set to 37°C.

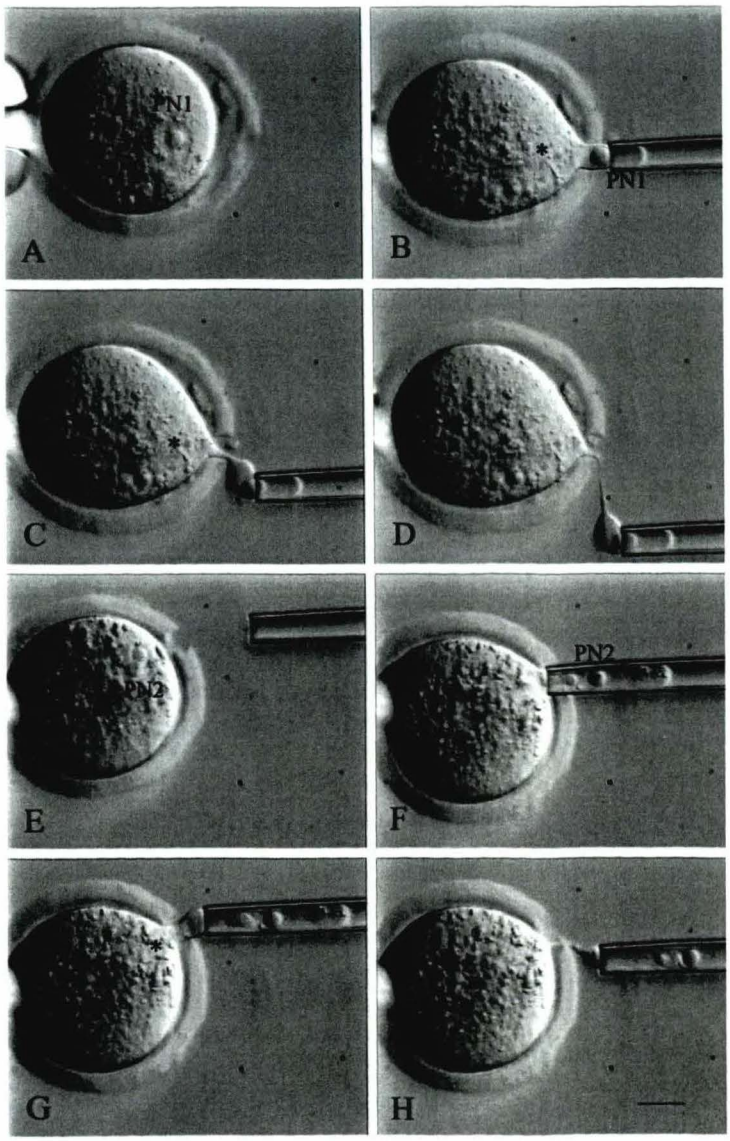


Figure 8.1 Method of enucleation of activated eggs after 4-5 hours of CCB exposure. The zona pellucida over one pronucleus (PN1) was breached by laser (A) and the pronucleus was aspirated into the enucleation pipette (B,C). It was then removed from the egg without removing cytoplasm (D). The egg was partially released from the holding pipette, turned, and a second hole was made over the second pronucleus (PN2) (E). The pronucleus was aspirated into the pipette (F), as before, and removed with minimal or no cytoplasm (G,H). The area containing the spindle remnant (asterisk) is particularly visible in B and C. Scale bar is approximately 20μm.

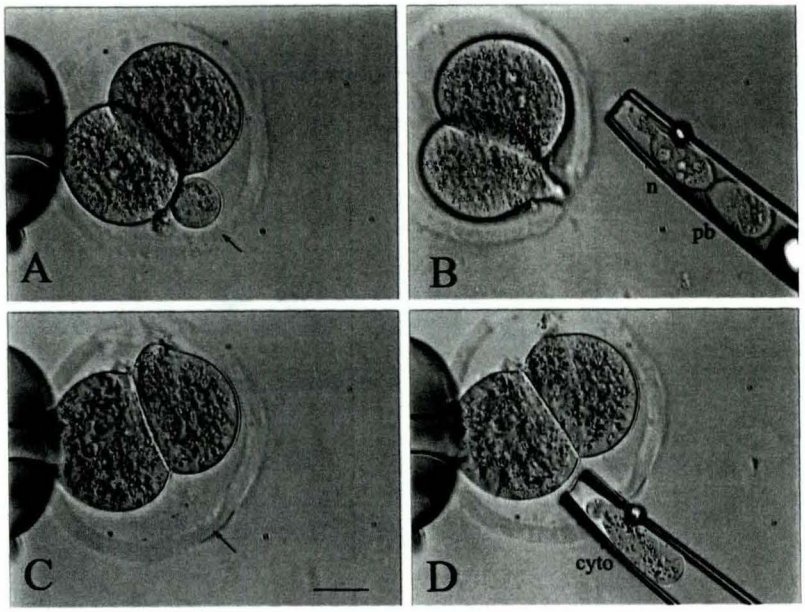


Figure 8.2 Method of enucleation of 2-cell embryos. (A) The zona pellucida over one blastomere was breached by laser (arrow). (B) The polar body (pb) along with the blastomere nucleus (n) was aspirated into the enucleation pipette. (C) The embryo was then turned and the zona pellucida over the second blastomere was breached (arrow). (D) Depending on the experiment, either a small volume of cytoplasm (cyto) or the nucleus itself (not shown) was removed. Scale bar is approximately 20μm.

8.3.4 Enucleation experiments

A schematic outline of the study (experiments I-V) is presented in Figure 8.3.

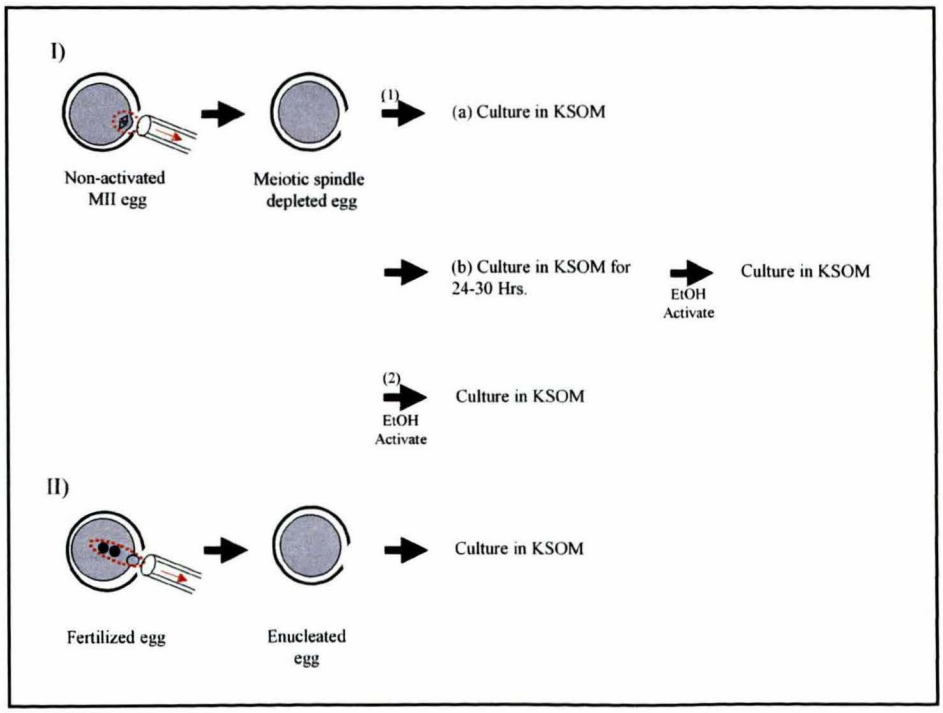
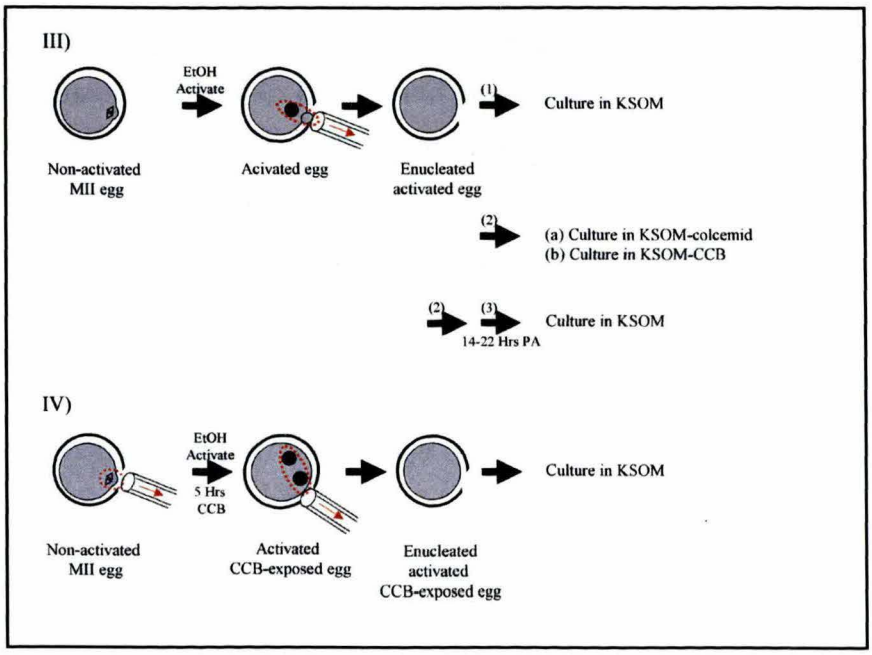
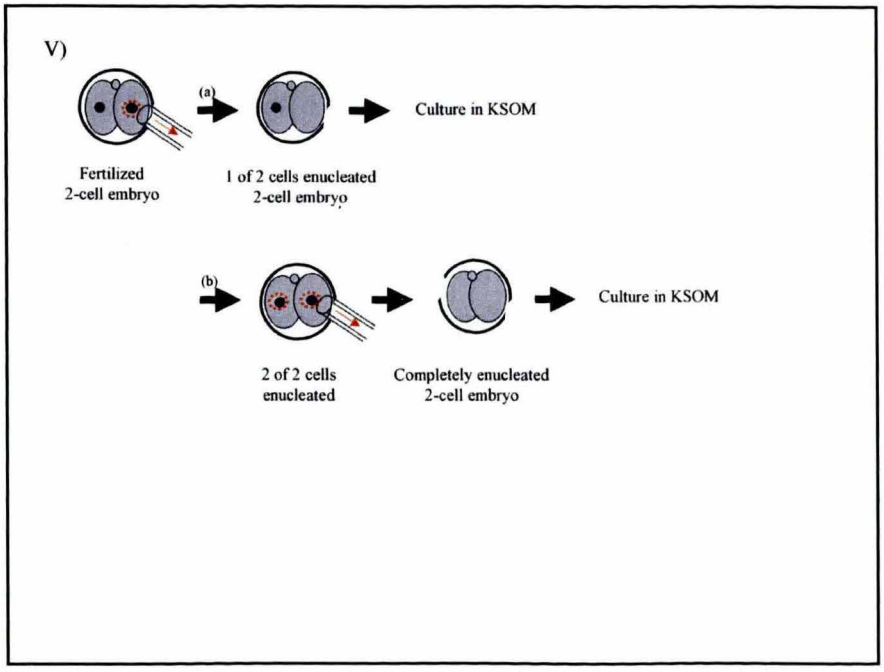


Figure 8.3 A schematic presentation of the experiments. EtOH = ethanol; PA = post activation.

Figure 8.3 continued





8.3.4.1 Experiment I: Non-activated MII eggs

The purpose of these experiments (and those under II) was to examine the relationship between fragmentation, MII arrest, and activation. The meiotic spindle-chromosome-complex was removed from these eggs within sixty minutes of collection.

8.3.4.1.1 Experiment I.1a: Removal of the spindle-chromosome complex and culture of cytoplasts

These eggs (N=82) were collected at 16 hours post hCG, manipulated to remove the spindle-chromosome-complex, placed in culture and checked every few hours for up to 30 hours in culture. Intact MII eggs (N=74) collected at the same time and cultured in KSOM served as control in this experiment.

8.3.4.1.2 Experiment I.1b: Delayed activation of cytoplasts from I.1a

A proportion of the eggs from I.1a (N=69) were exposed to activation conditions (7% ethanol for 4.5 minutes) after 24-30 hours in culture. They were then washed, placed in culture, and observed every 1-3 hours for 6 hours and thereupon less frequently, up to 24 hours post activation (PA). A proportion of the intact control eggs (N=57) were also activated at the same time.

8.3.4.1.3 Experiment I.2: Immediate activation of spindle-chromosome-depleted eggs

These eggs (N=14) were collected at 18 hours post hCG and were exposed to activation conditions immediately after removal of the spindle-chromosome-complex. They were then washed, placed in culture, and observed every 1-3 hours for 24 hours and thereupon less frequently for up to 48 hours PA.

8.3.4.2 Experiment II: Enucleation of normally fertilized pronuclear eggs

Thirty-one zygotes were enucleated 20-22 hours post hCG (an estimated 7-9 hours after fertilization), when both pronuclei could be clearly seen. Intact zygotes cultured in KSOM (N=25) served as control.

8.3.4.3 Experiment III: Enucleation of ethanol-activated eggs

The purpose of these experiments was to pinpoint the exact timing of fragmentation in relation to egg activation and to investigate both the reversibility and preventability of this process.

Enucleation was carried out five hours PA, when the second polar body and the female pronucleus could be clearly observed. Enucleated eggs were cultured under the following conditions and checked every 1-3 hours for 24 hours and, thereupon less frequently, up to 48 hours:

III.1) Culture in KSOM for the duration of the experiment (N=73), III.2) Culture in KSOM containing, (a) colcemid (N=72) or (b) CCB (N=30) for the duration of the experiment, III.3) Culture in (a) KSOM-colcemid (N=42) or (b) KSOM-CCB (N=30) for 14-22 hours, then transfer to KSOM.

Intact activated eggs cultured under the same conditions as above served as controls; respectively, these were nucleated eggs cultured in KSOM (N=55), KSOM-colcemid (N=24) or KSOM-CCB (N=25) for the duration of the experiment, and nucleated eggs cultured in KSOM-colcemid (N=15) or KSOM-CCB (N=15) and transferred to KSOM at 14-22 hours PA.

8.3.4.4 Experiment IV: Enucleation of ethanol-activated eggs exposed to CCB to prevent emission of the second polar body

The purpose of these experiments was to investigate the possibility of fragmentation during cytokinesis of meiosis II. Ethanol-activated eggs (N=51) were cultured in KSOM-CCB for 4-5 hours until two emerging pronuclei were observed. They were then subjected to enucleation and afterwards, washed, transferred to, and cultured in KSOM.

Nucleated eggs (N=15), exposed to CCB for 4-5 hours, washed, transferred to, and cultured in KSOM served as control.

8.3.4.5 Experiment V: Enucleation of fertilized 2-cell embryos in interphase

The purpose of these experiments was to investigate the possibility of fragmentation during cytokinesis of the second mitotic cycle. Enucleation in these experiments was done either (1) as early as 38 hours and up to 45 hours post hCG ("early-mid interphase" of the second cell cycle) or (2) from 46 to 50 hours post-hCG ("late interphase" of the second cell cycle), while interphase nuclei were still visible. Under each condition, either (a) one or (b) both blastomeres were enucleated. For early-mid-interphase enucleations, the experiments included 28 embryos in which one blastomere was enucleated and 83 embryos in which both blastomeres were enucleated. During late interphase enucleations, 25 embryos had one blastomere enucleated and 75 embryos had both blastomeres enucleated.

8.3.5 Fixation, immunostaining, and laser scanning confocal microscopy (LSM)

For LSM, eggs and embryos were fixed and extracted for 30 to 45 minutes at 37°C in 2% formaldehyde and 0.5% Triton X-100 in PIPES buffer (modified from Allworth and Albertini, 1993). Following fixation and extraction, they were washed and stored (at 4°C) in PBS supplemented with 3% BSA and 0.2% sodium azide (PBS/BSA/Az).

For indirect immunofluorescence labeling, monoclonal anti-acetylated-tubulin (de Pennart et al., 1988)(T6793, Sigma-Aldrich, Inc., Saint Louis, MO, USA), diluted 1:1200 and monoclonal anti-beta-tubulin (T4026, Sigma-Aldrich, Inc., Saint Louis, MO, USA) diluted 1:400, alone or in combination were used as primary antibodies. Eggs and embryos were incubated with primary antibodies for 60 minutes at 37°C; this was followed by two 15-minute washes in 0.2% Tween 20

(Sigma-Aldrich, Inc., Saint Louis, MO, USA) and a minimum of seven washes in PBS/BSA/azide to remove the Tween. The secondary antibody was an FITC-conjugated goat anti mouse IgG, whole molecule (F2012, Sigma-Aldrich, Inc., Saint Louis, MO, USA). Following 60 minutes incubation at 37°C with secondary antibody, the material was washed through a minimum of seven drops of PBS/BSA/azide. To counter-stain the nuclei/DNA, eggs/embryos were incubated for 20 minutes in 0.06mg/mL propidium iodide after which they were briefly washed. For examination, eggs and embryos were placed in 2mL drops of PBS/BSA/Az covered with mineral oil on a glass cover-slip set in a steel chamber (Attofluor cell chamber, Molecular Probes, Eugene, Oregon, USA).

Laser scanning confocal microscopy was carried out with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc, Thornwood, NY, USA) equipped with version 3.2 of the LSM software, an argon laser (emitting at wavelength 488nm) and an HeNe laser (emitting at wavelength 543nm).

8.4 Results

The results are summarized in Table 8.1.

Table 8.1 A summary of results in experiments I-V; the key to the experiments is presented in Figure 8.3

Experiment	No. Manipulated	No. Fragmented (%)	Time of Fragmentation or Period of Observation (Hours PA)	Status of Nucleated Controls
I (1a)	82	0 (0)	(17-48) [a]	Metaphase II Arrest
I (1b)	69	36 (52)	2	Fragmented
I (2)	14	12 (86)	15	Mitotic
II	31	21 (68)	~20-22 [b]	Mitotic or Cleaved
III (1)	73	50 (68)	15	Mitotic
III (2a)	72	0 (0)	9-48	Mitotic metaphase arrest
III (2b)	30	0 (0)	9-48	Interphase arrest
III (3a)	42	17 (40)	16-28 [c]	Cleaved
III (3b)	30	9 (30)	16-28 [c]	Cleaved
IV	51	27 (53)	9	Meiotic
		43 (84)[d]	16	Mitotic
V(1b)	83	6 (7)	~40b	Mitotic
V(2b)	75	56 (75)	~40b	Mitotic

Values in parentheses are percentages.

[a] Hours post hCG

[b] Time represents an approximation of hours post fertilization.

[c] Fragmentation occurred approximately 2-6 hours after removal of colcemid or CCB.

[d] This proportion reflects the second wave of fragmentation among the same cohort of 51 manipulated eggs.

8.4.1 Experiment I: Non-activated MII eggs

I.1a) Of the 82 eggs that were placed in culture following the removal of the spindle-chromosome-complex, none (0/82) showed plasma membrane ruffling, blebbing, or fragmentation at any time during a 24-30 hour culture period. Similarly, none of the intact controls (0/74) divided or fragmented during culture.

I.1b) When the spindle-chromosome-complex-depleted eggs were exposed to activation conditions after 24-30 hours in culture, many (36/69 or 52%) fragmented within 2 hours. A proportion of the control eggs (9/57 or 16%) also fragmented within that time.

I.2) When the spindle-chromosome-complex-depleted eggs were exposed to activation conditions, i.e., ethanol-treated, a large proportion (12/14 or 86%) fragmented although not until 15 hours after ethanol exposure, at which time nearly all of the nucleated eggs subjected to ethanol treatment, were found to be in mitosis (see control group for III.1).

Figure 8.4 shows LSM images representative of non-activated mature eggs, collected 18 hours post hCG, mature eggs collected at 18 hours post hCG after 30 hours of culture, and non-activated enucleated eggs after 30 hours of culture. These eggs were characterized by the absence of a network of microtubules. Intact eggs were also characterized by more or less exclusive localization of tubulin in the meiotic spindle except for the presence of about 4-8 cytoplasmic asters (Figure 8.4A). After *in vitro* ageing, the spindle showed extensive arrays of astral microtubules pointing away from the

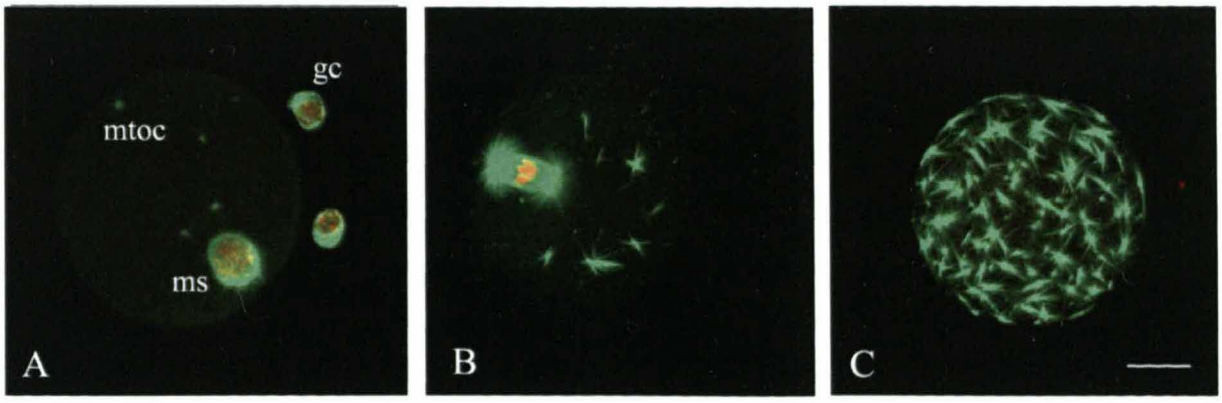


Figure 8.4 Representative LSM images of non-activated mature eggs, collected at 18 hours post hCG (A), and cultured for 30 hours (B), or enucleated and cultured for 30 hours (C), all in KSOM. The meiotic spindle (ms) is visible in A (front view) and in B; a few asters or microtubule organizing centers (mtoc) are visible in the freshly collected egg in A, but their complexity has increased with ageing (B and C). In C, without the spindle-chromosome-complex, many more aster-like microtubule bundles are visible after 30 hours of culture. Eggs have been stained for acetylated and beta tubulin (green) and DNA (red). The egg in C is negative for DNA. Images are projections reconstructed from several 3μm thick optical sections. Scale bar is 20μm. gc = granulosa cell

spindle poles and toward the cortex; the number of asters did not appear to increase in these eggs although they became more extensive in structure (Figure 8.4B). In the spindle-chromosome-complex-depleted eggs, the number of asters increased following 30 hours of culture, as did their size (Figure 8.4C).

8.4.2 Experiment II: Normally fertilized pronuclear eggs

Enucleated zygotes remained round with smooth membranes until 31 hours post hCG or approximately 18 hours post fertilization. By 33 hours post hCG (about 20 hours post fertilization), 29% (9/31) of the enucleated zygotes had fragmented or had ruffled membranes; two hours later, this proportion had increased to 68% (21/31).

At the same time points, i.e., 33 and 35 hours post hCG (20-22 hours post fertilization), respectively, 28% (7/25) and 44% (11/25) of the nucleated control zygotes had cleaved into two cells.

8.4.3 Experiment III: Ethanol-activated eggs

III.1) Activated enucleated eggs cultured in KSOM after enucleation remained round with smooth membranes for up to 13 hours PA while their nucleated counterparts were in interphase (single female pronucleus clearly visible). By 14 hours PA (32 hours post hCG), some cytoplasts showed cortical ruffling, and by 15 hours, 68% (50/73) were fragmenting completely; at this time, 45% (25/55) of the nucleated controls were in mitosis, that is, they had no visible nuclei.

By 24 hours PA, a small proportion of the previously fragmenting enucleated eggs had returned to a smooth spherical single-cell form; the others were still fragmented but the individual fragments had assumed a spherical or ellipsoidal form. Of the nucleated controls, 82% (45/55) had completed division and were in interphase. A pictorial summary of these results is presented in Figure 8.5. The status of the microtubules in intact and enucleated eggs at mitosis can be seen in Figure 8.6.

III.2a) None (0/72) of the activated enucleated eggs cultured in KSOM-colcemid fragmented. Likewise, nucleated control eggs did not divide or fragment in KSOM-colcemid (0/24). None exhibited pronuclei.

III.2b) None (0/30) of the activated enucleated eggs cultured in KSOM-CCB fragmented during culture. Likewise, nucleated control eggs did not divide or fragment in KSOM-CCB (0/25), although as expected, a high proportion exhibited two pronuclei.

III.3a) Following transfer of activated enucleated eggs to KSOM after 14-22 hours of culture in KSOM-colcemid, 40% (17/42) fragmented about two to six hours. Among the nucleated control eggs, 53% (8/15) divided during the same period. Division in these control groups was often accompanied by fragmentation.

III.3b) Transfer to KSOM following 14-22 hours of culture in KSOM-CCB led to fragmentation in 30% (9/30) of the enucleated eggs, again about two to six hours. When the intact controls were exposed to CCB for the same period then transferred to KSOM, 33% (5/15) divided to 2-cells within the same period. Division in the control group was often accompanied by fragmentation.

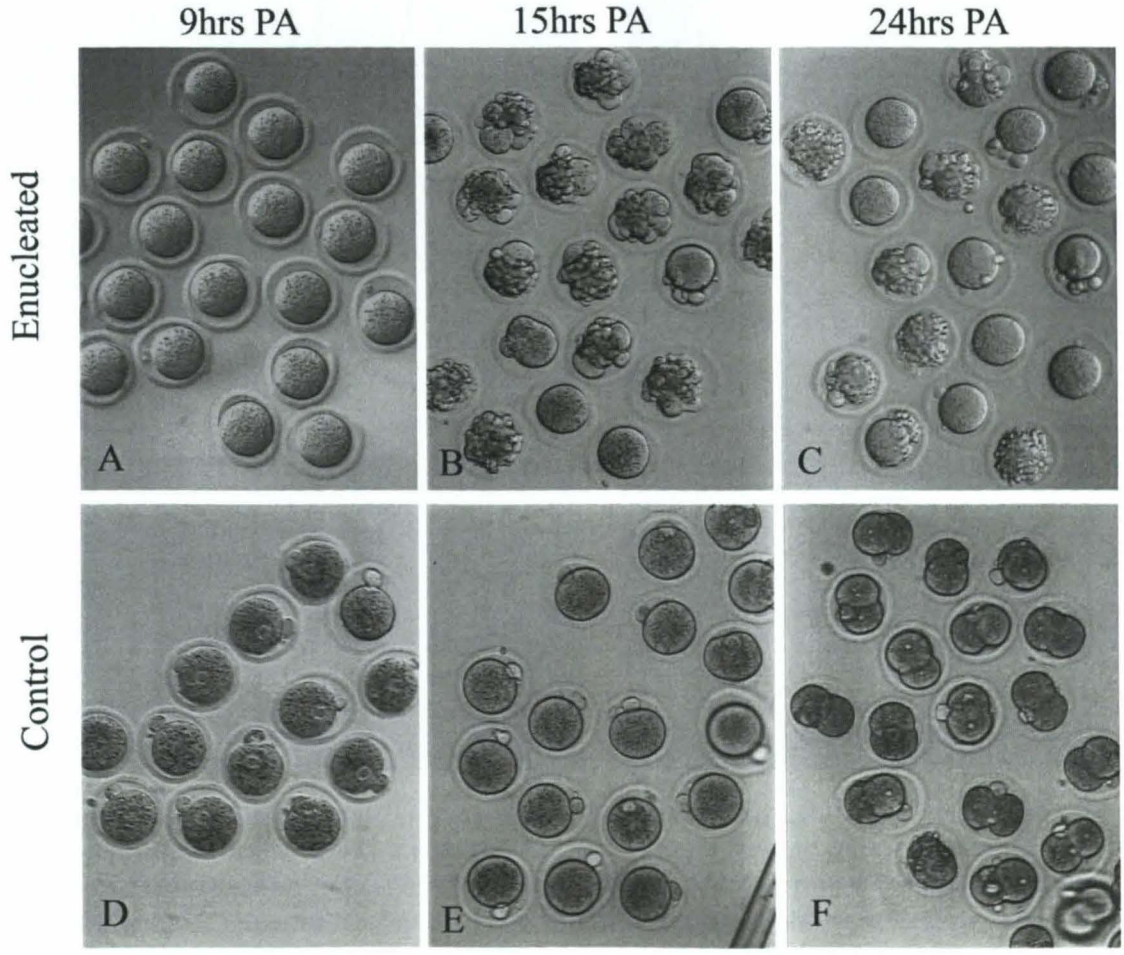


Figure 8.5 Representative DIC images of eggs in experiment III.1. Activated enucleated (A-C) and activated intact (D-F) eggs cultured in KSOM are shown at 9, 15, and 24 hours post activation (PA). Enucleated eggs remained round with smooth membranes at 9 hours PA (A). At 15 hours PA, many had fragmented (B) and at 24 hours PA, some of the previously fragmented cytoplasts had become ovoid again but in most, fragments remained in the PVS (C). At 9, 15, and 24 hours PA, respectively, the nucleated controls were in interphase (one female nucleus and one polar body visible)(D), mitosis (the pronucleus could not be seen) (E) and interphase of the second cell cycle (each of two cells show a prominent nucleus) (F).

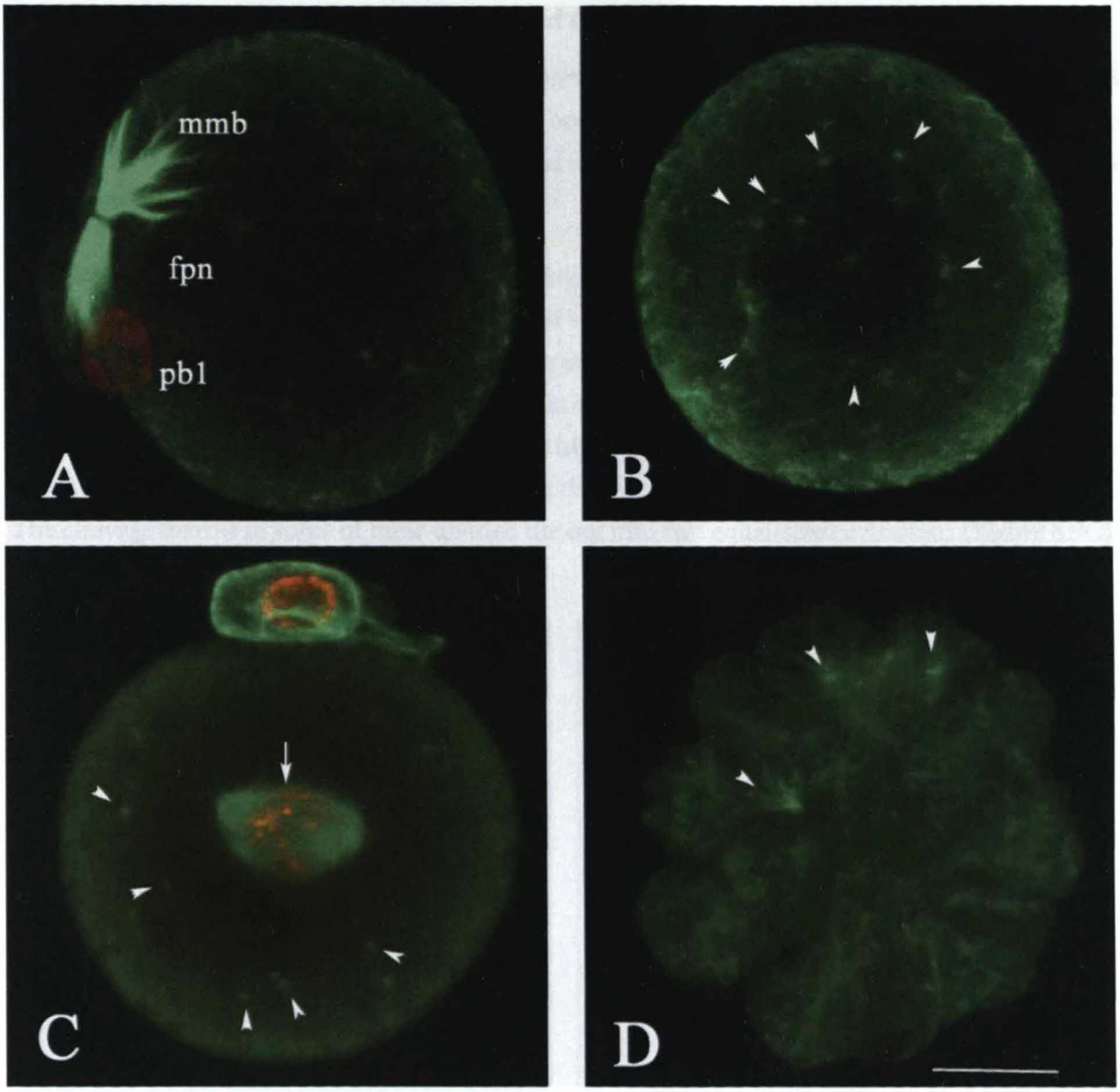


Figure 8.6 The status of microtubules in intact activated eggs and eggs from which the meiotic spindle-chromosome-complex was removed. At 5.5 hours post activation, the intact egg shows extrusion of the first polar body (pb1; A), evolution of the female pronucleus (fpn), and the meiotic midbody (mmb). The spindle-depleted egg, on the other hand, shows several microtubule organizing centers (arrowheads; B), organized around the center of the egg. At 14 hours post activation, in the intact egg, microtubules are exclusively invested in the first mitotic spindle, which rests in the center of the egg (C). Arrowheads in C point to microtubule organizing centers. At the same timepoint, the spindle-chromosome-complex-depleted egg is in the cytokinetic phase of the cell cycle and has undergone fragmentation (D). Arrowheads in D point to "midbody-like" microtubules. Eggs have been stained for acetylated and beta tubulin (green) and DNA (red). Images are projections reconstructed from several 3μm thick optical sections. Scale bar is 20μm.

8.4.4 Experiment IV: Activated eggs exposed to CCB

When emission of the second polar body was prevented by exposing intact activated eggs to CCB for 4-5 hours PA, two female pronuclei formed, both located peripherally in the cytoplasm at some distance from each other (Figure 8.7). A pictorial summary of the outcome of enucleation of these eggs is presented in Figures 8.8 and 8.9.

Four hours following removal of these two emerging female pronuclei, at 9 hours PA, a fragmentation event, morphologically distinct from that seen in previous experiments was noted in 53% (27/51) of the eggs: 20% (10/51) showed ruffling and blebbing of the membrane that culminated in the formation of one or several small fragments at or near the location where the second polar body would have formed (Figure 8.9A). In the remaining enucleated eggs, (17/51 or 33%), a cleavage furrow started to form, also in the region where the nuclei had been situated prior to enucleation. The cleavage furrow in some of these divided the egg into two equal-sized cytoplasts (Figure 8.9C).

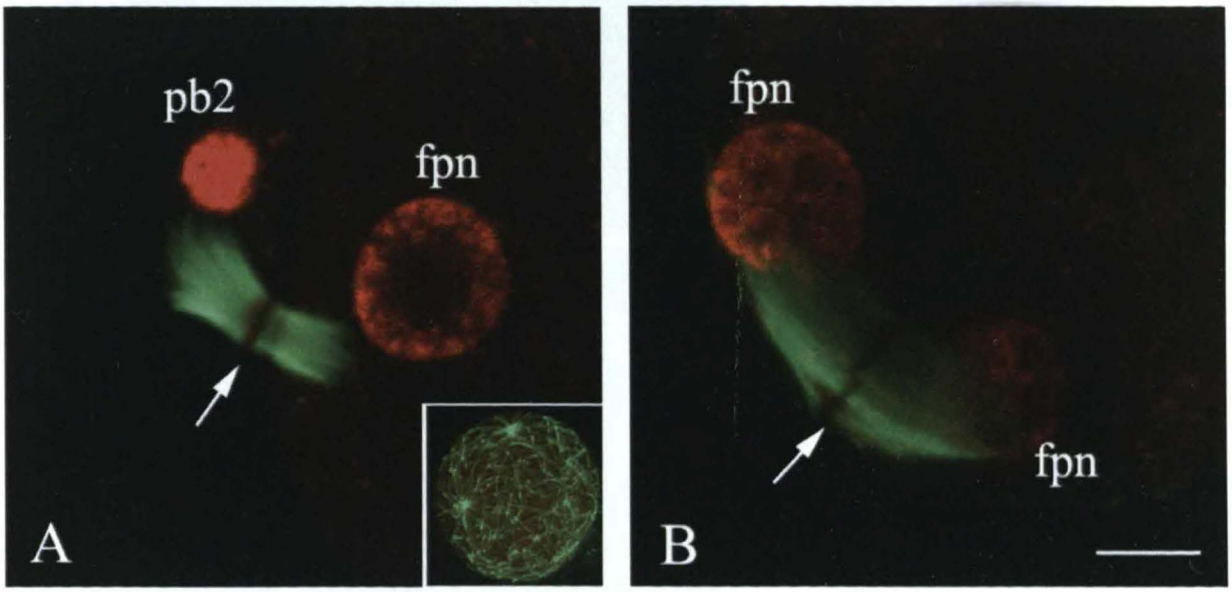


Figure 8.7 Representative LSM images of activated eggs following 5 hours of culture in KSOM (A) or KSOM-CCB (B), as part of experiment IV. The egg in A has extruded a polar body (pb2), and a female pronucleus (fpn) has formed. The meiotic midbody, in the center (arrow), is visible between the egg and the polar body. The inset shows detail of the extensive microtubule network and few microtubule organizing centers in the same egg. In the egg in B, two pronuclei, both female (fPN), have formed since the polar body is retained in the presence of CCB. The spindle remnant (arrow) is also visible between the two pronuclei. Eggs have been stained for acetylated and beta tubulin (green) and DNA (red). Images are projections reconstructed from several 3μm thick optical sections. Scale bar is 20μm.

At or about 16 hours PA (continuing up to 25 hours PA), a second and universal fragmentation wave occurred in 43/51 or 84% of the eggs and led to complete fragmentation of the cytoplasts (Figure 8.9D).

Among the nucleated control eggs, 13 of the 15 showed cortical ruffling and/or formation of "polar bodies" within 9 hours of activation (Figure 8.9B). At about 16 hours PA, coinciding with complete fragmentation of the enucleated eggs, the majority of these intact eggs had entered mitosis and some had already divided; by 25 hours PA, all had cleaved into two-cells.

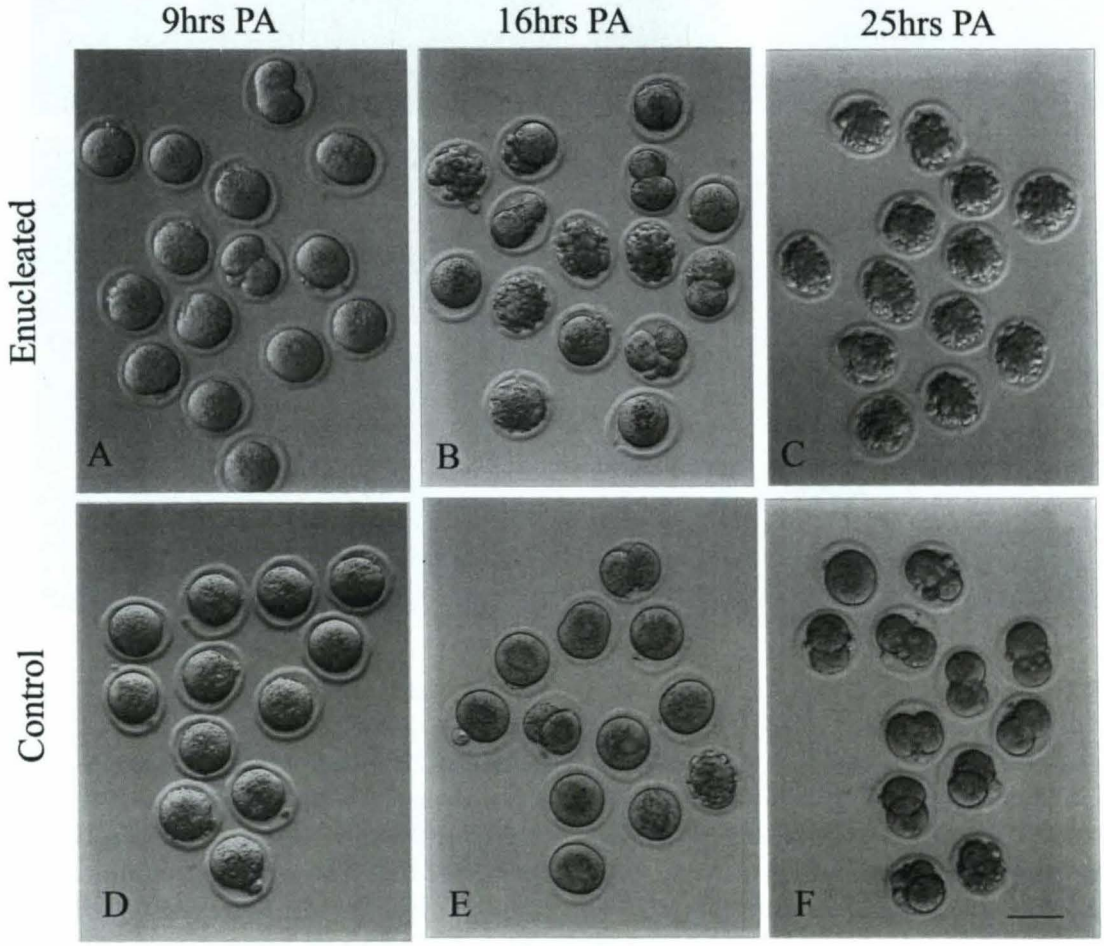


Figure 8.8 Activated enucleated (A-C) and activated intact (D-F) eggs that were maintained in KSOM-CCB for 4-5 hours immediately post activation (PA) then transferred to KSOM. Nine hours post activation, cytoplasts showed cortical ruffling and either produced one or more polar cytoplasts or "cleaved" more or less evenly (A). Beginning at sixteen hours PA and up to 25 hours PA, these enucleated eggs showed more extensive fragmentation affecting the entire egg (B, C). At the same time-points, respectively, nucleated control eggs showed, blebbing and formation of one or more "polar bodies" (D) and entry into mitosis or cleavage (E, F). Scale bar is 80μm.

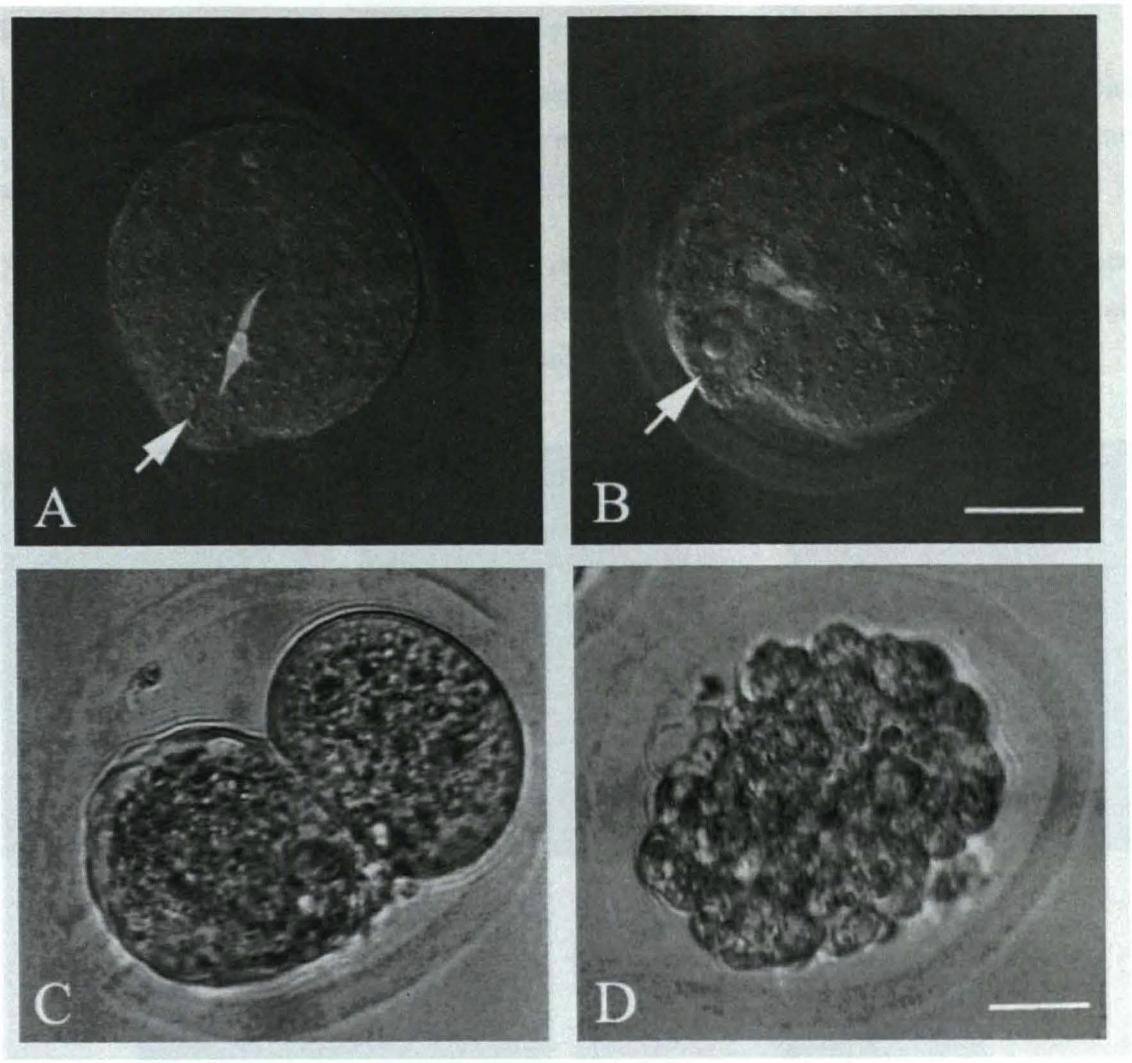


Figure 8.9 Representative LSM (A and B) and DIC (C and D) images of eggs in experiment IV in which activated eggs were maintained in KSOM-CCB for 4-5 hours immediately post activation (PA), were either enucleated or not, washed, and transferred to KSOM. In some enucleated eggs, the early fragmentation wave culminated in the formation of a polar body-like fragment or bleb (A, arrow). The meiotic spindle remnant is visible in this enucleated egg, indicating that removal of the nuclei did not guarantee removal of the meiotic spindle remnant. In B, the arrow points to a similar polar body-like structure in the nucleated control egg; the second female pronucleus is present but not visible in this optical section. In other enucleated eggs, the first wave of fragmentation at 9 hours PA led to a more or less even "cleavage" (C). By 16 hours PA, both anucleate fragments in the egg in C fragmented completely (D). Eggs in A and B represent a single optical section midway through the egg; the eggs have been stained for acetylated and beta tubulin (shown in grey-scale). Scale bar is 20μm.

8.4.5 Experiment V: Fertilized 2-cell embryos

V.1a) Among the 28 embryos in which only one blastomere was enucleated in early-mid interphase, none (0/28) showed any fragmentation after overnight culture, while their intact sister blastomeres cleaved normally during that period (Figure 8.10).

V.1b) A small proportion (6/83 or 7.2%) of the embryos in which both blastomeres were enucleated in early-mid interphase showed some membrane deformation when checked after overnight culture, but the great majority remained unruffled and ovoid throughout culture.

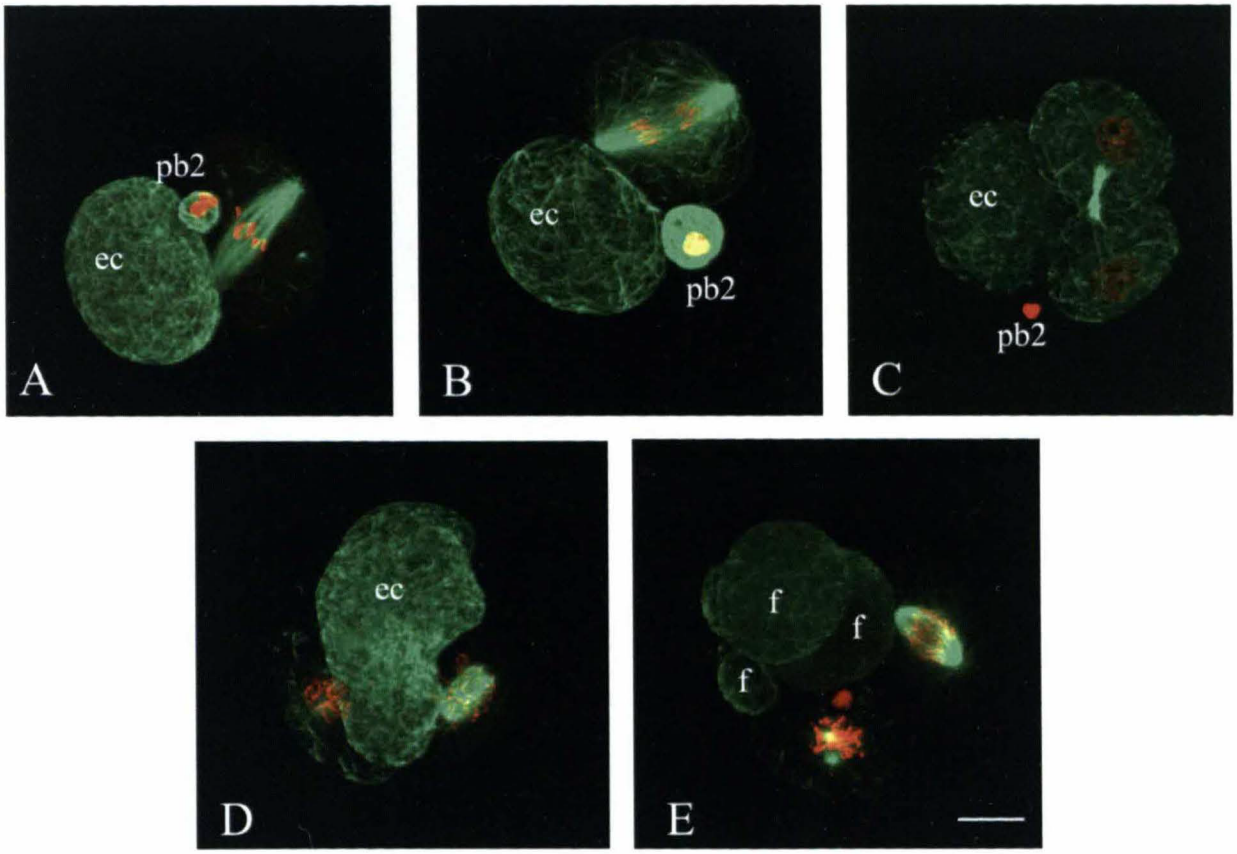


Figure 8.10 Representative LSM images of 2-cell embryos from experiment V in which one of the two cells was enucleated early (A-C) or late (D-E) in interphase. During subsequent culture, the enucleated cells (ec) in A, B, and C are unfragmented and show interphase microtubules. The nucleated cells in A and B are in metaphase and anaphase, of the second cell cycle, respectively. In C, the nucleated cell has divided and the resulting blastomeres are in interphase. The enucleated cell in D shows cortical deformations and that in E has fragmented; three fragments (f) are visible. The nucleated cells in these embryos are in prophase through anaphase of the third cell cycle. Embryos have been stained for acetylated and beta tubulin (green) and DNA (red). The enucleated cells are negative for DNA. Images are projections reconstructed from several 3μm thick optical sections. Scale bar is 20μm.

V.2a) Among the 25 embryos in which one late interphase blastomere was enucleated, 68% (17/25) were found to have fragmented after overnight culture, while the intact sister blastomeres cleaved normally during that period.

V.2b) Among the 75 embryos in which both blastomeres were enucleated in late interphase, 74.7% (56/75) were found to be fragmented after overnight culture (Figure 8.11). Rounding of the fragmented enucleated cells occurred within the following 24-48 hours of culture, but some fragments remained in the PVS in most instances.

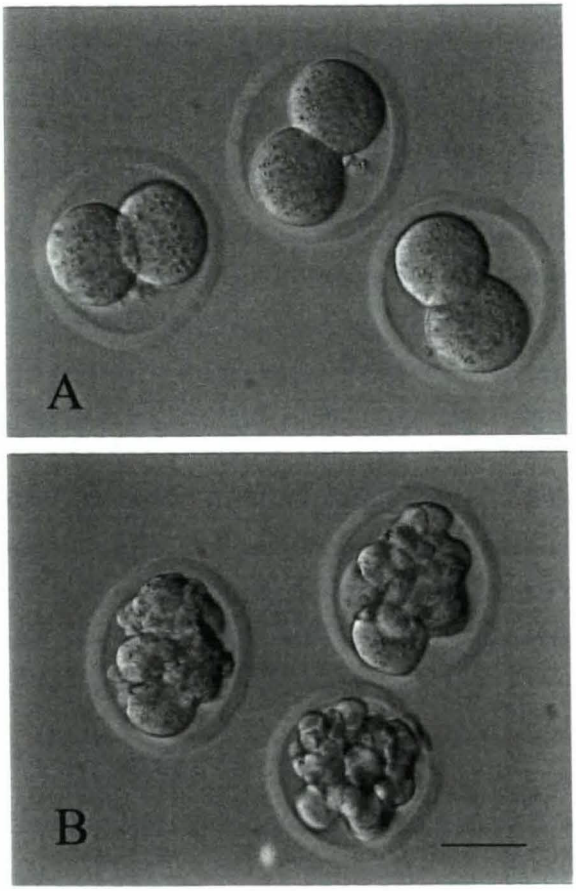


Figure 8.11 Representative DIC images of 2-cell embryos from experiment V. Both blastomeres were enucleated in early-mid interphase (A) or later (B) and cultured overnight. Blastomeres in (A) remained unfragmented after enucleation while blastomeres in (B) fragmented. Scale bar is approximately 50μm.

8.5 Discussion

In the present experiments, cytoplasmic fragmentation did not proceed unless the cells were in division mode, suggesting a general rule that fragmentation does not occur in mitotically inactive cells and therefore is not a predetermined fate of arrested or quiescent cells. The study further suggests that fragmentation in eggs and embryos occurs during the cytokinetic phase of the cell cycle, in response to the loss of normal interplay between the spindle complex and cortical microfilaments.

8.5.1 Timing of cytoplasmic fragmentation

Whether enucleation immediately followed or preceded artificial activation, the resulting fragmentation in the cytoplasts was a precisely timed event in the cell cycle, rather than a happenstance, and always coincided with entry of nucleated control cells into mitosis. In the cytoplasts, this timing represents the cytokinetic rather than the mitotic phase of the cell cycle, since the removal of the nuclear structure removes the necessity for coordination of nuclear and cytoplasmic divisions (and any delay normally imposed by the former on the latter).

When the nucleated control cells had completed cytokinesis and entered interphase, some blebs and fragments receded in the cytoplasts, suggesting further autonomous cell-cycling activity in the latter.

8.5.2 Fragmentation and the cytoskeleton

The dynamic relationship between spindle microtubules and cortical microfilaments is pivotal to normal cytokinesis. Therefore, deliberate depolymerization of microfilaments (by CCB treatment) or depolymerization of microtubules (by colcemid treatment) prevents cytokinesis altogether; here, the same treatments also prevented fragmentation. Drug removal, on the other hand, restored both microtubules and microfilaments, allowing cytokinesis and fragmentation to proceed.

It is noteworthy that both following extended exposure to cytoskeletal disruptive drugs, and delayed activation, limited to complete fragmentation was seen in control embryos. This behavior, which is generally uncharacteristic of normal mouse development suggests that once induced, cytoskeletal disorder may persist and become overtly expressed in the course of a subsequent cytokinesis, even in the presence of a nuclear apparatus.

8.5.3 Meiotic fragmentation

If our hypothesis that fragmentation is a deviant form of cytokinesis is correct, it should be possible to demonstrate that the phenomenon can also occur during cytokinesis of the second meiosis. We think that this was demonstrated in experiment IV, for in that experiment two distinct waves of fragmentation occurred. The first wave was limited to the region where the nuclei had been situated prior to enucleation and occurred several hours before mitosis 1 in nucleated controls. The second wave was universal and coincided with mitosis 1 in nucleated controls, similar to the events described in experiments I-III. In our opinion, the first of these waves represented cytokinesis of second meiosis, while the second represented cytokinesis of the first mitotic division.

We have further investigated meiotic fragmentation in more detail, and the results will form the subject of a separate report (Willadsen and Alikani, in preparation).

8.5.4 Fragmentation during second mitosis

We also investigated the possibility of inducing fragmentation during cytokinesis of the second cell cycle. In her experiments, Ciemerych (1995) did not observe cortical deformations or any other autonomous activity in the anucleate halves of blastomeres from 2- and 4-cell embryos. This lack of activity was attributed to diminished cytoplasmic autonomy and the low intensity of mitosis promoting factor (MPF) activation during the second (and subsequent) cell cycle(s) (Kubiak and Ciemerych, 2001).

In experiment V of the present study, some enucleated blastomeres of fertilized 2-cell embryos did fragment. However, the timing of enucleation was crucial to this outcome: if enucleation was done during early-mid interphase, further activity ceased in the anucleate blastomere; no surface deformations were seen and the microtubules maintained their interphase configuration. However, when enucleation was delayed until late interphase, a large proportion of the enucleated cells either showed cortical deformations or fragmented during subsequent culture.

We propose that the failure of the enucleated early blastomeres to undergo fragmentation is reminiscent of the "2-cell block" in the mouse (Goddard and Pratt, 1983) and supports the notion of fragmentation as atypical cytokinesis. The "2-cell-block" takes effect at the late G2/M phase of the second cell cycle when maternal transcripts run out and the zygotic genome must be activated for normal development to continue (Flach et al., 1982).

8.5.5 Relevance to human embryo fragmentation

We have assumed that the process of fragmentation in eggs and embryos is essentially the same whatever the triggering cause(s), i.e., that fragmentation is the diverse expression of a single phenomenon: the failure of the normal dynamic relationship between microtubules and microfilaments during one particular phase of the cell division cycle, i.e., cytokinesis. Our results support that assumption. However, we cannot yet be certain that the assumption has more general validity, and for this reason, we can only speculate what the relevance of the results of the present study might be to fragmentation and related phenomena in human eggs and embryos, not to mention other cell types.

In addition, the topography and organization of the cytoskeleton differ between species (Simerly et al., 1995). For instance, the sperm centrosome is thought to play an essential role as the microtubule organizing center in the human (Sathananthan et al., 1991; Palermo et al., 1994; Van Blerkom, 1996) but not in the mouse (Maro et al., 1985; Schatten et al., 1985).

In the human, the genome becomes activated sometime between the 4- and 8-cell stages (Braude et al., 1988) but most fragmentation occurs prior to that. Indeed, it appears that around the time of genomic activation, and particularly after compaction and the establishment of structural junctions (Nikas et al., 1996; Gualtieri et al., 1992), the tendency of human blastomeres to fragment is simply lost. Whether the impetus for this change is the activation of the genome, increased adhesion between the cells, reduced cell size or a combination of these factors remains to be determined. Nevertheless, the basic suggestion of the present experiments is that fragmentation in human embryos is a manifestation of abnormal cytokinesis rather than cell death. This proposal would support a causal relationship between cytoplasmic fragmentation and cytoskeletal changes associated with post-ovulatory ageing (Pickering et al., 1988) or environmental fluctuations during *in vitro* culture (Almeida and Bolton, 1995; Dale et al., 1998). It would also provide a plausible explanation for the high frequency of fragmentation and its association with nuclear abnormalities, most importantly, post zygotic chromosome mosaicism among human IVF embryos.

Monash University

Declaration for Thesis Chapter 9

In the case of Chapter 9, contributions to the work involved the following:

Name	% contribution	Nature of contribution
1. Mina Alikani	100	Writing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) there are no other authors of the publication according to these criteria;

(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Tyho-Galileo Research Laboratories, West Orange, NJ, USA

		Date
Signature 1	[redacted]	24 December, 2015

Chapter 9

General discussion, reflections, and conclusions

9.1 General discussion

A recent survey of the EggCyte database shows that about forty percent of some 75000 human embryos whose development was initiated in the Saint Barnabas IVF laboratory had lost one quarter or more of their cytoplasmic volume to fragmentation. A large proportion of the embryos affected by such extensive fragmentation showed one or more other abnormalities, including blastomere multi-nucleation, uneven cell division, developmental arrest, and chaotic chromosome mosaicism. The work described in the previous chapters was undertaken to uncover causes and consequences of cytoplasmic fragmentation and explain its association with other frequently observed embryo abnormalities.

This final chapter attempts a critical evaluation of each experimental and analytical approach that was chosen to fulfill the aim of the studies.

At the time these studies were initiated, it was already well known that fragmentation was, in general, associated with loss of embryo viability, but this knowledge was inadequate because little if any distinction was drawn among fragmented embryos, and the process itself was simply presumed to be degenerative in nature.

The first steps toward understanding the pathogenesis of fragmentation were the attempts to better define it and to delineate its implications for pregnancy and implantation. In Chapter 3, a fragmentation classification system was introduced. This was developed through non-invasive morphological evaluation of large numbers of embryos and was based on fragmentation patterns: the size and distribution of fragments relative to the size and position of nucleated cells. These patterns were shown to be of predictive value for clinical outcome and therefore useful for selection of embryos for transfer. Moreover, they provided support for the idea that fragmentation was unlikely to represent the end-point of a degenerative process for the embryo as a whole.

Notwithstanding its clinical merit, however, this classification system has limitations and, as it has become clear now, weaknesses. One obvious limitation is the relativity of its predictive property, a problem associated with human embryo viability assessment schemes in general. The main weakness is its disregard for the timing of fragmentation. To provide more insight into the origin of the different fragment types, a revised version of the classification system must link the timing of fragmentation to its developmental consequence.

Early cleavage stage classification systems are relevant if embryos were to be cultured for 2-3 days and replaced in the uterus. However, with the emergence of improved media that allow extension of the culture period, considerable interest in using blastulation as the single criterion for embryo selection also emerged, making the study of early cleavage stages more or less obsolete. The main advantage of embryo selection after extended culture is obvious: it is much easier to decide on day-5 whether an embryo is a blastocyst than it is to predict on day-3 whether an embryo can, or will, develop into a blastocyst.

In Chapter 4, the feasibility of maintaing embryos with one or more abnormalities in culture for extended periods was evaluated. The impact of fragmentation and other frequent cleavage abnormalities on blastulation after culture to day-5 of development was investigated by analysis of morphological data obtained during the course of development of embryos. These analyses showed that the developmental consequences of abnormal cleavage were already noticeable on the fourth day of development. Compaction either failed completely or was abnormal in such embryos leading to, on the following day, the failure of both cavitation and normal blastulation. In those embryos that did blastulate, abnormalities abounded: absence or disorganization of the inner cell mass, large and disorganized trophectoderm cells, excluded fragments or cells in the blastocoelic cavity or the PVS, and low overall cell numbers.

Thus extended culture of abnormally cleaving embryos of marginal viability under the conditions used in the study led to further loss of developmental potential even to the point of making the embryo in question non-viable. Clearly this outcome was partly a product of the specific culture conditions employed in this particular study; however, it is doubtful whether any *in vitro* culture conditions available at present can support the development of human embryos with abnormalities in the first three days in culture as well as the uterine environment often does.

In mammals, compaction of the embryo normally takes place after a certain number of cleavage divisions which varies between species. In the human, compaction normally takes place after the embryo has reached the 16 cell stage, while in the mouse it begins at the late 8-cell stage. The inside-outside hypothesis of Tarkowski (Tarkowski and Wroblewska, 1967) predicts that in the

course of compaction, at least one cell must become positioned in such a way that it is completely surrounded by other cells in order for an inner cell mass to form. If the total number of interacting cells is reduced, the number of inside cells and hence the number of cells in the inner cell mass of the resulting blastocyst are disproportionately reduced. Simple geometrical considerations suggest that for any cell to be "inside", the critical total number is about eight interacting cells in the embryo at the time of blastulation, although experimental evidence suggests that this may be a slight overestimate.

The observations of Chapter 4 are consistent with the general principles outlined above especially when it is taken into account that in many of these embryos asynchrony between blastomeres occurred frequently due to disturbances in early cleavage divisions. The observations were thus of considerable clinical interest in that they provided the basis for an argument against the indiscriminate use of blastocyst culture and transfer.

The analyses that formed the basis of Chapters 3 and 4 did not reveal how fragmentation or its associated anomalies interfere with development over time, either *in vivo* or *in vitro*. This question was taken up in the experiments described in Chapters 5 and 6.

Two approaches were used to address the issue. The first examined the potential effects of the fragments on the viable cells in a mouse model, and the second sought to evaluate the developmental potential of nucleated cells of fragmented human embryos cultured apart from their associated fragments.

Chapter 5 describes the experiments in which the first approach was used: Single blastomeres isolated from 2-cell mouse embryos were placed in host zonae pellucidae along with homologous (mouse) and heterologous (human) cell fragments and the micromanipulated embryos were cultured *in vitro*. For the most part, the development of the mouse blastomeres was unaffected by the presence of fragments, whether homologous or heterologous.

The main conclusion drawn from the study was that development failure in fragmented human embryos is unlikely to result from any negative influence of the fragments *per se*. Rather, the significance of the presence of fragments is that they indicate other disturbances within the embryo proper at a systemic level.

One problem that was encountered during these experiments was standardization of experimental parameters and conditions. Were these experiments to be repeated, the technical approach would have to be refined. The size of the hole in the zona pellucida and the number and type of inserted fragments would have to be better controlled. Moreover, the zona pellucida might have to be sealed

to avoid expulsion of the inserted fragments, which occurred frequently during culture of the micromanipulated embryos.

The second approach to investigating the possible role of fragments in the disruption of development is described in Chapter 6. The developmental capacity of surviving cells of fragmented embryos was evaluated after the cells were separated from the fragments. This was done in two ways. Disaggregation of fragmented human embryos and individual culture of the component cells led to mitotic arrest in some cases, but other cells divided and even attempted to blastulate, albeit with too few cells for a proper blastocyst to form. On the other hand, aggregation of mono-nucleated cells from several disaggregated fragmenting embryos, in some cases, led to formation of a chimaeric embryo that underwent compaction and blastulation. The chimaeric blastocysts were on the whole smaller than normal human blastocysts. However, this was explained partly by the fact that the aggregated cells rarely amounted to the full cell complement of a normal embryo, and partly by the fact that one or more of the cells originally aggregated was often found to have been excluded from the embryo proper.

Because cell sizes vary widely in most fragmented embryos due both to the uneven loss of cytoplasm and asynchrony in cleavage between cells within the same embryo, the precise developmental stage of the blastomeres used in the aggregation experiments was not ascertained. This problem could potentially be circumvented by measuring cell and nuclear diameters and comparing these with normal blastomeres from non-fragmented embryos. It was also not possible to know what the proportions of cytogenetically abnormal cells in the aggregates were. Examination (by fluorescence *in situ* hybridization) of some of the blastocysts that developed showed that they contained many normal cells as well as aneuploid cells, but at the same time, only abnormal cells were found to have been eventually excluded from the compacted aggregates. The role of chromosomal integrity of the blastomeres in the success of the aggregation therefore remains to be clarified. In the same context, it remains to be determined whether cells originating in abnormally fertilized eggs can be used as supportive cells within the aggregates.

What this work did demonstrate was the occurrence of viable cells with apparently normal regulatory capacity within non-viable embryos, thus emphasizing the importance of distinguishing between embryo viability and cell viability. Of course, the work also demonstrated the feasibility of producing chimaeric human embryos, which although not in itself surprising in view of the experience with chimaeric embryos in other species, is nonetheless important. Such embryos could play a useful role in investigations of cell allocation during preimplantation development in human embryos, and might also serve as a source of embryonic stem cells from clinically unusable embryos.

In the chapters discussed so far, focus was placed on morphological aspects of fragmentation and the consequences of fragments for development, but the nature and process of fragmentation and its underlying abnormalities were not addressed. With the study described in Chapter 7, a step was taken in this direction. The impetus for the study was provided by the observation that compaction and blastulation are often grossly disturbed in fragmented embryos.

In all other mammalian species studied so far, E-cadherin, a vital cell adhesion protein, is actively relocated in the course of embryogenesis. The first relocation incidence occurs at the time of compaction and concerns the cells that form the outer layer of the embryo. In these cells, E-cadherin is redistributed from free cell surfaces to areas of contact between cells. This relocation is necessary for the formation of junctional complexes between the trophectodermal cells upon which the integrity of the blastocyst depends. However, previous studies of E-cadherin distribution in human embryos had yielded equivocal results. The work described in Chapter 7 was undertaken in an attempt to resolve this discrepancy and to examine the distribution pattern of E-cadherin in fragmenting and abnormally cleaving human embryos.

Immuno-cytochemistry combined with confocal microscopy was used to detect the localization of E-cadherin in cleavage stage embryos and blastocysts. The results indicated that the occurrence and distribution pattern of E-cadherin in human embryos is similar to that described in other mammalian species. The results also suggested that in abnormally cleaving human embryos, the characteristic distribution pattern of E-cadherin is perturbed and erratic. Considering the association of these abnormalities with failed compaction (including compaction of a proportion of the cells and exclusion of other cells), it was logical to conclude that disturbances in the distribution of E-cadherin in abnormally cleaving embryos lead to failure of compaction which in turn leads to failed or abortive blastulation. The presence in the same embryo of non-interacting cells and fragments and interactive cells may contribute to the problem by disrupting cell signaling processes. At a more fundamental level, as experimental evidence in the mouse suggests, these disturbances may be caused by failed genomic activation.

When one considers the prevalence of fragmentation in human embryos produced *in vitro*, and the negative impact of fragmentation and its associated abnormalities on the outcome of IVF/ART, it is surprising that so relatively little research effort has been directed towards discovering the nature and the causes of the phenomenon. There can be little doubt that the main reasons are the practical difficulties and obstacles facing the use of human eggs and embryos for experimentation, as well as the relative unimportance of fragmentation in experimental mouse embryology. As a result, fragmentation has been much talked about, but mostly misunderstood, and rarely seriously investigated. Even the terminology has served to obscure the heart of the matter. Which is it: a frag-

mented or a fragmenting embryo? This simple question proved to be a good one, when at a certain point it was decided to change the emphasis of the work from description of effects to aggressive investigation of causes and mechanism.

When does fragmentation occur?

It had been noticed in the course of cell fusion experiments that enucleated activated mouse eggs would fragment if left in culture overnight, whereas this was rarely the case with non-activated enucleated eggs. Because of the regularity with which this occurred and the relative ease of producing enucleated eggs and activating them, mouse eggs were chosen as the primary material for the fragmentation experiments that followed. Later, early cleavage stage embryos were also included. These experiments are described in Chapter 8. The meiotic spindle or the interphase nuclear apparatus was partially or completely removed from mature oocytes and early cleavage stage blastomeres. The micromanipulated cells were cultured *in vitro* and their response to the manipulations was examined.

The most important insight into the nature and dynamics of fragmentation was gained when its timing in relation to the cell cycle was investigated. The precise timing of meiosis and early mitotic events in the mouse and the ease with which the major stages are identified and manipulated are great advantages of this experimental system.

In meiotic cells, the timing of fragmentation coincided with second polar body extrusion, and in mitotic cells, it coincided with mitosis and cell division. Therefore, far from being random, fragmentation occurred only during the M phase of the cell cycle. By taking into consideration the observation that the non-activated mature eggs neither divide nor fragment and that such eggs are arrested in metaphase, it was also possible to point to cytokinesis as the phase during which fragmentation occurs. Indeed, it seemed reasonable to hypothesize that fragmentation in its various manifestations represents different forms of uncoordinated cytokinesis. This would account for the numerous chromosomal and nuclear abnormalities that accompany fragmentation. The hypothesis was supported by the study of the cyclical reorganizations of the microtubular components of the cytoskeleton that occur in synchrony with the cell cycle in nucleated and enucleated eggs and blastomeres.

At present, it is still unknown to which extent the results of experiments with mouse eggs and blastomeres apply to their human counterparts. Human eggs and blastomereres are larger and differently configured with respect to microtubules and other cytoskeletal components. Therefore experiments with human eggs and blastomeres will be necessary to detect and reconcile possible

differences. But it must be kept in mind that while the starting material used in the mouse experiments could be assumed to have been normal, such an assumption can not be made with equal confidence when human material is used.

It seems reasonable to predict (as preliminary work suggests) that such differences will turn out to be in detail rather than principle. Regardless of the validity of this prediction, by firmly establishing a link between fragmentation and cell division, both nuclear and cytoplasmic, and by the choice of a relatively well definable yet versatile model, the work has definitively opened an important area for future research. The most obvious objective for this research is to determine factors that define and maintain cytoskeletal integrity in human eggs. The case for such research is compelling, since in its absence human beings rather than human cells will be the experimental subjects – as they have been hitherto in assisted human reproduction.

9.2 Conclusions

A systematic approach to understanding cytoplasmic fragmentation, a frequently observed feature of human early cleavage stages *in vitro*, has shown that its simple dismissal as a degenerative process is vastly unjustified. Basic experimental embryology in the mouse has revealed the key to the nature of fragmentation: it is a dynamic process reminiscent of cytokinesis in relation to its requirement for activation, timing in the cell cycle, and its mediation by reorganization of the cytoskeleton. Thus, fragmentation *per se* is not an abnormality, but its occurrence does indicate an underlying problem; its consequence for the cell could be death and for the embryo, depending on the extent, reduced or lost viability. Strategies for prevention of fragmentation and early loss of embryos in culture can now be developed based on this discovery.

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Bibliography

Abbott AL, Xu Z, Kopf GS, Ducibella T, Schultz RM (1998) *In vitro* culture retards spontaneous activation of cell cycle progression and cortical granule exocytosis that normally occur in *in vivo* unfertilized mouse eggs. *Biology of Reproduction* 59, 1515-1521.

Almeida PA and Bolton VN (1995) The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote* 3, 357-365.

Allworth AE and Albertini DF (1993) Meiotic maturation in cultured bovine oocytes is accompanied by remodeling of the cumulus cell cytoskeleton. *Developmental Biology* 158, 101-112.

Alikani M, Olivennes F, Cohen J (1993) Microsurgical correction of partially degenerate mouse embryos promotes hatching and restores their viability. *Human Reproduction* 8, 1723-1728.

Alikani M, Palermo G, Bertoli M, Blake M, Cohen J (1995) Intracytoplasmic sperm injection in dysmorphic human oocytes. *Zygote* 3, 283-288.

Alikani M (2001) Cytoplasmic fragmentation in human embryos *in vitro*: implications and the relevance of fragment removal. In Gardner D, Weissman A, Howles C, Shoham Z (eds) *Textbook of Assisted Reproductive Techniques, Laboratory and Clinical Perspectives.* Martin Dunitz, London, United Kingdom, pp. 169-182.

Alikani M, Cekleniak NA, Walters E, Cohen J (2003) Monozygotic twinning following assisted conception: an analysis of 81 consecutive cases. *Human Reproduction* 18, 1937-1943.

Antczak M and Van Blerkom J (1999) Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Human Reproduction* 14, 429-447.

Austin CR (1951) Observations on the penetration of the sperm in the mammalian egg. *Australian Journal of Scientific Research* 4, 581-596.

Austin CR (1952) The capacitation of the mammalian sperm. *Nature* 23, 170:326.

Austin CR (1961) Fertilization of mammalian eggs *in vitro*. *International Reviews in Cytology* 12, 337-359.

Balakier H and Cadesky K (1997) The frequency and developmental capability of human embryos containing multinucleated blastomeres. *Human Reproduction* 12, 800-804.

Barcroft LC, Hay-Schmidt A, Caveney A, Gilfoyle E, Overstrom EW, Hyttel P, Watson AJ (1998) Trophectoderm differentiation in the bovine embryo: characterization of a polarized epithelium. *Journal of Reproduction and Fertility* 114, 327-339.

Batta SX, Stark RA, Brackett BG (1978) Ovulation induction by gonadotropin and prostaglandin treatments of rhesus monkeys and observations of the ova. *Biology of Reproduction* 18, 264-278.

Bavister BD (1969) Environmental factors important for *in vitro* fertilization in the hamster. *Journal of Reproduction and Fertility* 18, 544-545.

Bavister BD, Edwards RG, Steptoe, PC (1969) Identification of the midpiece and tail of the spermatozoon during fertilization of human eggs *in vitro*. *Journal of Reproduction and Fertility* 20, 159-160.

Becker DL, Leclerc-David C, Warner A (1992) The relationship of gap junctions and compaction in the preimplantation mouse embryo. *Development Supplement*, 113-118.

Bedford JM and Chang MC (1962) Fertilization of rabbit ova *in vitro*. *Nature* 193, 808-809.

Begg DA and Rebhun LI (1979) PH regulates the polymerization of actin in the sea urchin egg cortex. *Journal of Cell Biology* 83, 241-248.

Behr B, Pool TB, Milki AA, Moore D, Gebhardt J, Dasig D (1999) Preliminary clinical experience with human blastocyst development *in vitro* without co-culture. *Human Reproduction* 14, 454-457.

Bertrand E, Van den Bergh M, Englert Y (1996) Clinical parameters influencing human zona pellucida thickness. *Fertility and Sterility* 66, 408-411.

Berx G, Staes K, van Hengel J, Molemans F, Bussemakers MJ, van Bokhoven A, van Roy F (1995) Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1). *Genomics* 26, 281-289.

Biggers JD, McGinnis LK, Raffin M (2000) Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biology of Reproduction* 63, 281-293.

Bloor DJ, Metcalfe AD, Rutherford A, Brison DR, Kimber SJ (2002) Expression of cell adhesion molecules during human preimplantation embryo development. *Molecular Human Reproduction* 8, 237-245.

Boediono A, Suzuki T, Li LY, Godke RA (1999) Offspring born from chimeras reconstructed from parthenogenetic and *in vitro* fertilized bovine embryos. *Molecular Reproduction and Development* 53, 159-170.

Bogner K, Hinsch KD, Nayudu P, Konrad L, Cassara C, Hinsch E (2004) Localization and synthesis of zona pellucida proteins in the marmoset monkey (Callithrix jacchus) ovary. *Molecular Human Reproduction* 10, 481-488.

Bolton VN Wren ME Parsons JH (1991) Pregnancies after in-vitro fertilization and transfer of human blastocysts. *Fertility and Sterility* 55, 830-832.

Bos-Mikich A, Mattos AL, Ferrari AN (2001) Early cleavage of human embryos: an effective method for predicting successful IVF/ICSI outcome. *Human Reproduction* 16, 2658-2661.

Braude P, Bolton V, Moore S (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332, 459-461.

Bryant DM and Stow JL (2004) The ins and outs of E-cadherin trafficking. *Trends in Cell Biology* 14, 427-434.

Buster JE, Bustillo M, Rodi IA, Cohen SW, Hamilton M, Simon JA, Thorneycroft IH, Marshall JR (1985) Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage. *American Journal of Obstetrics and Gynecology* 15, 211-217.

Cao W, Brenner CA, Alikani M, Cohen J, Warner CM (1999) Search for a human homologue of the mouse Ped gene. *Molecular Human Reproduction* 5, 541-547.

Chang MC (1951) Fertilizing capacity of spermatozoa deposited into the Fallopian tubes. *Nature* 168, 697-698.

Chang MC (1959) Fertilization of rabbit ova *in vitro*. *Nature* 184, 466-467.

Chang MC (1968) *In vitro* fertilization of mammalian eggs. *Journal of Animal Science* 27, 15-26.

Chatot CL, Ziomek CA, Bavister BD, Torres I (1989) An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* 86, 679-688.

Chatot CL, Lewis JL, Torres I, Ziomek CA (1990) Development of 1-cell embryos from different strains of mice in CZB medium. *Biology of Reproduction* 42, 432-440.

Ciemerych MA (1995) Chromatin condensation activity and cortical activity during the first three cell cycles of a mouse embryo. *Molecular Reproduction and Development* 41, 416-424.

Ciemerych MA, Tarkowski AK, Kubiak JZ (1998) Autonomous activation of histone H1 kinase, cortical activity and microtubule organization in one- and two-cell mouse embryos. *Biology of the Cell* 90, 557-564.

Clayton L, Stinchcombe SV, Johnson MH (1993) Cell surface localisation and stability of uvomorulin during early mouse development. *Zygote* 1, 333-344.

Cohen J, Simons RF, Edwards RG, Fehilly CB, Fishel SB (1985) Pregnancies following the frozen storage of expanding human blastocysts. *Journal of In Vitro Fertilization and Embryo Transfer* 2, 59-64.

Cohen J, Wiemer KE, Wright G (1988) Prognostic value of morphological characteristics of cryopreserved embryos: a study using video-cinematography. *Fertility and Sterility* 49, 827-834.

Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, Wiemer K (1990) Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Human Reproduction* 5, 7-13.

Cohen J, Alikani M, Trowbridge J, Rosenwaks Z (1992) Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Human Reproduction* 7, 685-691.

Cohen J, Alikani M, Ferrara T, Munné S, rein A, Schatman G, Tomkin G, Rosenwaks Z (1994) Rescuing abnormally developing embryos by assisted hatching. In Mori T, Aono T, Tominaga T, Hiroi M (eds) *Frontiers in Endocrinology, Perspectives on Assisted Reproduction* (Volume 4), Ares Serono Symposia, Rome, pp. 536-544.

Comiskey M, Goldstein CY, De Fazio SR, Mammolenti M, Newmark JA, Warner CM (2003) Evidence that HLA-G is the functional homolog of mouse Qa-2, the Ped gene product. *Human Immunology* 64, 999-1004.

Cooke S, Tyler JP, Driscoll GL (2003) Meiotic spindle location and identification and its effect on embryonic cleavage plane and early development. *Human Reproduction* 18, 2397-2405.

Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF (1986) A formula for scoring human embryo growth rates in *in vitro* fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *Journal of* In Vitro *Fertilization and Embryo Transfer* 3, 284-295.

Dale B, Gualtieri R, Talevi R, Tosti E, Santella L, Elder K (1991) Intercellular communication in the early human embryo. *Molecular Reproduction and Development* 29, 22-28.

Dale B, Tosti E, Iaccarino M (1995) Is the plasma membrane of the human oocyte reorganised following fertilisation and early cleavage? *Zygote* 3, 31-36.

Dale B, Menezo Y, Cohen J, Dimatteo L, Wilding M (1998) Intracellular pH regulation in human oocytes. *Human Reproduction* 13, 964-970.

De Pennart H, Houliston E, Maro B (1988) Post translational modifications of tubulin and the dynamics of microtubules in mouse oocytes and zygotes. *Biology of the Cell* 64, 375-378.

Desai NN, Goldstein J, Rowland DY, Goldfarb JM (2000) Morphological evaluation of human embryos and derivation of an embryo quality scoring system specific for day 3 embryos: a preliminary study. *Human Reproduction* 15, 2190-2196.

Dokras A, Sargent IL, Ross C, Gardner RL, Barlow, DH (1991) The human blastocyst: morphology and human chorionic gonadotrophin secretion *in vitro*. *Human Reproduction* 6, 1143-1151.

Dokras A Sargent IL Barlow DH (1993) Human blastocyst grading: an indicator of developmental potential? *Human Reproduction* 8, 2119-2127.

Dozortsev D, Ermilov A, El-Mowafi DM, Diamond M (1998) The impact of cellular fragmentation induced experimentally at different stages of mouse preimplantation development. *Human Reproduction* 13, 1307-1311.

Edwards RG and Gates AH (1959) Timing of the stages of the maturation divisions, ovulation, fertilization, and the first cleavage of eggs of adult mice treated with gonadotrophin. *Journal of Endocrinology* 18, 292-304.

Edwards RG, Bavister BD, Steptoe PC (1969) Early stages of fertilization *in vitro* of human oocytes matured *in vitro*. *Nature* 221, 632-635.

Edwards RG, Steptoe PC, Purdy JM (1970) Fertilization and cleavage *in vitro* of preovulatory human oocytes. *Nature* 227, 1307-1309.

Edwards RG and Steptoe PC (1974) Control of human ovulation, fertilization and implantation. *Proceedings of the Royal Society of Medicine* 67, 932-936.

Edwards RG, Steptoe PC, Purdy JM (1980) Establishing full-term human pregnancies using cleaving embryos grown *in vitro*. *British Journal of Obstetrics and Gynaecololgy* 87, 737-756.

Edwards RG and Steptoe PC (1980) *A matter of life.* William Morrow and Company, Inc., New York, USA.

Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, Steptoe PC, Webster JM (1984) Factors influencing the success of *in vitro* fertilization for alleviating human infertility. *Journal of* in vitro *Fertilization and Embryo Transfer* 1, 3-23.

Edwards RG and Beard HK (1997) Oocyte polarity and cell determination in early mammalian embryos. *Molecular Human Reproduction* 3, 863-906

Edwards RG and Beard HK (1999) Is the success of human IVF more a matter of genetics and evolution than growing blastocysts? *Human Reproduction* 14, 1-6.

Edwards RG and Pool T (2002) Human Chimaeras and understanding early development. *Reproductive Biomedicine Online* 5, 103.

Edwards RG and Hansis C (2005) Initial differentiation of blastomeres in 4-cell human embryos and its significance for early embryogenesis and implantation. *Reproductive Biomedicine Online* 11, 206-218.

Eichenlaub-Ritter U, Chandley AC, Gosden RG (1986) Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged *in vivo*: an immunofluorescence study. *Chromosoma* 94, 337-345.

Ellis RE, Jacobson DM, Horvitz HR (1991) Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics* 129, 79-94.

El Mestrah M, Castle PE, Borossa G, Kan FW (2002) Subcellular distribution of ZP1, ZP2, and ZP3 glycoproteins during folliculogenesis and demonstration of their topographical disposition within the zona matrix of mouse ovarian oocytes. *Biology of Reproduction* 66, 866-876.

El-Shershaby AM and Hinchliffe JR (1974) Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. *Journal of Embryology and Experimental Morphology* 31, 643-654.

Enders AC, Hendrickx AG, Binkerd PE (1982) Abnormal development of blastocysts and blastomeres in the Rhesus Monkey. *Biology of Reproduction* 26, 353-366.

Enders AC, Lantz KC, Schlafke S (1990) Differentiation of the inner cell mass of the baboon blastocyst. *Anatomical Record* 226, 237-248.

Epifano O, Liang LF, Familari M, Moos MC, Dean J (1995) Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* 121, 1947-1956.

Erenus M, Zouves C, Rajamahendran P, Leung S, Fluker M, Gomel V (1991) The effect of embryo quality on subsequent pregnancy rates after *in vitro* fertilization. *Fertility and Sterility* 56, 707-710.

Everett CA and West JD (1996) The influence of ploidy on the distribution of cells in chimaeric mouse blastocysts. *Zygote* 4, 59-66.

Everett CA and West JD (1998) Evidence for selection against tetraploid cells in tetraploid<-->diploid mouse chimaeras before the late blastocyst stage. *Genetical Research* 72, 225-228.

Fehilly CB, Willadsen SM, Tucker EM (1984) Experimental chimaerism in sheep. *Journal of Reproduction and Fertility* 70, 347-351.

Fehilly CB and Willadsen SM (1986) Embryo manipulation in farm animals. *Oxford Reviews of Reproductive Biology* 8, 379-413.

Fenwick J, Platteau P, Murdoch AP, Herbert M (2002) Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*. *Human Reproduction* 17, 407-412.

Flach G, Johnson MH, Braude PR, Taylor RA, Bolton VN (1982) The transition from maternal to embryonic control in the 2-cell mouse embryo. *EMBO Journal* 1, 681-686.

Fleming TP and Johnson M (1988) From egg to epithelium. *Annual Reviews in Cell Biology* 4, 459-485.

Fleming TP, Sheth B, Fesenko I (2001) Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis. *Frontiers in Bioscience* 6, D1000-1007.

Fowler RE and Edwards RG (1957) Induction of superovulation and pregnancy in mature mice by gonadotrophins. *Journal of Endocrinology* 15, 374-384.

Gabrielsen A, Bhatnager PR, Petersen K, Lindenberg S (2000) Influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following *in vitro* fertilization treatment. *Journal of Assisted Reproduction and Genetics* 17, 323-328.

Gabrielsen A, Lindenberg S, Petersen K (2001) The impact of the zona pellucida thickness variation of human embryos on pregnancy outcome in relation to suboptimal embryo development. A prospective randomized controlled study. *Human Reproduction* 16, 2166-2170.

Gardner DK and Lane M (1997) Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Human Reproduction Update* 3, 367-382.

Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB (1998a) Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertility and Sterility* 69, 84-88.

Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J (1998b) A prospective randomized trial of blastocyst culture and transfer in *in vitro* fertilization. *Human Reproduction* 13, 3434-3440.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB (2000) Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertility and Sterility* 73, 1155-1158.

Gardner RL (1997) The early blastocyst is bilaterally symmetrical and its axis of symmetry is aligned with the animal-vegetal axis of the zygote in the mouse. *Development* 124, 289-301.

Gardner RL (1999) Polarity in early mammalian development. *Current Opinion in Genetics and Development* 9, 417-421.

Gardner RL (2002) Experimental analysis of second cleavage in the mouse. *Human Reproduction* 17, 3178-3189.

Garello C, Baker H, Rai J, Montgomery S, Wilson P, Kennedy CR, Hartshorne GM (1999) Pronuclear orientation, polar body placement, and embryo quality after intracytoplasmic sperm injection and in-vitro fertilization: further evidence for polarity in human oocytes? *Human Reproduction* 14, 2588-2595.

Garside WT, Loret de Mola JR, Bucci JA, Tureck RW, Heyner S (1997) Sequential analysis of zona thickness during *in vitro* culture of human zygotes: correlation with embryo quality, age, and implantation. *Molecular Reproduction and Development* 47, 99-104.

Geber S, Winston RM, Handyside AH (1995) Proliferation of blastomeres from biopsied cleavage stage human embryos in vitro: an alternative to blastocyst biopsy for preimplantation diagnosis. *Human Reproduction* 10, 1492-1496.

Gemzell CA (1962) Induction of ovulation with human pituitary gonadotrophins. *Fertility and Sterility* 13, 153-168.

Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M, Valkenburg M (1999) Prevention of twin pregnancy after *in vitro* fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Human Reproduction* 14, 2581-2587.

Giorgetti C, Terriou P, Auquier P, Hans E, Spach JL, Salzmann J, Roulier (1995) Embryo score to predict implantation after *in vitro* fertilization: based on 957 single embryo transfers. *Human Reproduction* 10, 2427-2431.

Goyanes VJ, Ron-Corzo A, Costas E, Maneiro E (1990) Morphometric categorization of the human oocyte and early embryo. *Human Reproduction* 5, 613-618.

Gordo AC, Rodrigues P, Kurokawa M, Jellerette T, Exley GE, Warner C, Fissore R (2002) Intracellular calcium oscillations signal apoptosis rather than activation in *in vitro* aged mouse eggs. *Biology of Reproduction* 66, 1828-1837.

Goddard MJ and Pratt HP (1983) Control of events during early cleavage of the mouse embryo: an analysis of the '2-cell block'. *Journal of Embryology and Experimental Morphology* 73, 111-133.

Gualtieri R, Santella L, Dale B (1992) Tight junctions and cavitation in the human pre-embryo. *Molecular Reproduction and Development* 32, 81-87.

Gulyas BJ (1975) A reexamination of cleavage patterns in eutherian mammalian eggs: rotation of blastomere pairs during second cleavage in the rabbit. *Journal of Experimantal Zoology* 193, 235-248.

Hammond J (1949) Physiology of reproduction in relation to nutrition. *The British Journal of Nutrition* 3, 79-83.

Hansis C and Edwards RG (2003) Cell differentiation in the preimplantation human embryo. *Reproductive Biomedicine Online* 6, 215-220.

Hardarson T, Lundin K, and Hamberger L (2000) The position of the metaphase II spindle cannot be predicted by the location of the first polar body in the human oocyte. *Human Reproduction* 15, 1372-1376.

Hardarson T, Hanson C, Sjogren, Lundin K (2001) Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. *Human Reproduction* 16, 313-318.

Hardarson T, Lofman C, Coull G, Sjogren A, Hamberger L, Edwards RG (2002) Internalization of cellular fragments in a human embryo: time-lapse recordings. *Reproductive Biomedicine Online* 5, 36-38.

Hardy K, Handyside AH, Winston RML (1989) The human blastocyst: cell number, death and allocation during late preimplantation development *in vitro*. *Development* 107, 597-604.

Hardy K, Winston RML, Handyside AH (1993) Binucleate blastomeres in preimplantation human embryos *in vitro*: failure of cytokinesis during early cleavage. *Journal of Reproduction and Fertility* 98, 549-558.

Hardy K, Stark J, Winston RM (2003) Maintenance of the inner cell mass in human blastocysts from fragmented embryos. *Biology of Reproduction* 68, 1165-1169.

Harper JC, Robinson F, Duffy S, Griffin DK, Handyside AH, Delhanty JD, Winston RM (1994) Detection of fertilization in embryos with accelerated cleavage by fluorescence in-situ hybridization (FISH). *Human Reproduction* 9, 1733-1737.

Hartwell LH and Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* 246, 629-634.

Hawes SM, Gie Chung Y, Latham KE (2001) Genetic and epigenetic factors affecting blastomere fragmentation in two-cell stage mouse embryos. *Biology of Reproduction* 65, 1050-1056.

Hendrickx AG and Kraemer DC (1968) Preimplantation stages of baboon embryos. *The Anatomical Record* 162, 111-120.

Hertig AT, Rock J, Adams EC, Menkin MC (1954) On the preimplantation stages of the human ovum: a description of four normal and four abnormal specimens ranging from the second to the fifth day of development. *Contributions to Embryology* 35, 199-220.

Ho Y, Wigglesworth K, Eppig JJ, Schultz RM (1995) Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Molecular Reproduction and Development* 41, 232-238.

Hogan B, Beddington R, Costantini F, Lacy E (1994) *Manipulating the Mouse Embryo: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, USA.

Huisman GJ, Alberda AT, Leerentveld RA, Verhoeff A, Zeilmaker GH (1994) A comparison of *in vitro* fertilization after embryo transfer after 2, 3, and 4 days of embryo culture. *Fertility and Sterility* 61, 970-971.

Hurst PR, Wheeler AG, Eckstein P (1980) A study of uterine embryos recovered from rhesus monkeys fitted with intra-uterine devices. *Fertility and Sterility* 33, 69-79.

Jackson KV, Ginsberg ES, Hornstein MD, Rein MS, Clarke RN (1998) Multinucleation in normally fertilized embryos is associated with an accelerated ovulation induction response and lower implantation and pregnancy rates in in-vitro fertilization embryo-transfer cycles. *Fertility and Sterility* 70, 60-66.

Janny L and Menezo YJR (1996) Maternal age effect on early human embryonic development and blastocyst formation. *Molecular Reproduction and Development* 45, 31-37.

Johnson MH, Chakraborty J, Handyside AH, Willison K, Stern P (1979) The effect of prolonged decompaction on the development of the preimplantation mouse embryo. *Journal of Embryology and Experimental Morphology* 54, 241-261.

Johnson MH, McConnell J, Van Blerkom J (1984) Programmed development in the mouse embryo. *Journal of Embryology and Experimental Morphology* 83 (Supplement), 197-231.

Johnson MH, Maro B, Takeichi M (1986) The role of cell adhesion in the synchronization and orientation of polarization in 8-cell mouse blastomeres. *Journal of Embryology and Experimental Morphology* 93, 239-255.

Johnson MH and Selwood L (1996) Nomenclature of early development in mammals. *Reproduction, Fertility, and Development* 8, 759-764.

Johnson MH (2001) Mammalian development: axes in the egg? *Current Biology* 11, R281-284.

Jones GM, Trounson AO, Lolatgis N, Wood C (1998) Factors affecting the success of human blasto-

cyst development and pregnancy following in-vitro fertilization and embryo transfer. *Fertility and Sterility* 70, 1022-1029.

Jurisicova A, Varmuza S, Casper RF. Involvement of programmed cell death in preimplantation embryo demise (1995) *Human Reproduction Update* 1, 558-566.

Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach CL (1996a) HLA-G expression during preimplantation human embryo development. *Proceedings of the National Academy of Sciences USA* 93, 161-165.

Jurisicova A, Varmuza S, Caspar RF (1996b) Programmed cell death and human embryo fragmentation. *Molecular Human Reproduction* 2, 93-98.

Jurisicova A, Rogers I, Fasciani A, Casper RF, Varmuza S (1998) Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development. *Molecular Human Reproduction* 4, 139-145.

Jurisicova A, Antenos M, Varmuza S, Tilly JL, Casper RF (2003) Expression of apoptosis-related genes during human preimplantation embryo development: potential roles for the Harakiri gene product and Caspase-3 in blastomere fragmentation. *Molecular Human Reproduction* 9, 133-141.

Kaufman MH (1982) The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment. *Journal of Embryology and Experimental Morphology* 71, 139-154.

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* 26, 239-257.

Kidder GM, Rains J, McKeon J (1987) Gap junction assembly in the preimplantation mouse conceptus is independent of microtubules, microfilaments, cell flattening, and cytokinesis. *Proceedings of the National Academy of Sciences USA* 84, 3718-3722.

Killeen ID and Moore NW (1971) The morphological appearance and development of sheep ova fertilized by surgical insemination. *Journal of Reproduction and Fertility* 24, 63-70.

Kimura Y and Yanagimachi R (1995) Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction* 52, 709-720

Kligman I, Benadiva C, Alikani M, Munné S (1996) The presence of multinucleated blastomeres in human embryos is correlated with chromosomal abnormalities. *Human Reproduction* 11, 1492-1498.

Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, Smith K (1986) Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertility and Sterility* 46, 1118-1123.

Kubiak JZ and Ciemerych MA (2001) Cell cycle regulation in early mouse embryos. *Novartis Foundation Symposia* 237, 79-89.

Lanzendorf SE, Boyd CA, Wright DL, Muasher S, Oehninger S, Hodgen GD (2001) Use of human gametes obtained from anonymous donors for the production of human embryonic stem cell lines. *Fertility and Sterility* 76, 132-137.

Larue L, Ohsugi M, Hirchenhain J, Kemler R (1994) E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proceedings of the National Academy of Sciences USA* 91, 8263-8267.

Laverge H, De Sutter P, Verschraegen-Spae MR, De Paepe A, Dhont M (1997) Triple colour fluorescent *in situ* hybridization for chromosomes X, Y, and 1 on spare human embryos. *Human Reproduction* 12, 809-814.

Lawitts JA and Biggers JD (1993) Culture of preimplantation embryos. *Methods in Enzymology* 225, 153-164.

Levy R, Benchaib M, Cordonier H, Couchier C, Guerin JF (1998) Annexin V labelling and terminal transferase-mediated DNA end labelling (TUNEL) assay in human arrested embryos. *Molecular Human Reproduction* 4, 775-783.

Liu L, Trimarchi, JR, Smith PJ, Keefe DL (2002) Checkpoint for DNA integrity at the first mitosis after oocyte activation. *Molecular Reproduction and Development* 62, 277-288.

Loret De Mola JR, Garside WT, Bucci J, Tureck RW, Heyner S (1997) Analysis of the human zona pellucida during culture: correlation with diagnosis and the preovulatory hormonal environment. *Journal of Assisted Reproduction and Genetics* 14, 332-336.

Lundin K, Bergh C, Hardarson T (2001) Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Human Reproduction* 16, 2652-2657.

Magli MC, Gianaroli L, Munné S, Ferraretti AP (1998) Incidence of chromosomal abnormalities from a morphologically normal cohort of embryos in poor prognosis patients. *Journal of Assisted Reproduction and Genetics* 15, 297-301.

Majno G and Joris I (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *American Journal of Pathology* 146, 3-15.

Maro B, Howlett SK, Webb M (1985) Non-spindle microtubule organizing centers in metaphase II-arrested mouse oocytes. *Journal of Cell Biology* 101, 1665-1672.

Marquez C, Sandalinas M, Bahce M, Alikani M, Munné S (2000) Chromosome abnormalities in 1255 cleavage-stage human embryos. *Reproductive Biomedicine Online* 1, 17-26.

Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, van Schie RCAA, LaFace DM, Green DR (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *The Journal of Experimental Medicine* 182, 1545-1556.

Martin RH, Radenmaker AW, Hildebrand K (1987) Variation in the frequency and type of sperm chromosomal abnormalities among normal men. *Human Genetics* 77, 108-114.

Martinez ML, Fontenot GK, Harris JD (1996) The expression and localization of zona pellucida glycoproteins and mRNA in cynomolgus monkeys (Macaca fascicularis). *Journal of Reproduction and Fertility* 50 (Supplement), 35-41.

McLaren A and Biggers JD (1958) Successful development and birth of mice cultivated *in vitro* as early as early embryos. *Nature* 182, 877-878.

McLaren A (1967) Delayed loss of the zona pellucida from blastocysts of suckling mice. *Journal of Reproduction and Fertility* 14, 159-162.

McLaren A (1968) A study of balstocysts during delay and subsequent implantation in lactating mice. *Endocrinology* 42, 453-463.

McLaren A (1970) The fate of the zona pellucida in mice. *Journal of Embryology and Experimental Morphology* 23, 1-19.

Milki AA, Jun SH, Hinckley MD, Behr B, Giudice LC, Westphal LM (2003) Incidence of monozygotic twinning with blastocyst transfer compared to cleavage-stage transfer. *Fertility and Sterility* 79, 503-506.

Mintz B (1962) Experimental study of the developing mammalian egg; removal of the zona pellucida. *Science* 138, 594.

Mohr LR and Trounson AO (1982) Comparative ultrastructure of hatched human, mouse and bovine blastocysts. *Journal of Reproduction and Fertility* 66, 499-504.

Monks NJ, Turner K, Hooper MAK, Kumar A, Verma S, Lenton EA (1993) Development of embryos from natural cycle *in vitro* fertilization: impact of medium type and female infertility factors. *Human Reproduction* 8, 266-271.

Moore NW, Adams CE, Rowson LE (1968) Developmental potential of single blastomeres of the rabbit egg. *Journal of Reproduction and Fertility* 17, 527-531.

Moricard R (1950) Penetration of the spermatozoon *in vitro* into the mammalian ovum oxydo potential level. *Nature* 165, 763-764.

Morita Y and Tilly JL (1999) Oocyte apoptosis: like sand through an hourglass. *Developmental Biology* 213, 1-17.

Munné S, Weier HUG, Stein J, Grifo J, Cohen J (1993) A fast and efficient method for simultaneous X and Y in-situ hybridization of human blastomeres. *Journal of Assisted Reproduction and Genetics* 10, 82–90.

Munné S, Alikani M, Grifo J, Cohen J (1994) Monospermic polyploidy and atypical embryo morphology. *Human Reproduction* 91, 506-510.

Munné S, Alikani M, Tomkin G, Grifo J, Cohen J (1995) Embryo morphology, developmental rates and maternal age are correlated with chromosome abnormalities. *Fertility and Sterility* 64, 382-391.

Munné S and Cohen J (1998) Chromosome abnormalities in human embryos *Human Reproduction Update* 4, 842-855.

Munné S, Magli C, Bahçe M, Fung J, Legator M, Morrison L, Cohen J, Gianaroli L (1998) Preimplantation diagnosis of the aneuploidies most commonly found in spontaneous abortions and live births: XY, 13, 14, 15, 16, 18, 21, 22. *Prenatal Diagnosis* 18, 1459-1466.

Munné S, Sandalinas M, Cohen J (2001) Chromosome abnormalities in human embryos. In Gardner D, Weissman A, Howles C, Shoham Z (eds) *Textbook of Assisted Reproductive Techniques, Laboratory and Clinical Perspectives.* Martin Dunitz, London, United Kingdom, pp. 297-318.

Neganova IE, Sekirina GG, Eichenlaub-Ritter U (2000) Surface-expressed E-cadherin, and mitochondrial and microtubule distribution in rescue of mouse embryos from 2-cell block by aggregation. *Molecular Human Reproduction* 6, 454-464.

Nigg EA (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nature Reviews in Molecular and Cell Biology* 2, 21-32.

Nikas G, Ao A, Winston R, Handyside AH (1996) Compaction and surface polarity in the human embryo in-vitro. *Biology of Reproduction* 55, 32-37.

Ortiz ME and Croxatto HB (1979) Observations on the transport, aging, and development of ova in the human genital tract. In Talwar GP (ed) *Recent Advances in Reproduction and Regulation of Fertility.* Elsevier/North-Holland Biomedical Press, New York, USA, pp. 307-317.

Palacios F, Tushir JS, Fujita Y, D'Souza-Schorey C (2005) Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. *Molecular and Cellular Biology* 25, 389-402.

Palermo GD, Munné S, Cohen J (1994) The human zygote inherits its mitotic potential from the male gamete. *Human Reproduction* 9, 1220-1225.

Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z (1995) Development and implementation of intracytoplasmic sperm injection (ICSI). *Reproduction, Fertility, and Development* 7, 211-218.

Palmstierna M, Murkes D, Csemiczky G, Andersson O, Wramsby H (1998) Zona pellucida thickness variation and occurrence of visible mononucleated blastomers in preembryos are associated with a high pregnancy rate in IVF treatment. *Journal of Assisted Reproduction and Genetics* 15, 70-75.

Payne D, Flaherty SP, Barry MF, Mathews CD (1997) Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Human Reproduction* 12, 532-541.

Pelinck MJ, De Vos M, Dekens M, Van der Elst J, De Sutter P, Dhont M (1998) Embryos cultured *in vitro* with multi-nucleated blastomeres have poor implantation potential in human in-vitro fertilization and intracytoplasmic injection. *Human Reproduction* 13, 960-963.

Pellestor F (1991) Frequency and distribution of aneuploidy in human female gametes. *Human Genetics* 86, 283-288.

Pellestor F, Dufour MC, Arnal F, Humeau C (1994) Direct assessment of the rate of chromosomal abnormalities in grade IV human embryos produced by in-vitro fertilization procedure. *Human Reproduction* 9, 293-302.

Perez GI, Tao XJ, Tilly JL (1999) Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Molecular Human Reproduction* 5, 414-420.

Petzoldt U and Muggleton-Harris A (1987) The effect of the nucleocytoplasmic ratio on protein synthesis and expression of a stage-specific antigen in early cleaving mouse embryos. *Development* 99, 481-491.

Pey R, Vial C, Schatten G, Hafner M (1998) Increase of intracellular Ca2+ and relocation of E-cadherin during experimental decompaction of mouse embryos. *Proceedings of the National Academy of Sciences USA* 95, 12977-12982.

Pickering SJ, Johnson MH, Braude PR, Houliston E (1988) Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. *Human Reproduction* 3, 978-989.

Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J (1990) Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertility and Sterility* 54, 102-108.

Pieczenik G (2004) Are the universes of antibodies and antigens symmetrical? *Reproductive Biomedicine Online* 6, 154-156.

Pincus G (1930) Observations on the living eggs of the rabbit. *Proceedings of the Royal Society of London (B)* 107, 132.

Plachot M and Mandelbaum J (1990) Oocyte maturation, fertiliztion and embryonic growth *in vitro*. *British Medical Bulletin* 46, 675-694.

Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F (1987) Embryo scoring as a prognostic tool in IVF treatment. *Human Reproduction* 2, 705-708.

Quinn P, Warnes GM, Kerin JF, et al. (1985) Culture factors affecting the success rate of IVF and embryo transfer. *Annals of the New York Academy of Sciences* 442, 195-204.

Racowsky C, Jackson KV, Cekleniak NA, Fox JH, Hornstein MD, Ginsburg ES (2000) The number of eight-cell embryos is the key determinant for selecting day 3 or day 5 transfer. *Fertility and Sterility* 73, 558-564.

Racowsky C, Combelles CM, Nureddin A, Pan Y, Finn A, Miles L, Gale S, O'Leary T, Jackson KV (2003) Day 3 and day 5 morphological predictors of embryo viability. *Reproductive Biomedicine Online* 6, 323-331.

Raff JW and Glover DM (1989) Centrosomes, and not nuclei, initiate pole cell formation in Drosophila embryos. *Cell* 57, 611-619.

Rappaport R (1996) *Cytokinesis in Animal Cells.* Cambridge University Press, Cambridge, U.K.

Reima I, Lehtonen E, Virtanen I, Flechon JE (1993) The cytoskeleton and associated proteins during cleavage, compaction and blastocyst differentiation in the pig. *Differentiation* 54, 35-45.

Riethmacher D, Brinkmann V, Birchmeier C (1995) A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proceedings of the National Academy of Sciences USA* 92, 855-859.

Rijnders PM and Jansen CA (1998) The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. *Human Reproduction* 13, 2869-2873.

Rossant J (1976) Postimplantation development of blastomeres isolated from 4- and 8-cell mouse eggs. *Journal of Embryology and Experimental Morphology* 36, 283-290.

Rufas O, Fisch B, Ziv S, Shalgi R (2000) Expression of cadherin adhesion molecules on human gametes. *Molecular Human Reproduction* 6, 163-169.

Runner MN and Gates A (1954) Conception in prepubertal mice following artificially induced ovulation and mating. *Nature* 174, 222-223.

Sadowy S, Tomkin G, Munne S, Ferrara-Congedo T, Cohen J (1998) Impaired development of zygotes with uneven pronuclear size. *Zygote* 6, 137-141.

Sakkas D, Shoukir Y, Chardonennes D, Bianchi PG, Campana A (1998) Early cleavage of human embryos to the 2 cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. *Human Reproduction* 13, 182-187.

Sakkas D (2001) Evaluation of embryo quality: a strategy for sequential analysis of embryo development with the aim of single embryo transfer. In Gardner D, Weissman A, Howles C, Shoham Z (eds) *Textbook of Assisted Reproductive Techniques, Laboratory and Clinical Perspectives.* Martin Dunitz, London, United Kingdom, pp. 223-232.

Salumets A, Hyden-Granskog C, Makinen S, Suikkari AM, Tiitinen A, Tuuri T (2003) Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Human Reproduction* 18, 821-825.

Sandalinas M, Sadowy S, Alikani M, Calderon G, Cohen J (2001) Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. *Human Reproduction* 16, 1954-1958.

Sathananthan AH, Wood C, Leeton J (1982) Ultrastructural evaluation of 8-16 cell human embryos developed *in vitro*. *Micron* 13, 193-203.

Sathananthan AH, Bongso A, Ng SC, Ho J, Mok H, Ratnam S (1990) Ultrastructure of preimplantation human embryos co-cultured with human ampullary cells *Human Reproduction* 5, 309-318.

Sathananthan AH, Kola I, Osborne J, Trounson A, Ng SC, Bongso A, Ratnam SS (1991) Centrioles in the beginning of human development. *Proceedings of the National Academy of Sciences USA* 88, 4806-4810.

Sathananthan H, Ng SC, Bongso A, Trounson A, Ratnam S (1993) *Visual Atlas of early human development for assisted reproductive technology.* National University of Singapore, Singapore.

Schatten G, Simerly C, Schatten H (1985) Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. *Proceedings of the National Academy of Sciences USA* 82, 4152-4156.

Schmiady H, Kentenich H (1993) Cytological studies of human zygotes exhibiting developmental arrest. *Human Reproduction* 8, 744-751.

Schoolcraft WB, Gardner DK, Lane M, Schlenker T, Hamilton F, Meldrum DR (1999) Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two in-vitro fertilization programs. *Fertility and Sterility* 72, 604-609.

Scott LA and Smith S (1998) The successful use of pronuclear embryo transfers the day following oocyte retrieval. *Human Reproduction* 13, 1003-1013.

Scott L, Alvaro R, Leondires M, Miller B (2000) The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Human Reproduction* 15, 2394-2403.

Scott RT and Navot D (1994) Enhancement of ovarian responsiveness with micro-doses of GnRH-agonist during ovarian induction for *in vitro* fertilization. *Fertility and Sterility* 61, 880-885.

Sefton M, Johnson MH, Clayton L, McConnell JM (1996) Experimental manipulations of compaction and their effects on the phosphorylation of uvomorulin. *Molecular Reproduction and Development* 44, 77-87.

Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U, Tinneberg HR (2005) High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Human Reproduction* 20, 1596-1606.

Shoukir Y, Campana A, Farley T, Sakkas D (1997) Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. *Human Reproduction* 12, 1531-1536.

Shulman A, Ben-Nun I, Ghetler Y, Kaneti H, Shilon M, Beyth Y (1993) Relationship between embryo morphology and implantation rate after *in vitro* fertilization treatment in conception cycles. *Fertility and Sterility* 60, 123-126.

Silva C, Komminenia K, Oldenbourg R, Keefe DL (1999) The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. *Fertility and Sterility* 71, 719-721.

Simerly C, Navara C, Wu GJ, Schatten G (1995) Cytoskeletal organization and dynamics in mammalian oocytes during maturation and fertilization. In Grudzinskas JG and Yovich JL (eds) *Reviews in Human Reproduction. Gametes: The Oocyte.* Cambridge University Press, Cambridge, UK. pp. 54-94.

Sinowatz F, Kolle S, Topfer-Petersen E (2001) Biosynthesis and expression of zona pellucida glycoproteins in mammals. *Cells, Tissues, Organs* 168, 24-35.

Smith R and McLaren A (1977) Factors affecting the time of formation of the mouse blastocoele. *Journal of Embryology and Experimental Morphology* 41, 79-92.

Soyal SM, Amleh A, Dean J (2000) FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* 127, 4645-4654.

Squirrell JM, Lane M, Bavister BD (2001) Altering intracellular pH disrupts development and cellular organization in preimplantation hamster embryos. *Biology of Reproduction* 64, 1845-1854.

Staessen C, Janssenswillen C, Devroey P, Van Steirteghem AC (1993) Cytogenetic and morphological observations of single pronucleated human oocytes after *in vitro* fertilization. *Human Reproduction* 8, 221-223.

Staessen C, Janssenwillen C, Van Den Abbeel E, Devroey P, Van Steirteghem AC (1993) Avoidance of triplet pregnancies by elective transfer of two good quality embryos. *Human Reproduction* 8, 1650-1653.

Staessen C and Van Steirteghem A (1998) The Genetic Constitution of multinuclear blastomeres and their derivative daughter blastomeres. *Human Reproduction* 13, 1625-1631.

Steptoe PC and Edwards RG (1976) Reimplantation of a human embryo with subsequent tubal pregnancy. *Lancet* 1, 880-882.

Steptoe PC and Edwards RG (1978) Birth after the reimplantation of a human embryo. *Lancet* 2, 366.

Stone BA, Greene J, Vargyas JM, Ringler GE, Marrs RP (2003) Embryo fragmentation as a determinant of blastocyst development in vitro and pregnancy outcomes following embryo transfer. *American Journal of Obstetrics and Gynecology* 192, 2014-2019.

Straight AF and Field CM (2000) Microtubules, membranes and cytokinesis. *Current Biology* 10, R760-770.

Surani MA, Barton SC, Burling A (1980) Differentiation of 2-cell and 8-cell mouse embryos arrested by cytoskeletal inhibitors. *Experimental Cell Research* 125, 275-286.

Takase K, Ishikawa M, Hoshiai H (1995) Apoptosis in the degeneration process of unfertilized mouse ova. *Tohoku Journal of Experimental Medecine* 175, 69-76.

Tang PC, Ritchie WA, Wilmut I, West JD (2000) The effects of cell size and ploidy on cell allocation in mouse chimaeric blastocysts. *Zygote* 8, 33-43.

Tarkowski AK (1959) Experiments on the development of isolated blastomers of mouse eggs. *Nature* 184, 1286-1287.

Tarkowski AK (1966) An air-drying method for chromosome preparation mouse eggs. *Cytogenetics* 5, 394-400.

Tarkowski AK and Wroblewska J (1967) Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *Journal of Embryology and Experimental Morphology* 18, 155-180.

Tarkowski AK (1998) Mouse chimaeras revisited: recollections and reflections. *International Journal of Developmental Biology* 42, 903-908.

Tarkowski AK, Ozdzenski W, Czolowska R (2001) Mouse singletons and twins developed from isolated diploid blastomeres supported with tetraploid blastomeres. *International Journal of Developmental Biology* 45, 591-596.

Tatusova TA and Madden TL (1999) BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiology Letters* 174, 247-250.

Tesarik J, Kopecny V, Plachot M, Mandelbaum J (1987) Ultrastructural and autoradiographic observations on multinucleated blastomeres of human cleaving embryos obtained by *in vitro* fertilization. *Human Reproduction* 2, 127-136.

Tesarik J, Kopecny V, Plachot M, Mandelbaum J (1988) Early morphological signs of genome expression in human preimplantation development as revealed by quantitative electron microscopy. *Developmental Biology* 128, 15-20.

Tesarik J and Greco E (1999) The probability of abnormal development can be predicted by a single static observation on pronuclear stage morphology. *Human Reproduction* 14, 1318-1323.

Thie M, Fuchs P, Denker HW (1996) Epithelial cell polarity and embryo implantation in mammals. *International Journal of Development* 40, 389-393.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147.

Thomson JA and Solter D (1989) Chimeras between parthenogenetic or androgenetic blastomeres and normal embryos: allocation to the inner cell mass and trophectoderm. *Developmental Biology* 131, 580-583.

Tomkin G and Cohen J (2001) Data management and interpretation- computerized database for an ART clinic: hardware and software requirements and solutions. In Gardner D, Weissman A, Howles C, Shoham Z (eds) *Textbook of Assisted Reproductive Techniques, Laboratory and Clinical Perspectives.* Martin Dunitz, London, United Kingdom, pp. 367-380.

Trounson A and Sathananthan AH (1984) The application of electron microscopy in the evaluation of two- to four-cell human embryos cultured *in vitro* for embryo transfer. *Journal of* In vitro *Fertilization and Embryo Transfer* 1, 153-165.

Van Blerkom J (1991) Extrinsic and Intrinsic influences on human oocyte and early embryonic developmental potential. In Wassarman PM (ed) *Elements of Mammalian Fertilization.* CRC Press, Boca Raton, Florida, USA, pp. 81-109.

Van Blerkom J (1993) Development of human embryos to the hatched blastocyst stage in the presence or absence of a monolayer of Vero cells. *Human Reproduction* 8, 1525-1539.

Van Blerkom J (1996) Sperm centrosome dysfunction: a possible new class of male factor infertility in the human. *Molecular Human Reproduction* 2, 349-354.

Van Blerkom J, Antczak M, Schrader R (1997) The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Human Reproduction* 12, 1047-1055.

Van Blerkom J, Davis P, Alexander S (2000) Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. *Human Reproduction* 15, 2621-2633.

Van Blerkom J, Davis P, Alexander S (2001) A microscopic and biochemical study of fragmentation phenotypes in stage-appropriate human embryos. *Human Reproduction* 16, 719-729.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W, Gerris J (1999) Characterization of a top quality embryo, a step towards single-embryo transfer. *Human Reproduction* 14, 2345-2349.

Van Royen E, Mangelschots K, Vercruyssen M, De Neubourg D, Valkenburg M, Ryckaert G, Gerris J (2003) Multinucleation in cleavage stage embryos. *Human Reproduction* 18, 1062-1069.

Veeck LL (1988) Oocyte assessment and biological performance. *Annals of the New York Academy of Sciences* 541, 259-274.

Veiga A, Calderon G, Barri PN, Coroleu B (1987) Pregnancy after the replacement of a frozen-thawed embryo with less than 50% intact blastomeres. *Human Reproduction* 2, 321-323.

Velilla E, Escudero T, Munne S (2002) Blastomere fixation techniques and risk of misdiagnosis for preimplantation genetic diagnosis of aneuploidy. *Reproductive Biomedicine Online* 4, 210-217.

Vestweber D, Gossler A, Boller K, Kemler R (1987) Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos. *Developmental Biology* 124, 451-456.

Waksmundzka M, Krysiak E, Karasiewicz J, Czolowska R, Tarkowski AK (1984) Autonomous cortical activity in mouse eggs controlled by a cytoplasmic clock. *Journal of Embryology and Experimental Morphology* 79, 77-96.

Walmsley R (2002) *A Comprehensive Analysis of Multinucleated Cleavage Stage Human Embryos Cultured In Vitro.* Thesis submitted to University of Leeds, UK for the Degree of Master of Science in Clinical Embryology.

Warner CM, Exley GE, McElhinny AS, Tang C (1998) Genetic regulation of preimplantation mouse embryo survival. *Journal of Experimental Zoology* 282, 272-279.

Warner CM, Cao W, Exley GE, McElhinny AS, Alikani M, Cohen J, Scott RT, Brenner C (1998) Genetic regulation of egg and embryo survival. *Human Reproduction* 13 (supplement), 178-190.

Whitten WK (1956) Culture of tubal mouse ova. *Nature* 177, 96.

Whittingham DG (1968) Fertilization of mouse eggs *in vitro*. *Nature* 220, 592-593.

Wiemer KE, Dale B, Hu Y, Steuerwald N, Maxson WS, Hoffman DI (1995) Blastocyst development in co-culture: development and morphological aspects. *Human Reproduction* 10, 3226-3232.

Willadsen SM (1979) A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature* 277, 298-300.

Willadsen SM (1981) The development capacity of blastomeres from 4- and 8-cell sheep embryos. *Journal of Embryology and Experimental Morphology* 65, 165-172.

Willadsen SM and Polge C (1981) Attempts to produce monozygotic quadruplets in cattle by blastomere separation. *Veterinary Record* 108, 211-213.

Willadsen SM (1989) Cloning of sheep and cow embryos. *Genome* 31, 956-962.

Winston NJ, Braude PR, Pickering SJ, George MA, Cant A, Currie J, Johnson MH (1991) The incidence of abnormal morphology and nucleocytoplasmic ratios in 2-, 3-, and 5-day human pre-embryos. *Human Reproduction* 6, 17-24.

Wright G, Wiker S, Elsner C, Kort H, Massey J, Mitchell D, Toledo A, Cohen J (1990) Observations on the morphology of pronuclei and nucleoli in human zygotes and implications for cryopreservation. *Human Reproduction* 5, 109-115.

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. *International Reviews in Cytology* 68, 251-306.

Yanagimachi R and Chang MC (1963) Fertilization of hamster eggs *in vitro*. *Nature* 200, 281-282.

Xu Z, Abbott A, Kopf GS, Schultz RM, Ducibella T (1997) Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-trisphosphate sensitivity. *Biology of Reproduction* 57, 743-750.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS (1998) Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Human Reproduction* 13, 998-1002.

Zaninovic N, Veeck L, Xu K, Rosenwaks Z (1999) Microsurgical fragment removal on day two enhances preembryo quality and increases pregnancy rates in poor prognostic patients. Annual meeting program supplement, ASRM, Toronto, Canada, Volume 72, Supplement 1, S13 (abstract).

Zhao M and Dean J (2002) The zona pellucida in folliculogenesis, fertilization and early development. *Reviews in Endocrine and Metabolic Disorders* 3, 19-26.

Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Andersen AN (1997) Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. *Human Reproduction* 12, 1545-1549.

List of abbreviations

A

AA	amino acid
ANOVA	analysis of variance between groups
ART	assisted reproductive technologies
AT	acidified Tyrode's solution
ATP	adenosine triphosphate
Az	sodium azide

B

BSA	bovine serum albumin

C

°C	degree Celcius
CCB	cytochalasin B
C. elegans	*caenorhabditis elegans*
CMF-CZB	calcium-magnesium-free CZB medium
CO_2	carbon dioxide
CRL	crown-rump length
CZB	Chatot Ziomek Bavister medium

D

DIC	differential interference contrast
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

E

EC	early cleaving zygotes
E-cadherin	epithelial cadherin
ES cell	embryonic stem cell
ET	embryo transfer
EtOH	ethanol

F

F1	first filial generation
FHB	fetal heart beat
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone

G

g	gravitational force
G_1	gap 1 phase of interphase
G_2	gap 2 phase of interphase
G1.2	Gardner's phase 1 growth medium, version 2
G2.2	Gardner's phase 2 growth medium, version 2
GC	granulosa cell

H

hCG	human chorionic gonadotrophin
HeNe	helium-neon
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hrs	hours
HSA	human serum albumin
HTF	human tubal fluid medium

I

ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IgG	immunoglobulin G
IVF	*in vitro* fertilization
IU	international units

K

KSOM	potassium simplex optimized medium
$KSOM^{AA}$	amino acid-supplemented KSOM

L

LSM	laser scanning microscope

M

μ	micron/micrometer
M-CZB	modified CZB
MI	metaphase I
MII	metaphase II
mg	milligram
mL	milliliter
MM	micromanipulation medium
μg	microgram
μL	microliter
μm	micrometer
MNB	multi-nucleate blastomere
MPF	mitosis promoting factor
M-phase	mitotic phase
mRNA	messenger ribonucleic acid
MTOC	microtubule organizing center

N

n	number
nm	nanometer
NEBD	nuclear envelope breakdown

P

p	probability value
PA	post activation
PB1	first polar body
PBS	phosphate buffered saline
PCD	programmed cell death
pH	pondus hydrogenii
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PMSG	pregnant mare's serum gonadotrophin
PN	pronucleus/pronuclei
PVP	polyvinylpyrrolidone
PVS	perivitelline space

R

rpm	rounds per minute

S

SD	standard deviation
SEM	scanning electron microscope
S phase	synthesis phase of interphase
SQL	structured query language
SSS	synthetic serum substitute

T

TE	trophectoderm
TEM	transmission electron microscope
TUNNEL	transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling

Z

ZP	zona pellucida
ZPT	zona pellucida thickness
ZPTV	zona pellucida thickness variation

Appendix A

Consent for *In Vitro* Fertilization/Assisted Reproduction

The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center

I / we, _____ and _____, desire to participate in the Assisted Reproduction Program at The Institute for Reproductive Medicine and Science of Saint Barnabas. We understand that there are a number of steps to this procedure and that starting this process does not guarantee that we will complete the process, achieve pregnancy or delivery of a healthy child. One of our physicians has discussed with you the etiology of your condition, and alternative therapies, if any, that are available. We understand that the female partner will receive medication to induce the maturation of several eggs and during this period she will undergo a surgical procedure to retrieve her eggs. The steps may be performed by any of the following physicians: David Sable, Margaret Garrisi, Serena Chen, Patricia Hughes, Natalie Cekleniak, Larry Grunfeld and Benjamin Sandler. The egg retrieval procedure will be done by needle aspiration, usually under ultrasound guidance or perhaps by laparoscopy. We understand that the eggs will be prepared and inseminated in marked dishes with a sample of the male partner's sperm after preparation, which removes the sperm from the seminal fluid. The embryos, which may result from fertilization, will be placed into the female partner's uterus by means of a small catheter, which passes through her cervix under ultrasound guidance. Following this transfer, blood hormone levels will be monitored in the female partner to make sure that there are adequate hormonal levels to support a developing pregnancy and then to determine if a pregnancy has resulted. We understand that each of these steps may fail and carries known risks as well as theoretical concerns as detailed in the following paragraphs.

A - Ovulation induction

We understand that a variety of medications are available for the induction of ovulation including clomiphene citrate (Clomid, Serophene), human menopausal gonadotropins (Pergonal, Humegon) pure follicle stimulating hormone (urofollitropin, Fertinex, FSH), human chorionic gonadotropin (hCG), recombinant FSH (Gonal-F or Follistim) and GnRH-agonists (leuprolide acetate, Lupron). We understand that some of these medications must be given by intra-muscular injection, which

may cause bruising or discomfort at the injection site. These medications may cause the ovaries to become over stimulated, leading to a condition called ovarian hyper stimulation syndrome (OHSS). We understand that in its most severe form this condition might require hospitalization for intravenous fluids and monitoring until the syndrome resolves. Worldwide there have been rare reports of death following severe OHSS. We therefore understand the importance of maintaining close contact with the IVF team during the time that these medications are being used and for two weeks afterwards. Some studies have linked injectable gonadotropins and clomiphene citrate to an increased risk of ovarian cancer, while other studies have not. At this time, the long term effects of these drugs are not known. The increased risk may be due to infertility and not to the drugs used to treat infertility. As a results of the use of gonadotropins, the stimulated ovary can twist on itself, cutting off its blood supply. Surgery may be required to untwist or remove the ovary.

We understand that before the start of a cycle, the male partner will be asked to supply a semen sample for analysis by the andrology laboratory. He may be asked to take a specific antibiotic during the first part of the stimulation cycle to treat bacteria that may be present in order to increase chances for a successful fertilization. In certain cases, semen may also be frozen in advance to be certain of its availability at the time of egg retrieval

B - Monitoring protocol

We understand that while receiving the medications listed above, the female partner will be closely monitored by the IVF team. We understand that this monitoring will include daily blood drawing, which can cause mild discomfort and bruising at the puncture site. We understand that ultrasound examination of the ovarian follicles and the uterus will be performed frequently. These examinations may at times be uncomfortable, but have no known risks of any kind. The female partner may also be asked to collect urine samples for further hormone analysis. We understand that if monitoring suggests a low probability for successful egg retrieval, that the stimulation cycle will be stopped and no egg retrieval will occur. We also understand that we may be given the option of starting the ovarian stimulation procedure again in a subsequent cycle.

C - Egg retrieval

We understand that, at a time determined by the IVF team, the female partner would be admitted to the egg retrieval procedure room and IVF laboratories at Saint Barnabas Medical Center. We understand that in the vast majority of cases, ultrasound directed needle puncture of the follicles will be done. Rarely, the retrieval may be done by laparoscopy under general anesthesia. We understand that the procedure involves the small risk of general anesthesia as well as injury to bowel, bladder, or blood vessels, which might require a large incision (laparotomy) to repair. We understand that

a separate informed consent will be obtained for a laparoscopic retrieval if it becomes necessary. With either type of egg retrieval, we understand that in rare cases there could be bleeding from the site where the ovaries were punctured. This may require laparotomy (an incision in the abdomen) if the bleeding cannot be controlled through the laparoscope. The risks of the procedure are similar to the risks of laparoscopy, including general anesthesia.

We understand that we cannot be guaranteed that the number of eggs predicted prior to retrieval will indeed be recovered or that any of the eggs will be normal or ripe. Some follicles may not yield eggs and rarely none of the follicles will yield eggs. The egg retrieval involves equipment such as incubators, suction apparatuses and ultrasound machines that may fail because of technical malfunction. We also understand that once the eggs are isolated in the laboratory, that blood and abnormal nursing cells are removed from around the egg using dissection needles and that although unlikely, some or all of the eggs may be damaged in the process. Eggs may also be damaged because of shock due to differences in conditions.

Initial egg yield numbers are counted once and rapidly in order to place the eggs inside the incubators and stabilize conditions as soon as possible. Eggs themselves are not visualized during egg retrieval, but only the nursing cells surrounding the eggs. The normality of the eggs cannot be assessed at egg retrieval. The exact number of eggs is only determined later on at insemination or ICSI.

C - Insemination, fertilization and embryo growth

Once retrieved, the eggs will be incubated in a special solution (culture medium) and evaluated for timing of insemination by the embryology team of the IVF program. We understand that a sample of semen from the male partner, obtained by masturbation in a private collection room near the laboratory, will be evaluated, prepared, and used for insemination. Semen collection in this way can be unsuccessful and if there are any doubts, a sample can be prepared and frozen in advance for thawing at this time. However, in case of unexpected failure it is possible to obtain spermatozoa from a testicle using a minor operative procedure (testicular sperm retrieval). Separate consent is needed for this procedure.

The seminal fluid that surrounds the spermatozoa must be removed prior to insemination. Sperm processing involves high centrifugal force, washing with an artificial colloidal suspension called PurespermTM or by swim-up. We understand that the consistency of highly viscous semen will be reduced by an enzyme. The prepared semen may be exposed to substances intended to promote sperm movement or materials intended to remove toxic substances. The zygotes or fertilized eggs are changed over into a culture solution. This solution may be changed every 48 hours or more frequently. Solutions may be specially tailored to embryonic stage. The embryos are checked at least

once daily and their development is determined. Embryos will remain in the solution(s) for 48-120 hours and then transferred.

Should a pregnancy occur, we understand that no risk to the fetus is presently known to medical science arising from the materials and methods used in the preparation and handling of eggs, semen and embryos. We understand that not all eggs recovered can be fertilized, and that it is possible that none of the eggs may fertilize. We also understand that not all eggs may be ripe and that ripe eggs may be fertilized multiple times by sperm or even self-fertilize without the sperm participating. Zygotes and later stage embryos may develop abnormally at any time.

D - Blastocyst culture, alternative culture and embryo transfer

We understand that between three to four days after egg retrieval our embryos will be placed into the uterine cavity of the female partner. Alternatively you may be asked to consider having embryos transferred at the blastocyst stage five or six days after egg retrieval, using commercial culture solutions that support growth for a longer period. This protocol may be especially advantageous to couples who are at risk of multiple pregnancy, since the extended culture increases the opportunity for embryologists to select the highest quality embryos. A potential disadvantage is that some embryos may be more sensitive to prolonged presence in the laboratory with the result that cryopreservation and /or embryo transfer may not occur.

We know that some patients have embryos that develop slowly, or show different forms of microcopic anomalies such as fragmentation and multi-nucleation and may not become pregnant. This may be due to a sensitivity of the embryos to the standard culture conditions in the laboratory. If this is a repeat attempt at assisted reproduction, we may be asked to have our eggs and zygotes cultured in a different culture medium in order to determine whether this sensitivity can be overcome. We realize that alternative culture is only effective in a proportion of the patients. The alternative culture may not have any benefit in our case, and we understand that pregnancy and success is not guaranteed.

For any embryo transfer, a thin catheter will be passed through the cervix and into the uterus so the embryo may be deposited there. We understand that this may involve some cramping and discomfort, and possibly a small amount of bleeding. Infection could be introduced at the time of the catheter insertion into the uterus, requiring antibiotic therapy. We understand there is not guarantee that any of the embryos thus transferred will result in a pregnancy.

We understand that the success of IVF can often relate directly with the number of embryos transferred to the uterus. We also understand that IVF significantly increases the risk for multiple ges-

tation (more than one baby), and that this risk also correlates directly with either the number of embryos transferred, their development, the age of the female partner (or egg donor), the number of prior attempts and other unknown factors. We also understand that in rare cases, embryos may split in two or three, resulting in multiple fetuses; on occasion this can mean that there are more fetuses than embryos transferred. There are distinct obstetric risks to multiple gestations, the most serious of which are pre-term labor and the delivery of premature infants who require intensive care. It is the policy of this program to replace anywhere from one to six embryos in a given cycle all depending on availability and factors such as your age, cycle attempt and embryonic parameters. Any additional viable embryos may be cryopreserved (frozen) for possible replacement in a subsequent cycle. We understand that a separate consent for the cryopreservation must be completed if the embryos are to be cryopreserved.

E - Post-transfer management

We understand that, in conjunction with the transfer of embryos, the female partner may be given natural progesterone by intramuscular injection, vaginal suppository, or oral capsule in an attempt to increase the chances for successful implantation. Sometimes an additional hCG injection may be given instead of the progesterone. Should a pregnancy result, we understand that no harmful effects to the mother of the fetus are presently known to medical science from the use of this natural progesterone or hCG supplementation. During this period, we understand that various blood hormone levels may be evaluated.

F - Disposition of unwanted or unsuitable cells, fluids, spermatozoa, eggs and embryos

Bloods, blood products and cells as well as follicular and seminal fluids and cells contained therein obtained during follicular monitoring, egg or sperm retrieval, may be used for scientific observations. In the event that we have unused or unripe spermatozoa, these may be subjected to scientific observations or discarded without any further observations. Under no circumstances will these spermatozoa be used for fertilization purposes or donation to other individuals, couples, corporations or institutions. In the event that we have unripe, unfertilized or abnormally fertilized eggs, these may be subjected to scientific observations or discarded without further studies. We understand that these eggs will never be used for fertilization purposes or donation to other individuals, couples, corporations or institutions and that further growth of them will be ceased immediately after the observation. We also understand that these eggs are unwanted and considered abnormal. Embryos that arrest after 1-6 days after egg retrieval, that are partially degenerate or for any other reasons considered unsuitable for embryo transfer or cryopreservation may be observed to determine cellular inclusions, genes, gene mutations, proteins and chromosomes. The studies use pro-

tocols that will cease the immediate growth of individual cells. We understand that these embryos or their cells will never be used for purposes other than those described and will never be offered to other individuals, couples, corporations or institutions. We also understand that these embryos or their cells are unwanted and considered abnormal.

A separate research consent form is being offered to you, by which you can grant permission for the use of your unwanted and unsuitable spermatozoa, eggs, embryos and their cells in research studies for the development of new reproductive technologies. This separate research protocol is being supervised by the Internal Review Board (IRB) of Saint Barnabas Medical Center, and you are not in any way required to give your permission. Your further and ongoing treatment at The Institute for Reproductive Medicine and Science and Saint Barnabas Medical Center will not be affected if you do not wish to participate in that or any other IRB-reviewed protocol.

G - Use of blood products

We understand that maternal blood serum, derived from blood collected from the female partner shortly before egg collection will be prepared for culture with your eggs and embryos in order to promote growth. Sometimes we will not be able to use your serum. It has been shown in rare instances that maternal serum may inhibit embryos developing in-vitro. Human serum albumin, a commercially prepared blood product for clinical laboratory use, is added to the egg collection fluid, micromanipulation, and semen preparation fluids. Careful screening is done by the manufacturers to reduce the likelihood of transmission of infectious diseases such as HIV, Hepatitis B and C. To date there have been no documented cases of disease transmission linked to human serum albumin usage in our Center. We understand and accept the risk that use of these blood products could result in the transmission of HIV, Hepatitis and/or other viral or possibly as yet unknown non-viral diseases.

H - Use of chemical substances, disposable items and mechanical devices during the procedures

A large number of chemical substances (sugars, salts, enzymes, proteins), mechanical devices (incubator chambers, microscopes, air handling systems, filters, standard laboratory equipment) and disposable items (pipettes, petri dishes, flasks, microtools) are used during the laboratory procedures. There may be unknown risks associated with the use of any of these items that cause your procedure to fail, even though checks and quality control measures are performed on a regular basis. Thus far we do not know of any association between the use of these materials and anomalies of pregnancy and fetal development, but underlying unidentified problems may nevertheless

exist. An enzyme made from cow testis called hyaluronidase is routinely used to remove nursing cells from around the eggs, and there is a chance that this enzyme may inadvertently remove the zona pellucida (the layer surrounding eggs) and cause your procedure to fail. Another enzyme called chymotrypsin made from cow pancreas is used to reduce the viscosity of seminal fluid. This enzyme may also in very rare instances inadvertently remove the zona pellucida and also may cause your procedure to fail.

I - Risks associated with procedures

Based on current medical knowledge, we understand there does not appear to be a higher incidence of birth defects associated with IVF procedures. However, there is not at present sufficient statistical data available to definitively conclude that this is so. Therefore, we understand that IVF may impose risks to the fetus during development. We also understand that because more than one embryo or egg may be transferred, there may be a higher incidence of multiple births. An embryo may split when inside the uterus, forming monozygotic twins and there may be other associated anomalies . In certain cases, fetal reduction may be considered if more embryos implant that can be medically (or personally) deemed advisable to carry through a pregnancy. We also understand that ectopic or tubal pregnancies may occur in the procedure. These associated procedures can also produce increased financial and emotional burdens.

We understand and accept that the use of ovarian fertility drugs may be associated with an increased risk of ovarian diseases in later life, including cancer. We recognize that the exact risk, if any, has yet to be established and may not be known for many years.

J - Success rate and outcome

We understand that failure to obtain a pregnancy may result from many reasons, including the following:

Maturation of the egg(s) may not occur, or the time of the egg maturation may be misjudged, may not be predictable or may not take place in the monitored cycle.

Pelvic adhesions may prevent access to the ovary with the follicles, thus causing the procedure to obtain the egg from the ovary to fail.

The egg(s) obtained from the wife may be abnormal.

Normal spermatozoa may not be available.

Normal fertilization of the egg(s) by the sperm may not occur.

Cleavage or growth of the embryo(s) may not occur at any day of development or the embryo(s) may not develop normally.

The embryo(s) may become infected in the laboratory or an unforeseen laboratory accident may result in loss or damage to the eggs, sperm, or embryo(s).

The embryo(s) may become contaminated by infection in the semen or bacteria from the vagina.

Some embryos may not develop well in approved commercial culture medium despite standard testing.

Implantation of the embryo(s) in the uterus after embryo transfer may not occur or an early pregnancy may be lost after an initial positive result

Even if a pregnancy is established, we understand that delivery of a child may not occur due to miscarriage, ectopic pregnancy (outside the uterus), stillbirth, or other complications associated with pregnancy and delivery.

There may be unknown side-effects from any of the procedures used resulting in abnormal pregnancy or abnormal fetal development

We understand that the members of the IVF team cannot guarantee that a pregnancy will result from this procedure. Even in normally fertile couples, the chance of pregnancy is approximately 25% in a given natural cycle. If no pregnancy occurs, we may be offered participation in future cycles when assessment by the IVF team reveals no contra-indications. We understand that the IVF team cannot guarantee the normality of any infant that results from this procedure.

We understand that we may at any time decide to withdraw from participation in this program without prejudice. Any information obtained during this procedure and identified with us will remain confidential and will be disclosed only with our permission. Any publication resulting from this procedure will not identify us individually. Representatives of The Food and Drug Administration (FDA), The Center for Disease Control (CDC) and The Department of Health of New Jersey may inspect the records.

We have been encouraged to ask questions and any that we have asked have been answered to our satisfaction. A member of the IVF team will answer future questions.

Signed: Name female partner _____ Signature_____

Signed: Name male partner _____ Signature_____

Witnessed: Name _____ Signature_____

MD: Name _____ Signature_____

Date: _____

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Appendix B

The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center

Research utilization of discarded and abnormal gametes and embryos

A – You are invited to participate in research studies that make use of any extra spermatozoa, non-viable unfertilized eggs and non-viable embryos that may result from your attempt at in-vitro fertilization. Participation would in no way affect the normal progress of your procedure, nor your chances of pregnancy, nor later replacement of thawed embryos, as the study of sperm, eggs and embryos would not commence until completion of each of the steps necessary to realize your procedure, and intends to use only those spermatozoa which were not used and would otherwise be discarded, eggs that were not fertilized after insemination (and intra-cytoplasmic sperm injection or ICSI when applicable) and those embryos which could not be used for freezing or embryo replacement, all of which would otherwise be discarded.

B – Researchers at The Gamete and Embryo Research Laboratory of the Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center hope to develop new methods for gamete and embryo freezing, pre-conception and pre-implantation genetic diagnosis, new ways for gametes and embryos to develop and grow in-vitro, and new ways to isolate and grow cells from abnormal eggs and embryos in order to study the potential of embryos to produce so-called stem-cells. Stem-cells are cells that are not yet differentiated or changed into specialized cells such as muscle and blood cells. Embryos, even abnormal ones, may contain such cells. Their isolation and experimental growth is considered to be of fundamental importance for the progress of medicine, not least reproductive medicine, in the decades ahead. Stem cell based research offers the prospect of cures to many devastating diseases such as Juvenile Diabetes and Muscular Dystropohy. Further studies are urgently needed to improve the efficiency of assisted reproductive technology and other fields in medicine, and this is why we ask you to participate in these research studies.

C – None of the experiments will give rise to normal embryos at any stage, as only extra spermatozoa and unwanted ("non-viable"), eggs and embryos will be used. Indeed, were this possible, they would not have been "unwanted" in the first place, and would have formed part of your *in*

vitro fertilization procedure, either being immediately transferred or frozen. The research concerns only non-viable gametes and embryos, which are unusable and normally discarded. The research studies might include new processes of cell manipulation, surgery and culture that may extend the time in culture of gamete and embryonic cells for five days or more from the time of retrieval. This period is in fact unlikely to exceed seven days, when interaction with the uterus would normally be required for further development. None of the material in whole or part will ever be used to impregnate another woman or assist any other individual or couple than yourselves in any way to become pregnant. None of the isolated cells will ever be used for production of clinically normal stem-cells or be donated for use in other centers, corporations or for use in other individuals for the purpose of becoming pregnant or for curing genetic or other diseases.

D – Physicians and scientists at Saint Barnabas Medical Center expect that the research studies will result in development of beneficial new procedures for predicting IVF outcome, improving success rates and efficiency of assisted reproduction as well as the potential of cells, even abnormal ones, to develop into stem-cells. The discarded material will be used for one or more of the following studies:

The test of better cell surgery or micromanipulation techniques such as cell drills, lasers or tweezers or substances used to enhance the effect of cell surgery. These new tools are needed to either optimize or change current standard techniques such as assisted hatching, ICSI and biopsy.

The development of new methods needed to optimize the determination of genes and chromosomes in gametes and embryos

The development of better methods for sperm, unfertilized egg and embryo freezing

The development of new methods for combining male and female reproductive cells for couples that are otherwise unable to produce their own functional gametesThe development of new methods that allow for higher survival of gametes and embryos in-vitro and promote attachment to the uterus.

The development of new methods for the study of stem-cells.

E – Participation in these studies by donation of unwanted material will cause no additional physical discomfort whatsoever during the conduct of your procedure, nor will your chances of becoming pregnant be affected in any way. It is also unlikely that you will receive any personal benefits from participation at this time; however, it is likely that future patients will benefit from the information we will obtain from these studies.

F – This consent form is being offered to all couples treated with IVF at Saint Barnabas Medical Center. Any information obtained during this study and identified with you will remain confidential. The Food and Drug Administration (FDA) and The Center for Disease Control (CDC) in association with the Society for Assisted Reproduction (SART) may inspect the records. There are no added costs related to your participation in this study. Your decision whether or not to participate will not prejudice your future relations with Saint Barnabas Medical Center and the treatment you now undergo in this institute. If you decide to participate, you are free to discontinue participation at any time. Your participation is voluntary and your refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. If you have any questions regarding your rights as a research subject, please call the office of the IRB chairman, Dr. Goodman at (973) 322-5637. The principle investigator of this protocol is Dr. Jacques Cohen, who can be reached at 973-3226310. You may request a copy of this form.

G – **I**/We hereby attest that we have read the entire consent form, or that it has been read to us, so that we understand it completely. I/we further attest that any and all questions of mine/us regarding this form or this study have been answered to my/our complete satisfaction.

Signed: name female partner _____ Signature _____

Signed: name male partner_____ Signature _____

Witnessed: name _____ Signature _____

Date: _____

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IRB Consent Form

Publications

Alikani M, Cohen J, Tomkin G, Garrisi GJ, Mack C, Scott R. (1999) Human embryo fragmentation *in vitro* and its implications for pregnancy and implantation. *Fertility and Sterility* 71, 836-842.

Alikani M, Calderon G, Tomkin G, Garrisi J, Kokot M, Cohen J (2000) Cleavage anomalies in early human embryos and survival after prolonged culture *in vitro*. *Human Reproduction* 15, 2634-2643.

Alikani M (2001) Cytoplasmic fragmentation in human embryos *in vitro*: implications and the relevance of fragment removal. In Gardner D, Weissman A, Howles C, Shoham Z (eds) *Textbook of Assisted Reproductive Techniques, Laboratory and Clinical Perspectives.* Martin Dunitz, London, United Kingdom, pp. 169-182.

Alikani M, Sadowy S, Cohen J (2002) Human embryo morphology and developmental capacity. In Van Soom A and Boerjan M (eds) *Assessment of Mammalian Embryo Quality, Invasive and non-invasive techniques.* Kluwer Academic Publishers, The Netherlands, pp. 1-24.

Alikani M and Willadsen SM (2002) Human blastocysts from aggregated mononucleated cells of two or more non-viable zygote-derived embryos. *Reproductive Biomedicine Online* 5, 56-58.

Alikani M, Schimmel T, Willadsen SM (2005) Cytoplasmic fragmentation in activated eggs occurs in the cytokinetic phase of the cell cycle, in lieu of normal cytokinesis, and in response to cytoskeletal disorder. *Molecular Human Reproduction* 11, 335-344.

Alikani M (2005) Epithelial cadherin distribution in abnormal human pre-implantation embryos. *Human Reproduction* 20, 3369-3375.