Ultrastructural observations of enzymatically treated human blastocysts: zona-free blastocyst transfer and rescue of blastocysts with hatching difficulties

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Enzymatic treatment of the zona pellucida to either soften or remove totally the zona before blastocyst transfer has resulted in high implantation rates. The zona is usually completely dissolved after 1.5 min exposure with 10 IU pronase at 37°C. Since there may be concerns that pronase treatment for periods of 1.5 min or longer may cause adverse effects on the trophectoderm (TE) and inner cell mass (ICM), the changes to human blastocysts exposed to different time intervals of pronase were investigated. Of 18 blastocysts exposed to pronase for 1.5 min, the zona was completely dissolved and no changes were observed by light microscopy (LM) or transmission electron microscopy (TEM), compared with 11 naturally hatched untreated blastocysts (controls). In another five blastocysts exposed to pronase for 2 min, no LM changes were observed but subtle TEM changes such as fewer bundles of tonofibrils attached to desmosomes were observed. When three other blastocysts were exposed to pronase for 5 min, the blastocoele collapsed, and the TE cells started to show blebbing under LM. Under TEM, the cytoplasm of TE cells was extensively vacuolated and many TE cells showed cytoplasmic blebbing towards the blastocoele. However, the epithelium was uninterrupted with intact tight junctions and desmosomes. Of a separate group of 44 blastocysts cultured in vitro, 54.5% had hatching difficulties when monitored from day 5 to day 8 and 80% of these could be rescued by removal of the zona with pronase for 1.5 min prior to extensive degeneration taking place. The results confirm that the optimal time for softening or complete removal of the zona before transfer was around 1.5 min and that enzymatic treatment was a safe, non-invasive procedure to remove the zona of blastocysts. The human embryonic TE is a very hardy, robust epithelium that withstands pronase treatment.

Key words: blastocyst/hatching/human/pronase/zona-free

Introduction

There has been tremendous interest recently in improving the results of assisted reproduction through transfer of blastocysts after extended culture. Recent evidence suggests that pregnancy rates are quite high with blastocyst transfer ranging from 40 to 60% (Ménézo et al., 1992; Scholtes and Zeilmaker, 1996; Gardner et al., 1998a; Jones et al., 1998; Bongso et al., 1999). Blastocyst transfer also appeared to increase implantation and pregnancy rates in patients with many previously failed IVF cycles without increasing the risk of multiple pregnancy (Cruz et al., 1999). The transfer of two instead of three blastocysts reduced the multiple pregnancy rate and eliminated the risk of triplets. Both two- and three-blastocyst transfers produced similar pregnancy rates of 60% and similar implantation rates of 47%. Thus pregnancy outcome was not compromised by reducing the number of transferred blastocysts from three to two (Milki et al., 1999). Interestingly, more male than female infants were born after blastocyst transfer since male embryos had a faster cleavage rate, and no significant differences in birthweight were observed between infants born after blastocyst transfer compared with those born after spontaneous conception (Ménézo et al., 1999). However, blastocyst transfer necessitates the use of improved in-vitro systems that could consistently generate high blastulation rates so as to avoid disappointment to patients undergoing a day 5 transfer. This has been possible through the use of co-culture systems and the new generation sequential culture media combinations. We recently demonstrated that a culture protocol using the sequential IVF50-S2 media combination was a good substitute for Vero cell co-culture for the transfer of viable day 3-6 human embryos (Fong and Bongso, 1999). Blastulation rates as high as 68.5% in an IVF50-S2 sequential combination (Fong and Bongso, 1999) and 69.0% in another sequential media combination (G1.2-G2.2) (Gardner et al., 1998b) have been reported. Very high total cell numbers (TCN) ranging from 161 to 311 were reported for expanded and hatching blastocysts in these new in-vitro systems, suggesting an improvement in embryo viability (Fong and Bongso, 1999).
Blastocyst transfer after enzymatic treatment of the zona pellucida, to either soften or remove the zona totally before transfer, did not reduce the high implantation rates observed with zona-intact blastocyst transfer but has been expected to bring about closer contact and communication of the trophectoderm (TE) with the endometrium thereby improving implantation. In 19 women who had several repeated attempts and a mean of 2.5 ± 0.7 zona-softened or zona-free blastocysts replaced, clinical pregnancy rate/cycle, implantation and multiple pregnancy rates of 53, 33 and 40% were recently observed by our group (Fong et al., 1997, 1998). Similar improved results were shown with zona-free blastocyst transfers by another group (Jones et al., 1998). The results reflected improved embryo viability, better in-vivo hatching when the zona was softened and perhaps closer contact and communication of blastocyst and endometrium in the absence of the zona, thus improving implantation. In all these studies the zona pellucida was softened or totally removed with pronase at concentrations of 10 IU/ml for exposure periods of no longer than 1.5 min. Interestingly, in the mouse it was postulated that prior to implantation in vivo, a protease-like enzyme was released either from the blastocyst itself or the endometrium, to assist hatching and subsequent implantation (McLaren 1970; Cohen et al., 1992).

Observations by light microscopy (LM) showed that when the zona was completely removed with 10 IU/ml of pronase, none of the cells of the TE was disrupted prior to or while loading the catheter at the time of transfer and the pronase-treated blastocyst remained expanded. Furthermore, a significant number of human blastocysts have hatching problems with the subsequent risk of collapse of the blastocoele (A.Bongso et al., unpublished data), and these could hopefully be rescued by immediately removing the zona completely with pronase with subsequent re-expansion of the blastocoelic cavity. Since there may be inherent fears that the exposure of blastocysts to pronase for periods 1.5 min or longer may cause adverse effects on the embryo and disturb subsequent implantation, a study was undertaken to investigate the LM and ultrastructural changes of human blastocysts exposed to pronase for different time periods.

Materials and methods

Patients and embryos

A total of 37 blastocysts from 30 patients were donated to study the effects of pronase treatment at different time intervals. A separate group of 44 blastocysts was monitored under LM for growth and development from day 5 to day 8 to observe hatching problems and rescue with pronase treatment. All blastocysts were spare embryos after adequate numbers were replaced and frozen for the patients. The patients were treated by ovulation induction with a down-regulation protocol of gonadotrophin-releasing hormone agonist (Suprefact; Frankfurt, Germany) followed by recombinant FSH (rFSH) (Gonal F; Serono, Geneva, Switzerland). Folliculogenesis was monitored by ultrasonography and rising oestradiol concentrations. When one dominant follicle reached 17 mm and two reached 16 mm, a dose of 5000 IU of human chorionic gonadotrophin (HCG) (Profasi; Serono) was administered to trigger ovulation. At 36 h after HCG, oocytes were recovered transvaginally using ultrasound guidance and ASP250 flushing medium (Scandinavian IVF Science AB, Gothenburg, Sweden). ASP250 is a human tubal fluid basal medium supplemented with heparin and HEPES buffer. All mature cumulus-oocyte complexes (COC) were washed in IVF50 medium (Scandinavian IVF Science AB) and each COC pre-incubated for 3–6 h in 0.5 ml IVF50 medium without oil in 5 ml loosely capped tubes at 37°C in a 5% CO₂ in air atmosphere.

Motile spermatozoa separated by a 40:70-90 discontinuous colloidal silica gel triple gradient (PureSperm, Nidacon International, Gothenburg, Sweden) were used to inseminate oocytes or for intracytoplasmic sperm injection (ICSI) (Ng et al., 1996). For insemination, each COC was introduced into 100 µl sperm droplets in IVF50 medium containing 10 000 motile sperm under oil (Ovoil; Scandinavian IVF Science AB, Gothenburg, Sweden) in small Petri dishes and incubated at 37°C in a humidified modular chamber (Billups–Rothenburg, Delmar, CA, USA) with a controlled environment of a 5% CO₂, 5% O₂ and 90% N₂ gas atmosphere. The modular chambers were housed in large 5% CO₂ in air incubators (Heraeus, Hanau, Germany).

At 1 h after insemination, the COC with minimal cumulus were washed in droplets of IVF50 medium (Scandinavian IVF Science AB) and each then transferred to 20 µl of IVF50 under oil and incubated overnight. At 16–20 h post insemination, the COC were mechanically denuded and fertilization determined by the presence of two pronuclei and two polar bodies.

Growth of embryos and embryo scoring

Bipronucleate (2PN) embryos were cultured singly in 10 µl of G1.2 medium (Scandinavian IVF Science) under oil. Prior to incubation, each 2PN embryo was scored for polarized parameters such as pronuclear abutment, nucleoli alignment and presence of cytoplasmic halo according to the criteria of Scott and Smith (1998) and Tesarik and Gregoor (1999). At 24 h post insemination, each 2PN embryo was scored for the first cleavage to the 2-cell stage and later at 48 h scored for the incidence of fragmentation and blastomeric regularity at the 4–5-cell stage. Every 24 h the embryos received fresh triple gas in the modular chambers. On the morning of day 3, each embryo was rinsed twice in drops of G2.2 medium (Scandinavian IVF Science) and incubated in 10 µl of fresh G2.2 medium for a further 48 h. The embryos were scored on day 3 for 8-cell and compacting stages, and on day 4 for compacting, compacted and early cavitating stages (Fong and Bongso, 1999).

On the morning of day 5, prior to blastocyst transfer, each embryo was transferred to fresh 10 µl drops of G2.2 medium. Good quality, late cavitating embryos and blastocysts (early, expanding) generated from high 2PN scores were given priority in selection for transfer. Other morphological markers for blastocysts such as the size of the inner cell mass (ICM), thinness of the zona pellucida and ‘sickle-shaped’ appearance of the TE cells (Bongso et al., 1999) were also used when selecting the best blastocysts for transfer. A total of up to three embryos was replaced on day 5 and spare blastocysts were frozen. Patients who did not opt for freezing donated their blastocysts for this study. All spare blastocysts used in this study were of very good quality.

Pronase treatment

Of a total of 37 blastocysts, 26 were exposed to pronase for different time durations for LM and transmission electron microscopy (TEM) studies and 11 naturally hatched blastocysts used as controls without pronase treatment. All embryos were carefully monitored under Hoffman’s inverted optics with a 37°C heated stage. On day 5 afternoon, each blastocyst from the pronase treatment group was exposed to 10 IU/ml pronase (Protease P8811, embryo tested; Sigma, St Louis, MO, USA) in G2.2 medium under oil at 37°C in a 5% CO₂
in air atmosphere to remove the zona pellucida (Fong et al., 1997). Eighteen blastocysts were exposed to pronase for 1.5 min, five for 2 min, and three for 5 min. The zonae of all pronase-treated blastocysts had dissolved at the end of the treatment and the zona-free blastocysts were washed four times in G2.2 medium before being fixed. All observations of the effects of pronase treatment were also recorded using Hoffman’s inverted optics.

A separate group of 44 blastocysts was monitored under LM for growth and development on days 5 to 8 to observe hatching problems. Those that showed hatching problems (see below) were immediately exposed to 10 IU/ml of pronase for 1.5 min to remove the zona pellucida before extensive degeneration occurred, then washed in G2.2 medium under oil and monitored for further development.

**Transmission electron microscopy**
The 26 blastocysts exposed to pronase for different time intervals were washed four times in G2.2 medium before being fixed routinely in 3% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.3) at 22°C (Sathananthan, 2000). The blastocysts were postfixed in 1% aqueous osmium tetroxide, dehydrated rapidly through a series of alcohols and acetone, and embedded in Araldite. Semithin (1 μm) and ultrathin (~70 nm) sections were cut with glass and diamond knives. Semithin sections were stained with 1% toluidine blue while ultrathin sections were stained with alcoholic uranyl acetate and Reynolds’ lead citrate solutions respectively and examined by TEM.

**Results**

**Light microscopy**

**Blastocyst hatching in vitro**

Approximately 54.5% (24/44) of blastocysts in patients aged 27–39 years showed hatching difficulties on the morning of days 6, 7 and 8 in extended culture. Of the 45.5% (20/44) that hatched with no problems, 13.6% (6/44) completely hatched on day 6 while 31.8% (14/44) completely hatched on day 7. The difficulty in hatching did not necessarily arise from a thin or thick zona pellucida. Blastocysts having difficulties in hatching were grouped as follows: (i) expanded blastocysts where the zona did not open and the TE retracted from the inner lining of the zona; the blastocoele shrank in size with subsequent arrest and degeneration; (ii) blastocysts where the zona opened but the TE retracted; the blastocoele shrank and the embryo underwent arrest and degeneration; (iii) blastocysts with initial hatching followed by no further development but arrest and degeneration; (iv) blastocysts that showed ≥50% or more of hatching followed by arrest and subsequent degeneration. As soon as difficulties in hatching were observed and before degeneration occurred, the blastocysts were immediately treated with pronase to remove the zona, and 80% of such blastocysts re-expanded in 4–5 h and were rescued. Approximately 2% (1/44) of blastocysts had abnormal hatching at two or three points in the zona pellucida. When this blastocyst was monitored to day 8, it degenerated.

**Blastocysts treated with pronase**

Of the 18 good quality blastocysts treated with pronase for 1.5 min to remove the zona pellucida, all of them retained their blastocoelic cavities, the zona gradually dissolved, and the blastocoele did not collapse until fixation. Also, there were no obvious changes observed in the TE and ICM. No drastic changes were observed in the five blastocysts exposed to pronase for 2 min. However, in all three blastocysts exposed to pronase for 5 min, the zona pellucida had dissolved and slight blebbing of the TE cells was observed. All three blastocysts had blastocoele collapse. Of the 11 blastocysts that hatched naturally in culture without pronase treatment (controls), nine remained expanded while two collapsed in culture. However, both naturally hatched expanded and collapsed blastocysts appeared very healthy.

**Transmission electron microscopy**

Naturally hatched blastocysts (no pronase treatment) (Figures 1 and 2)

These were used as controls in this study. The two collapsed blastocysts had folded or invaginated TE epithelium. The TE cells were cuboidal or low columnar, similar to those at the embryonal pole. The nine expanded blastocysts in this group had predominantly flat squamous TE cells forming a continuous epithelium with specialized cell junctions. The ICM of expanded and collapsed blastocysts was small to large and had cells closely attached to one another with occasional desmosomes and gap junctions. The specialized cell junctional complexes in the TE were typical of epithelia, consisting of outer tight and adherent junctions, desmosomes and sometimes gap junctions, extending towards the blastocoele. Intermediate filament bundles anchored onto desmosomes and also appeared within thin, attenuated extensions of TE cells, adjoining the cell junctions. Bundles of intermediate filaments were also evident in ICM cells. Primary endoderm cells, delaminated from the ICM, were usually vacuolated (phagocytic vacuoles) and had begun to migrate downwards along the sides of the blastocoele. TE cells had few too many microvilli (MV) on their free surfaces, which frequently interdigitated to increase cell-cell attachments at cell junctions. All cells including endoderm cells beneath the ICM had nuclei with granular, reticulated nucleoli and oval to tubular mitochondria with well-defined cristae, characteristic of blastocysts. Other organelles were rough endoplasmic reticulum (RER), Golgi complexes that were sometimes extensive, lysosomes and multivesicular bodies and occasional centrioles associated with microtubules (MT). The remaining cytoplasm was finely granular with occasional translucent patches. Ribosomes were free or attached to the RER. Spherical lipid globules were present in many cells. Dividing cells in mitosis were rare and had dense chromosomes on bipolar spindles associated with polar centrioles with pericentriolar material, nucleating MT (typical centrosomes). A few cells were degenerating in the ICM, while fragments of early cleavage stages were evident in the blastocoele. These had rounded to oval, dense mitochondria, quite distinct from those in blastocyst cells.

**Blastocysts treated with pronase for 1.5 min (Figures 3 and 4)**

All 18 blastocysts showed little or no change in fine structure compared with the controls. Surface MV and cell junctions were well preserved and so were the cells of the TE.

**Blastocysts treated with pronase for 2 min (Figures 5 and 6)**

Like those treated with pronase for 1.5 min, the surface MV and cell junctions of these five blastocysts were well preserved...
Figures 1 and 2. Transmission electron micrographs of naturally hatched blastocysts. Figure 1. Blastocyst with a small inner cell mass (i). The trophectoderm (TE) cells (t) have few to many surface microvilli (MV) while endoderm cells (e) have clear vacuoles. All cells have nuclei with reticulated nucleoli. Magnification ×3500. Figure 2. Extended squamous TE cell showing cellular organelles. Note few surface MV. g = Golgi complex; m = mitochondria; n = nucleus. Magnification ×3500.

Figures 3 and 4. Transmission electron micrographs of blastocysts treated with pronase (1.5 min). Figure 3. Squamous trophectoderm (TE) cell of an expanded blastocyst. Note surface MV. l = lipid globule; m = mitochondria; n = nucleus. Magnification ×3500. Figure 4. Normal cell junction between extended TE cells composed of a tight junction associated with a microvillus, desmosome anchoring intermediate filaments (arrow) and interdigitating MV. b = blastocoele; l = lipid globule. Magnification ×6500.

and so were the cells of the TE. Microfilaments formed a terminal web in the TE epithelium and also formed an actin core in surface MV.

Blastocysts treated with pronase for 5 min (Figures 7 and 8) To appreciate the changes in cell structure that might occur after prolonged pronase treatment, blastocysts were examined after 5 min exposure to the enzyme. This treatment was too drastic, as expected. Cytoplasm of some trophoblast cells was extensively vacuolated while many cells showed severe blebbing of cytoplasm towards the blastocoele. A few TE cells had lesions damaging the cell membrane and outer cytoplasm, including cellular organelles. Furthermore, some of the cell junctions were somewhat disorganized. However, the epithelium was uninterrupted since tight junctions and desmosomes are difficult to separate, unless traumatized during embryo handling. Most of the TE cells of one expanded blastocyst were minimally effected by this treatment and hence the effects of enzyme treatment were variable.

Discussion
Observations on the problems of in-vitro hatching in human embryos on days 5-8 are reported for the first time. The high percentage of blastocysts with hatching difficulties in vitro
Figures 5–8. Transmission electron micrographs of blastocysts treated with pronase (2 and 5 min). Figure 5. Typical expanded blastocyst showing thin extended trophectoderm (TE) epithelium (2 min pronase). b = blastocoele; j = cell junction; n = nucleus. Magnification ×3500. Figure 6. Slightly contracted blastocyst showing cuboidal TE cells in epithelium (2 min pronase). Note surface MV and cell junctions. b = blastocoele; m = mitochondria; n = nucleus. Magnification ×3500. Figure 7. Blastocyst showing internal blebbing of TE with extensive vacuolization (v) of cells (5 min pronase). Note MV on surface. b = blastocoele. Magnification ×3500. Figure 8. TE cells (5 min pronase) with cell junctions and MV intact. Note extensive vacuolization (v) of cytoplasm. b = blastocoele; d = degenerating cell; j = cell junction. Magnification ×3500.

(54.5%) suggests that the improved sequential media culture conditions are unable to support this physiological phenomenon, although pronase-treated or naturally hatched blastocysts continued to grow quite well from day 6 onwards as confirmed from increasing total cell numbers (Fong and Bongso, 1999) and invasion of feeder layers for embryonic stem cell production (Bongso et al., 1999). Pronase appears to be a safe and harmless enzyme than can assist the process of hatching and rescue embryos for IVF patients and for studies on embryonic stem cells when used at the right concentration and duration. It appears that further improvements in the existing sequential culture media formulations may be necessary to support blastogenesis from day 5 to day 8. Perhaps future studies on the inclusion of pronase or other enzymes at appropriate concentrations into the existing sequential culture media formulations may facilitate this process and will be less labour-intensive than the manipulation of a day 5 blastocyst for 1.5 min pronase exposure, followed by washing of the embryos before transfer.

The first delivery of a normal healthy baby after enzymatically treated zona-free blastocyst transfer was by our group (Fong et al., 1997). Following this report, the results of two independent studies on larger groups of patients clearly demonstrated that implantation and delivery rates were not compromised and were higher than the conventional day 2 transfers (Fong et al., 1998; Jones et al., 1998). However, no
prospective randomized controlled studies have been undertaken to date to compare the implantation rates of zona-intact with zona-softened/zona-free blastocyst transfers.

In ongoing separate studies on the propagation of embryonic stem cells, where pronase-treated expanded zona-free blastocysts (with ICM and TE intact) were grown on a variety of feeder monolayers (vexo cells, human tubal epithelial, human endometrial epithelium, murine fibroblasts), ~93% of them attached tightly, spread out and invaded the feeder layers simulating the implantation process (Bongso et al., 1999).

This, therefore, suggested that enzymatic treatment of the blastocyst with pronase did not compromise the mitotic activity and growth of the ICM and TE cells on the feeder layers.

The concept of assisted hatching was first suggested by Cohen et al. (1990). Assisted hatching by zona drilling using acidic Tyrode’s solution and micromanipulation carried out during three randomized trials on day 3 on 330 IVF patients showed that implantation in the selectively zona-drilled group was significantly higher than in the control group (Cohen et al., 1992). Furthermore, zona-slitting increased the rate and number of blastocyst hatching compared to non-manipulated controls (Dokras et al., 1994). The laser beam was also used successfully to thin and drill holes in the zonae of mouse oocytes to improve hatching (Antinori et al., 1996). Recently, Ding et al. (1999) successfully carried out intracytoplasmic sperm injection on zona-free oocytes and obtained growth in vitro on ensuing embryos up to the blastocyst stage. However, despite all these favourable reports, the negative aspects of assisted hatching were reported recently where the incidence of monozygotic twinning was shown to be increased significantly by 1.2% per embryo transfer compared with natural pregnancies (Hershlag et al., 1999). It was postulated by these authors that after the assisted hatching process was carried out on day 3, the ICM subsequently splits into two when attempting to hatch out of the narrow hole drilled with the acid Tyrode’s solution or the laser beam. It is interesting to note that in the series of 40 patients who underwent pronase softening or total removal of the zona before blastocyst transfer in our programme (which in fact is not true assisted hatching), no incidence of monozygotic twins was noted. It thus appears that the complete removal of the zona may overcome the problem of the increased incidence of monozygotic twinning.

The naturally hatched blastocysts (controls) in this study showed similar ultrastructural fine details previously described for co-cultured and non-co-cultured blastocysts (Sathananthan et al., 1993, 1999). It is very clear from the observations of TEM in this study that treatment of human blastocysts with pronase is a very safe procedure. The TEM investigations clearly showed that the surface of TE cells were not affected by the routine pronase treatment for 1.5 min for zona softening or removal which has produced high success rates in terms of ongoing pregnancies (Fong et al., 1997, 1998). Even exposure to 2 min of pronase treatment caused only subtle changes. The re-expansion after collapse of the blastocoel cavity in some pronase-treated embryos followed by subsequent ongoing pregnancies after transfer demonstrates the powerful healing and repair processes in the TE. It appears that the TE of the human blastocyst is a robust, tight epithelium quite resistant to adverse conditions in its environment, thus protecting the ICM within. Since we had some concerns about the integrity of this surface epithelium (TE), morphological confirmation of good preservation of blastocyst microstructure after enzyme treatment was needed. The integrity of the trophoblast is vital to the development of the syncytiotrophoblast, which ultimately invades the endometrium on days 7–10 of development during implantation. During its sojourn in the uterus the blastocyst presumably expands, stretching the zona as it imbibles more and more fluid into its blastocoel. Blastocoel expansion evidently causes stretching of the TE epithelium, which becomes thin and squamous with bundles of intermediate filaments extending towards the cell extremities coupling at cell junctions via desmosomes. These filaments are thought to preserve the structural integrity of the epithelia and have different functions. The full expansion of the blastocyst facilitates a breach in the zona, thus initiating hatching. The process is probably completed as in the mouse by the release of a protease enzyme similar to pronase from either the blastocyst itself or the maternal endometrium subsequently encouraging implantation. (McLaren et al., 1970; Cohen et al., 1992). In the absence of such enzymes in vitro, it is possible that changes in the composition of the zona occur which result in hardening and difficulties in hatching.

Although blastocyst transfer may have several advantages, there have been recent suggestions that human embryos are polarized and that selection of embryos with high polarized scores may also eventually improve pregnancy outcome (Antczak and Van Blerkom, 1997; Edwards and Beard, 1997). The parameters usually used to score human embryos have been pronuclear abutment, nucleoli alignment, cytoplasmic halos and the early first cleavage to the 2-cell stage (Scott and Smith, 1998; Tesark, 1999). Although implantation rates of blastocyst and pronuclear stage transfer are similar, Scott and Smith (1998) confirmed that the only negative aspect of pronuclear or early 2-cell stage transfer was the fact that more polarized embryos had to be transferred (Scott and Smith, 1998). If assisted reproduction treatment is to reduce higher order multiple pregnancies by the transfer of only two embryos, then blastocyst transfer may be more advantageous than pronuclear or early 2-cell stage transfers. Until such time as prospective randomized controlled studies have proven the benefits of pregnancy outcome by either protocol, it has been decided to merge the two systems in our assisted reproduction programme by selecting for transfer individual blastocysts that are generated from high pronuclear, 2-cell stage polarized scores. Thereby we have been successful in maintaining a consistently viable implantation rate of 30–35% and the blastocysts used in this study were actually generated from such merged scoring systems.

References


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