Article

Pronuclear scoring as a predictor of embryo development

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Abstract

Many strategies have been proposed for the selection of viable embryos for transfer in human assisted reproduction. These have included morphological scoring criteria for day 1, 2, 3 and 5 embryos or combinations of these. Other strategies have used predictors such as timing of certain key events, as with early cleavage to the 2-cell, development to the 8-cell stage or patterns of fragmentation. All have shown some correlations with implantation. However, the overall success of these methods is still limited, with over 50% of all transferred embryos failing to implant. The use of pronuclear oocyte morphology has shown correlations with implantation and development to the blastocyst stage. The key aspects of pronuclear scoring, namely the presence of a cytoplasmic halo, the orientation of the nuclei in relation to the polar bodies and the size, number and pattern of distribution of nucleolar precursor bodies (NPB) in the nuclei were related to day 2,3 and 5 development, rate of development and day 3 and 5 morphology in a retrospective study. The pattern of the NPB or Z-score and the presence/absence of a halo had a significant effect on the rate of development on day 3 and day 5 and on the overall embryo morphology score. Low Z-score resulted in slow development, poor blastocyst formation and low morphology scores. The absence of a halo also resulted in slow and poor development, poor morphology, increased fragmentation and increased numbers of poor Z-scored embryos. The use of PN scoring can help predict embryos that have poor developmental potential, aid in early selection and may indicate the health of the oocyte.

Keywords: assisted reproduction, cytoplasmic halo, development, human embryos, nucleolar precursor bodies, pronuclear morphology

Introduction

The practice of human assisted reproduction is wide spread both in geographical location and in the types of conditions being treated. Over the last 20 years the treatments offered under the title of assisted reproduction have expanded from simple bypass of tubal blockage to all forms of female and male reproductive dysfunction and third party assisted reproduction. The use of assisted reproduction technology coupled with genetics is being used to alleviate tragic genetic conditions and allow couples the comfort of a healthy pregnancy. Along with this expansion have been many improvements in the drugs used for ovarian stimulation, the harvesting of oocytes and the replacement of embryos. Understanding the metabolic needs of the gametes and embryos has also lead to great improvements in the culture conditions utilized in the laboratories. These improvements have contributed to an increasing clinical pregnancy and delivery rate and, to a limited extent, an increased implantation rate. However, with these increases has come a growing number of twin and high order multiple pregnancies (Racowsky, 2002). This is due to multiple embryos being used in transfers coupled to the improvements. Limitation of the number of twin and high order multiple (HOM) pregnancies (>2) requires single embryo transfers.

To achieve this goal, a reliable method of selecting the most viable embryo from a cohort that results from stimulation cycles is required. To date, this has not been particularly successful. The reported implantation rates per embryo transferred range from the single digits to a maximum of 40% in overall cycles or 60% in highly selected groups of patients. This implies that between 40 and 90% of all embryo transferred are non-viable.

With the development of media that allowed embryos to progress *in vitro* to the blastocyst stage (Gardner and Lane, 1997), there was initially a reported increase in implantation (Dokras *et al.*, 1993; Gardner and Lane, 1997; Gardner *et al.*, 1998; Scholtes and Zeilmaker, 1998; Huisman *et al.*, 2000; Scott *et al.*, 2000; Shapiro *et al.*, 2002). However, this was in a highly selected group of patients. In wider or unselected groups, the clinical pregnancy rates may be higher but there is little difference in implantation (Gardner *et al.*, 1998; Marek *et al.*, 1999; Coskun *et al.*, 2000; Wilson *et al.*, 2002). Further, the rate of aneuploidy in blastocysts is high in many instances (Magli *et al.*, 2000; Sandalinas *et al.*, 2001), which may account for this. In a study in which natural cycle oocytes were retrieved and transferred on either day 2 or day 5, there was an increase in implantation rate per embryo transferred on day 5 versus day 2 (40 versus 24%) but only 55% of embryos attained the blastocyst stage and the implantation rate per oocyte was no different (14 versus 12%) (Vlaisavljevic *et al.*, 2001).

All these data suggest that very few oocytes actually have the potential to implant. If this were indeed the case, it would be ideal to select these at the earliest time. The first point at which developmental potential can be screened is the aspirated oocyte. Prior to this, the development of the follicle is used as a measure of maturity of the oocyte. However the parameters used, namely follicle size and oestradiol production, are very inexact and cannot predict any individual oocyte's potential. This is well documented with the retrieval of immature oocytes, failed fertilization (Bedford and Kim, 1993), and the lack of activation of the oocyte with sperm entry (Van Blerkom *et al.*, 1987). Further, during culture the embryos may display differential developmental capabilities, again pointing to differences in the oocytes or spermatozoa.

When oocytes are aspirated, they are encased in the cumulus cells, prohibiting much morphological evaluation. The degree of cumulus expansion has been used as an estimate of maturity, although the only definitive indicator is the presence of the first polar body, which results from the completion of the first meiotic division. During ICSI cycles the cumulus cells are removed, allowing visualization of the oocyte. There have been a number of reports correlating various oocyte morphology parameters with developmental potential (Serhal *et al.*, 1997; Loutradis *et al.*, 1999), while others report no correlation due to the heterogeneity of oocytes (De Sutter *et al.*, 1996; Balaban *et al.*, 1998). The first polar body naturally begins to disintegrate at about 20 h after extrusion. Early disintegration, probably due to early release of the polar body through disrupted events during meiosis I (Scott, 2002c), has been implicated in decreased development and implantation (Ebner *et al.*, 1999, 2000, 2002). During IVF cycles, none of these parameters can be observed. The earliest stage at which the newly formed embryo can be assessed is at the pronuclear oocyte stage.

Differential morphology of human 1-cell pronuclear oocytes was first described in terms of the nuclei morphology and the cytoplasmic movements, and retrospectively correlated with implantation potential (Payne *et al.*, 1997; Scott and Smith, 1998). The concept of 1-cell pronuclear oocyte scoring was further developed and patterns were described that directly and prospectively correlated with implantation (Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al.*, 2000). The use of either day 1 (Scott and Smith, 1998) or day 2/3 embryo transfers using embryos selected by pronuclear morphology resulted in increased implantation rates with fewer embryos used in transfer (Ludwig *et al.*, 2000; Wittemer *et al.*, 2000). Pronuclear scoring was further used to enhance day 3 morphology scoring (Scott *et al.*, 2000; De Placido *et al.*, 2002; Rienzi *et al.*, 2002; Zollner *et al.*, 2002), and correlated with blastocyst development (Scott *et al.*, 2000; Balaban *et al.*, 2001; Rienzi *et al.*, 2002; Zollner *et al.*, 2002). The source of spermatozoa in intracellular sperm injection (ICSI) cases was also shown to influence the pronuclear oocyte score and thus implantation potential (Demirel *et al.*, 2001; Tesarik *et al.*, 2002).

Pronuclear oocyte scoring relies on three major features: the size and location of the nuclei, the appearance of the cytoplasm and the numbers, sizes and distribution patterns of the nucleolar precursor bodies (NPB) in the nuclei (Tesarik and Greco, 1999; Scott *et al.*, 2000; Scott, 2002, 2003). This paper will present the developmental data of >3000 pronuclear embryos scored for all three characteristics. The relevance of the different parameters will be discussed in terms of the biology of development, the developmental potential of the different forms of pronuclear oocytes and how this is correlated with day 3 and day 5 morphological scoring.

Pronuclear scoring

Abnormal nuclei

When scoring a pronuclear oocyte, the nuclei should be of equal size and centrally located. If they are not of equal size, the resulting embryos have little developmental potential (Scott *et al.*, 2000; Scott, 2002c, 2003) and have a high degree of aneuploidy (Munné and Cohen, 1998; Sadowy *et al.*, 1998). Lack of a central location could indicate a failure of some mechanism in the fertilization process, such as aster or microtubule formation (Sathanathan *et al.*, 1988; Schatten, 1994). Pronuclear oocytes that present with any one of unequal sized nuclei, nuclei displaced to the periphery of the cell or nuclei that have not come together by 16–18 h after insemination are scored as abnormal and rarely used in transfer (**Figure 1**).

Cytoplasmic streaming or halo

It has been recognized that there is differential cytoplasmic appearances in pronuclear oocytes from many species. In both the mouse and the hamster, the dense areas that appear after fertilization have been attributed to redistribution of mitochondria (Muggleton-Harris and Brown, 1988; Barnett *et al.*, 1996). A similar pattern of differential cytoplasmic distribution was reported for human pronuclear embryos (Payne *et al.*, 1997; Scott and Smith, 1998) and shown to be associated with mitochondria redistribution (Van Blerkom and Runner, 1984; Van Blerkom *et al.*, 2000, 2002). The normal

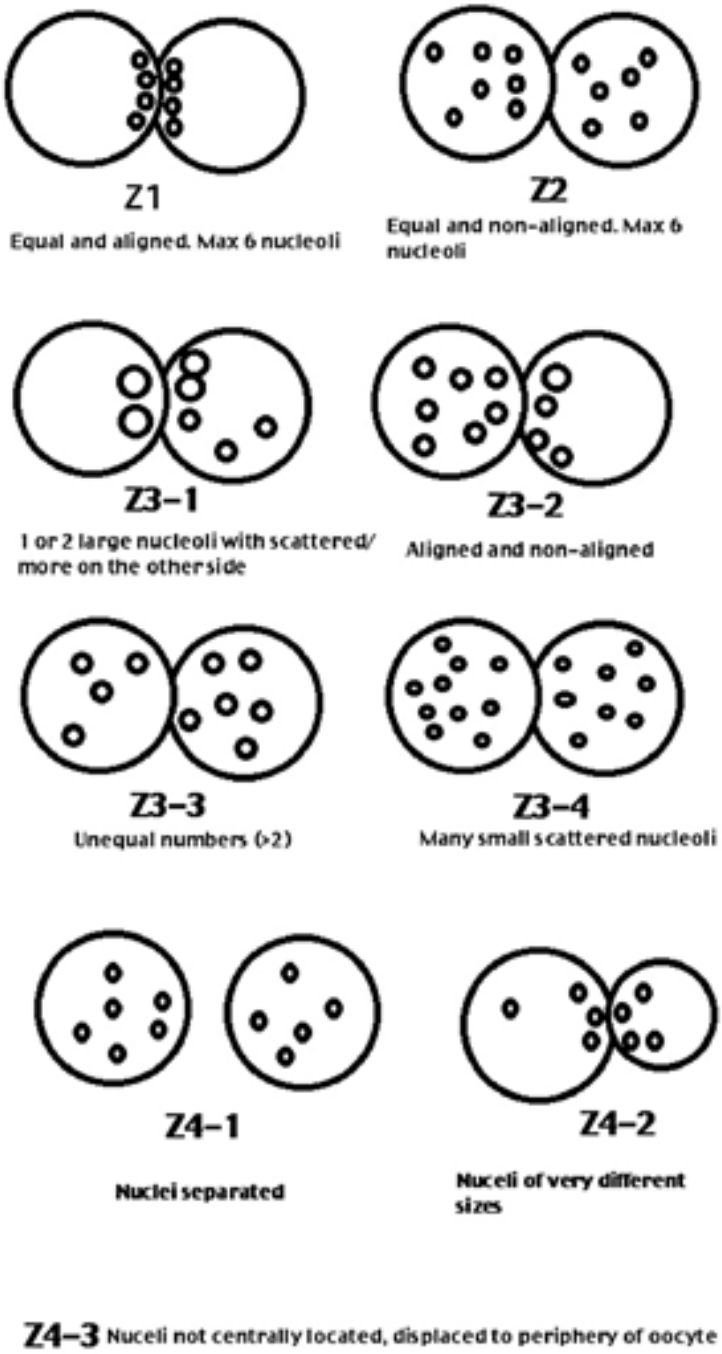

Figure 1. Schematic representation of Z-scoring in which the numbers, sizes and alignment of the nucleolar precursor bodies are considered.

pattern of mitochondria distribution has the bulk of mitochondria around the nuclei with a clearing in the pericortical area (Van Blerkom *et al.*, 1995a).

After decondensation of the fertilizing spermatozoon, the aster forms from the sperm centrosome, directing microtubule growth, which is also accompanied by a 'flare' in the cytoplasm (Payne *et al.*, 1997) (**Figure 2A**). When the male pronucleus forms, the chromatin decondenses on the outside, or the side facing the oolemma. The female has condensation on the side facing into the oocyte (**Figure 2B**). For correct alignment of the male and female chromatin, the male nucleus rotates onto the female pronucleus, also placing the centrosome into the furrow between the 2 nuclei (Van Blerkom *et al.*, 1995a) (**Figure 2B**). This is necessary for separation of the chromatin during the first mitotic division (**Figure 2**). Finally, the nuclei need to be aligned onto the polar axis for the completion of the mitotic division, resulting in the 2-cell embryo and completion of fertilization (Edwards and Beard, 1997, 1999; Payne *et al.*, 1997; Garello *et al.*, 1999; Scott, 2001) (**Figure 2C**). This alignment is essential, since the position of the second polar body defines the polar axis and the plane of the first cleavage division (Gardner, 1996, 1999, 2001) (**Figure 2D** and **Figure 3**).

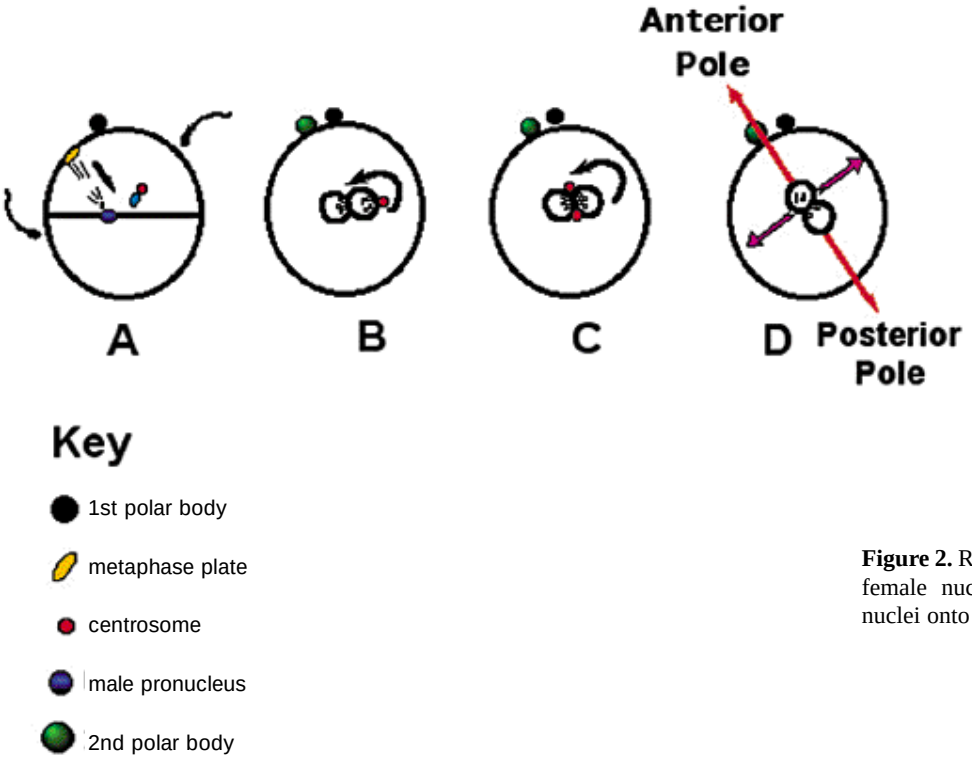

Figure 2. Rotation of male nucleus onto female nucleus and rotation of both nuclei onto the cleavage axis.

All of these movements and rotations could be contributing to the 'halo' or flare effect seen in human pronuclear oocytes in assisted reproduction technology labs (**Figure 3**). The consequences of a lack of movement could be multiple. If the mitochondria do not localize to the correct area, they could be unequally distributed to blastomers in the cleaving embryo, resulting in depleted ATP production in some cells (Van Blerkom *et al.*, 1995b, 2000). If the male pronucleus does not rotate, the chromatin will not be aligned and/or the centrosomes will be in the incorrect position (Tesarik *et al.*, 2002), again leading to abnormal development. Finally, if the nuclei are not correctly positioned on the polar axis, abnormal development will result.

The existence of the halo as evidence of this movement was recorded at 16–18 h post-insemination. The orientation of the nuclei on the polar axis (**Figure 3**) was recorded as perpendicular thus requiring rotation (**Figure 3a**) or parallel, having already rotated onto the axis (**Figure 3b**). Sometimes the nuclei were in a transition state, and were neither parallel nor perpendicular (**Figure 3c**, **d**). If the rotation had just begun (<30°off the horizontal), they were designated perpendicular or right angles. If they had rotated halfway or more, they were designated parallel. Both parameters were related to development on days 2, 3 and 5.

Nucleolar precursor bodies (NPB)

Within the nucleus of all mitotically active cells are nucleoli, the sites where ribosomal genes are transcribed and which are therefore essential for protein synthesis. Nucleoli appear and disappear with the cell cycle, there are generally between 2–7 per human nucleus with equal numbers in the 2 daughter cells in a mitotic division. The nucleoli develop at sites known as 'nucleolus organizing regions' (NOR), which are located on the chromosomes at points where the genes coding for ribosomal RNA (rDNA) are located. These genes are in tandem repeated sequences and function as the sites for pre-RNA and pre-ribosome synthesis. Nucleoli are comprised of a dense fibrillar component (DFC), a fibrillar centre (FC) and a granular component (GC). The transcription of rDNA requires the DF but not the FC. Transcription of the rDNA is restricted to foci on the DNA and the FC act as structural centres or sites for transcription and store inactive transcription factors, which initiate rDNA transcription. The GC is a group of pre-ribosomes (Goessens, 1984; Schwarzacher and Mosgoeller, 2000).

During a mitotic cell cycle, there are more nucleoli present at the beginning of the cell cycle (G1 phase); they then fuse as the cell cycle progresses and at the S1 phase there are only 1–2 large nucleoli per nucleus (Goessens, 1984). There is synchrony in both number and form of fusion of the nucleoli in the daughter cells, with asynchronous fusion being the result of aberrant chromosomal function (Goessens, 1984). The differentiation of normal from abnormal cells in cervical cancer was first described as an asynchrony in NPB in daughter cells. Many other forms of cancer present with more than the expected numbers of NOR, or differing, abnormal or unequal sizes of NOR in daughter cells. Further, during ageing, nucleoli begin to fragment, which results in increased numbers of dense bodies in the cells (Guarente, 1997). There are also many mitogenic and growth regulatory proteins, which are involved in cell-cycle control, located in the nucleolus (Pedersen, 1998).

Oocytes in antral follicles have nucleoli which actively synthesis RNA and proteins for oocyte growth (Motlik *et al.*, 1984a,b). With maturation synthesis ceases and the nucleoli disappear (Crozet *et al.*, 1986) and at the metaphase II and pronuclear stage they are present as only the FC portion, and are known as NPB. The development of a full nucleolus occurs

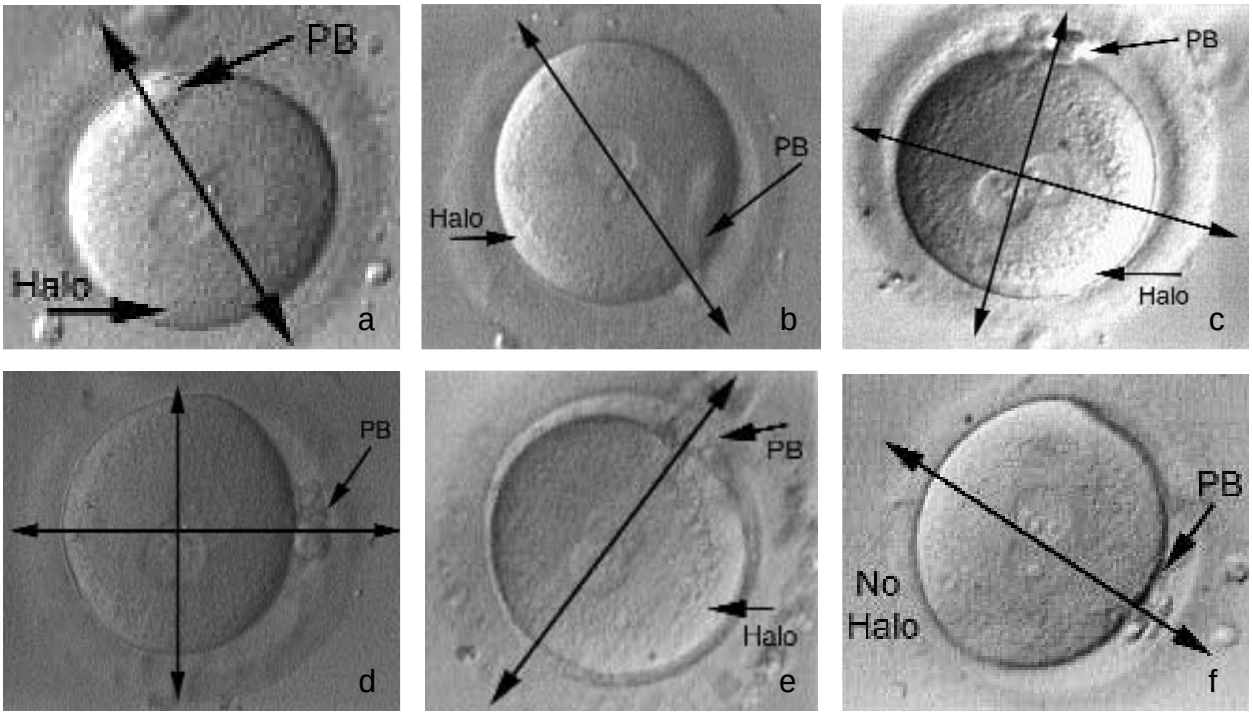

Figure 3. Pronuclear oocyte score and nuclei orientation. **a.** Z1 oocyte with halo and nuclei at right angles to the polar axis, requiring rotation. **b.** Z1 pronuclear oocyte with halo with nuclei aligned on polar axis, no rotation required. **c.** Z1 pronuclear oocyte with halo and nuclei rotation onto polar axis. **d.** Z2 pronuclear oocyte with halo and nuclei beginning to rotate onto polar axis. **e.** Z2 pronuclear oocyte with halo and nuclei aligned on polar axis. **f.** Z3 pronuclear oocyte with no halo and nuclei at right angles to polar axis.

over the first few mitotic cycles (Laurincik *et al.*, 2000) as the embryo cleaves (Tesarik and Kopecny, 1989, 1990) such that fully competent nucleoli are present when protein synthesis resumes with activation of the embryonic genome (Goessens, 1984; Flechon and Kopecny, 1998; Hyttel *et al.*, 2000).

A number of models have been proposed for scoring the NPB (Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al.*, 2000). The most commonly used ones rely on a single static observation at 16–18 h post-fertilization. In these models, oocytes presenting with equal numbers of equal sized NPB that are aligned on the furrow between the 2 nuclei are ideal. Equality with non-alignment is also acceptable. Any form of inequality, in size, numbers of NPB or in the pattern of alignment, is associated with poor outcome.

The scoring system used for the current data is presented in **Figure 1** (Scott, 2002, 2003). Pronuclear oocytes with equal numbers of NPB aligned at the furrow between the nuclei were designated Z1 (see **Figure 3a, b**), those with equality but not aligned as Z2 (See **Figure 3e**) and all others as Z3 (see **Figure 3f**). Within the Z3 group there were differences. Pronuclear oocytes in which there were only one or two large NPB on one side, with four to six that were scattered in the other were designated Z3–1. Those with NPB that were aligned in one nucleus and scattered in the other were scored as Z3–2. Pronuclear oocytes in which there was inequality in numbers, with a difference of >2 NPB were designated Z3–3 and those with many (>8) small pinpoint NPB scattered through the nucleus as Z3–4. In the data set, all Z3 pronuclear embryos are grouped as Z3, non-aligned, non-polarized. As indicated earlier, pronuclear oocytes with unequal sized or non-aligned nuclei were designated Z4.

Materials and methods

All assisted reproduction cycles from a 14-month period were included in the study. All women who were <43 years of age with an FSH concentration of <12 were accepted into the programme, regardless of infertility aetiology. All male factor diagnosis was treated with ICSI.

Ovarian stimulation

Women <30 years of age were treated with a standard down regulation long luteal leuprolide acetate (LA) protocol (Lupron: TAP Pharmaceuticals, North Chicago, IL, USA). Following down-regulation, ovarian stimulation was accomplished with FSH and/or human menopausal gonadotrophin (HMG) (Fertinex or Metrodin; Serono, Randolph, MA, USA: Humegon; Organon, West Orange, NJ, USA: or Repronex; Ferring, White Plains, NY, USA). All other women were pre-treated with oral contraceptives (30 µg ethinyl oestradiol/300 µg norgestrel). The day on which the oral contraceptives stopped was designated day 1. For women >30 years a microdose lupron flare of 40 µg twice daily was used followed by 4–8 ampoules of FSH and/or HMG per day, beginning on day 3 (Papageorgiou *et al.*, 2001). The stimulation protocol was determined by age, FSH level and previous history. Final oocyte maturation was triggered with 10,000 IU of human chorionic gonadotrophin (HCG) (Profasi, Serono) when the leading two follicles were >17 mm or the leading cohort measured 15–16 mm in diameter (Leondires *et al.*, 1999).

Oocyte retrieval, insemination and culture

Transvaginal ultrasound guided oocyte retrieval was performed under conscious sedation at 35 h post-HCG. Follicular aspirates with oocytes were placed directly into a gassed (5% CO_2 in air), humidified warmed isolette chamber (Hoffman Surgical Equipment Co., Conshohocken, PA, USA). All further oocyte and embryo manipulations were performed in the chamber.

Oocytes were placed in 100 µl drops of P1 medium (Irvine Scientific, Irvine, CA, USA) supplemented with 5% HSA (In Vitro Care, San Diego, CA, USA) under mineral oil (M-3516; Sigma Chemical Co., St Louis, MO, USA). Sperm preparations were in HTF (In Vitro Care) supplemented with 5% HSA.

Standard ICSI procedures were used. Inseminations and ICSI were performed at 40–41 h post-HCG. Fertilization checks were performed at 16–18 h post-insemination/ICSI. Fertilized pronuclear oocytes were placed in individual drops of P1 medium under oil and scored for the presence or absence of nuclei, their orientation, the presence or absence of a halo and the alignment of the NPB, the Z-score (Scott *et al.*, 2000).

Embryos were checked on day 2 at 24 h post-insemination (64 h post-HCG) for the evenness of cleavage, the presence of two elliptical cells in a 2-cell embryo or a tetrahedron in a 4-cell embryo (Scott, 2001, 2002). The total number of cells was recorded for each embryo and abnormal embryos noted. The embryos were scored again on day 3 at 66–68 h post-insemination (80 h post-HCG) in cases of determined day 3 transfer or where a decision between day 3 and day 5 was required. At this stage, embryos should have between 6 and 8 cells with only two cell sizes in those embryos with <8 cells (Roux *et al.*, 1995; Scott, 2002). Any embryos with more cell sizes were graded low, as these were probably anucleate fragments or blebs. Day 3 grading was according to both cell number and the degree of fragmentation. Embryos intended for day 5 transfer were scored at 72 h post-insemination (~112 h post-HCG) and moved into extended culture at this time. Extended culture media was CCM (VitroLife, Goteborg, Sweden), which was supplemented with HSA at source. Day 5 scoring was done at 114–166 h post-insemination (128–130 h post-HCG).

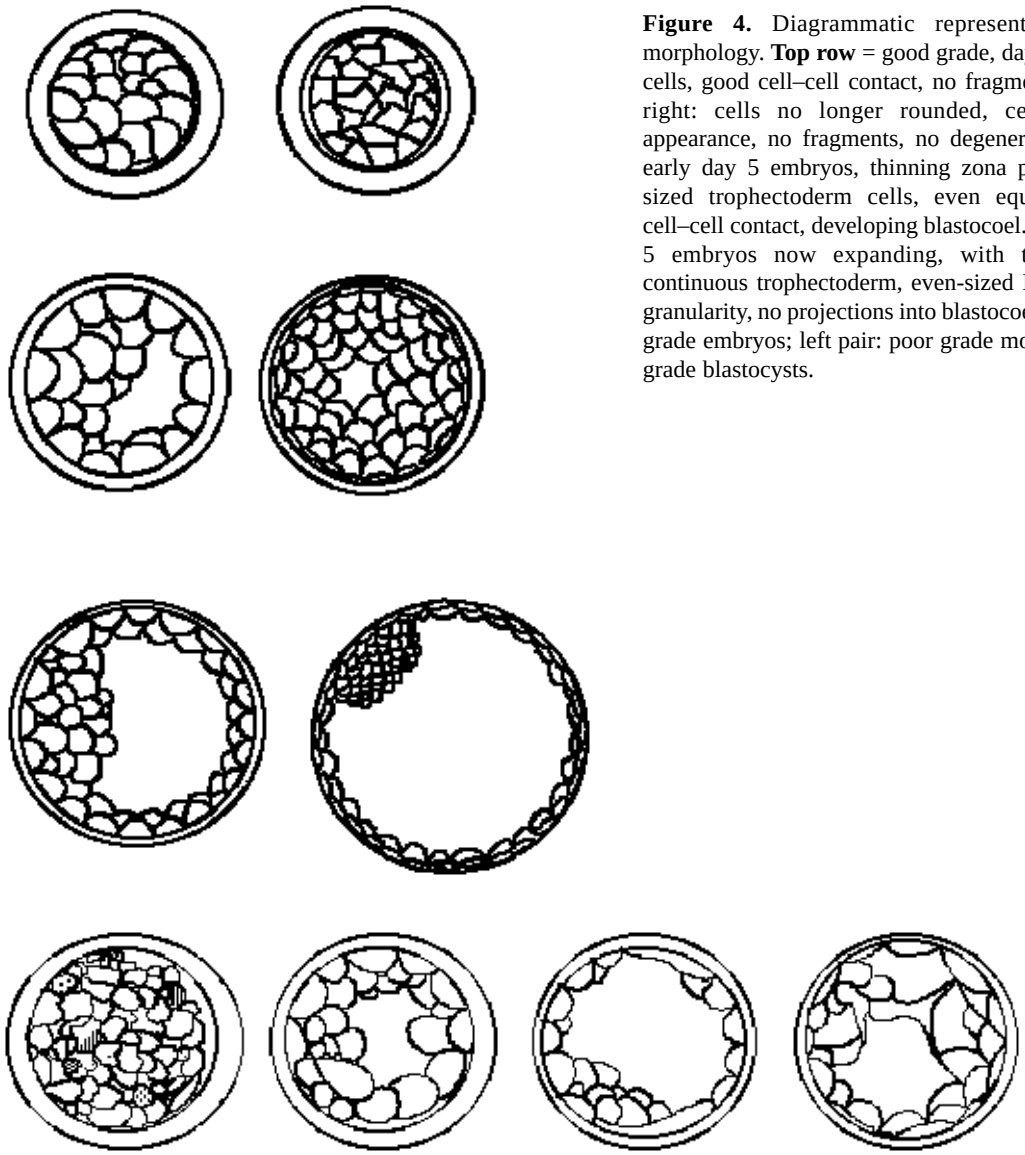

Figure 4. Diagrammatic representation of blastocyst morphology. **Top row** = good grade, day 4 embryos; left: >20 cells, good cell–cell contact, no fragments, no degeneration; right: cells no longer rounded, cells flattened, paved appearance, no fragments, no degeneration. **Second row** = early day 5 embryos, thinning zona pellucida, even equal-sized trophectoderm cells, even equal-sized cells, good cell–cell contact, developing blastocoel. **Third row** = late day 5 embryos now expanding, with thin zona pellucida, continuous trophectoderm, even-sized ICM cells, no cellular granularity, no projections into blastocoel. **Bottom row** = poor grade embryos; left pair: poor grade morulae, right pair: poor grade blastocysts.

Day 3 scoring

The number of cells in an embryo was noted on day 3. This was used in the data for developmental progression, since embryos are on a time clock (Johnson and Day, 2000) and deviations from this have been correlated with decreased implantation (Racowsky *et al.*, 2000; Shapiro *et al.*, 2000). On day 3, embryos were additionally scored as grade 1–5 according to morphology, which took into account cell number as well as evenness of cell division and degree of fragmentation. grade 1: 8 cells, <10% fragmentation, good cell–cell contact, no multinucleated blastomers; grade 2: 8 cells, 10–20% fragmentation or lacking good cell–cell contact, no multinucleated blastomers; grade 3: 6–7 cells or 8 cells with 20% fragmentation or uneven blastomer size, no multinucleated blastomers; grade 4: >8 cells or 4–6 cells or 8 cells with >20% fragmentation or uneven blastomer size or multinucleated blastomers; grade 5: <4 cells or grossly fragmented or with half of the blastomers being multinucleated.

Blastocyst scoring

A good grade blastocyst needed to have: progression from 16-cell to compacted morula on day 4; and on day 5 the presence of a blastocoel or the signs of one beginning; defined trophectoderm with enough cells to form a continuous layer without a single cell stretching or flattening on the surface, a well defined organized inner-cell mass; >60 cells. The mere presence of a blastocoel was not sufficient for scoring as a blastocyst. Good grade morulae had a paving stone appearance, good cell integrity and cell contact, and no necrosis. Embryos were scored as morula/early blastocysts or blastocysts/expanded blastocysts (Scott, 2002) (**Figure 4**).

Statistical analysis

Data were analysed using Fisher's exact tests, the Mann–Whitney test, or analysis of variance with a *P*-value of <0.5 being considered statistically significant. Each parameter was recorded separately. The developmental data were considered only on cell number. The day 3 embryo score data did not take into account the pronuclear score.

Results

NPB and development

A total of 3882 pronuclear oocytes were obtained in the laboratory over a 14-month period. These originated from 429 women who yielded 7145 oocytes of which 5335 were mature (75% mature), giving a 73% fertilization rate. The data were not analysed according to either age or treatment type (IVF or ICSI), as it has been shown previously that neither of these factors influenced the pronuclear score (Scott *et al.*, 2000). Of these, 3807 (98%) had accurate scoring. A total of 1550 (40.7%) Z1; 969 (25.4%) Z2; 879 (23.1%) Z3 and 409 (10.7%) Z4 embryos were obtained, which compares favourably with previous reports (Scott *et al.*, 2000).

For day 2 development, the embryos were scored as arrested if they were 1 cell, or as 2-cell, 3-cell, 4-cell or greater and no score. The data are presented in **Figure 5a**. There was no difference in development at 24 h post-insemination for Z1, 2 or 3 pronuclear embryos. However, Z4 embryos were delayed and had significantly more arrested development.

The development on day 3, at 66–68 h post-insemination, showed differences between the pronuclear scores (**Figure 5b**). There was an increase in slow embryos with a concomitant decrease in 8-cell embryos in the Z2 group compared with the Z1 group ($P < 0.5$) with a further increase in this pattern for Z3 embryos ($P < 0.5$). Z4 pronuclear embryos had a significant number of arrested and slow developing embryos on day 3 ($P < 0.1$).

Day 3 embryo score

All embryos were scored for day 3 morphology, regardless of Z-score. The score for each Z-score group was then analysed. The data are presented in **Figure 5d**. There was a significant difference in the day-3 morphology between the four Z-groups. From Z1 to Z2 there was an increase in grades 2 and 3 embryos and decrease in grade 1 embryos, which can be accounted for in both the increase in slower growing embryos and an increase in fragmentation ($P < 0.5$). This was pattern was amplified in the Z3 group ($P < 0.1$). The Z4 pronuclear embryos had very poor day 3 morphology and >40% arrested embryos.

Day 5 development

A total of 1015 Z1 derived embryos, 640 Z2, 708 Z3 and 379 Z4 embryos were placed in extended culture. The rate of blastocyst development on day 5, at 114–116 h post-insemination (128–130 h post-HCG) for the different Z-scores is shown in **Figure 5c**. Only blastocysts that were of a quality for transfer or freezing were included in the data. There was a significant reduction of blastocysts and expanded blastocysts in Z2 ($P < 0.5$) and Z3 ($P < 0.1$) groups and in the number of blastocysts on day 5 and 6 for the Z3 group ($P < 0.5$) and Z4 group ($P < 0.1$).

Overall, embryos resulting from Z1 pronuclear oocytes had more cells on day 3, a better day 3 morphology score and a higher number of blastocysts/expanded blastocysts on day 5 and 6. The embryos from Z2 pronuclear oocytes were slightly slower in development on both day 3 and 5 and had lower day 3 morphology scores. The Z3 pronuclear oocytes had even slower development on days 3, 5 and 6 and lower embryo scores. Z4 pronucelar oocytes had significant amounts of developmental arrest and low blastocyst formation.

Cytoplasmic halo and embryo development

The presence or absence of a cytoplasmic halo was scored at 16–18 h post-insemination or ICSI. The development of these embryos on day 2, day 3 and day 5, the day 3 morphology score were recorded (**Figure 6**).

In all, 3336 pronuclear oocytes had accurate scoring (86%)of which 2608 presented with a halo (78.2%) and 728 without (21.8%). The development on day 2 was no different between the two groups. There was a significant difference in the day 3 development (**Figure 6a**) and morphology scores (**Figure 6c**).

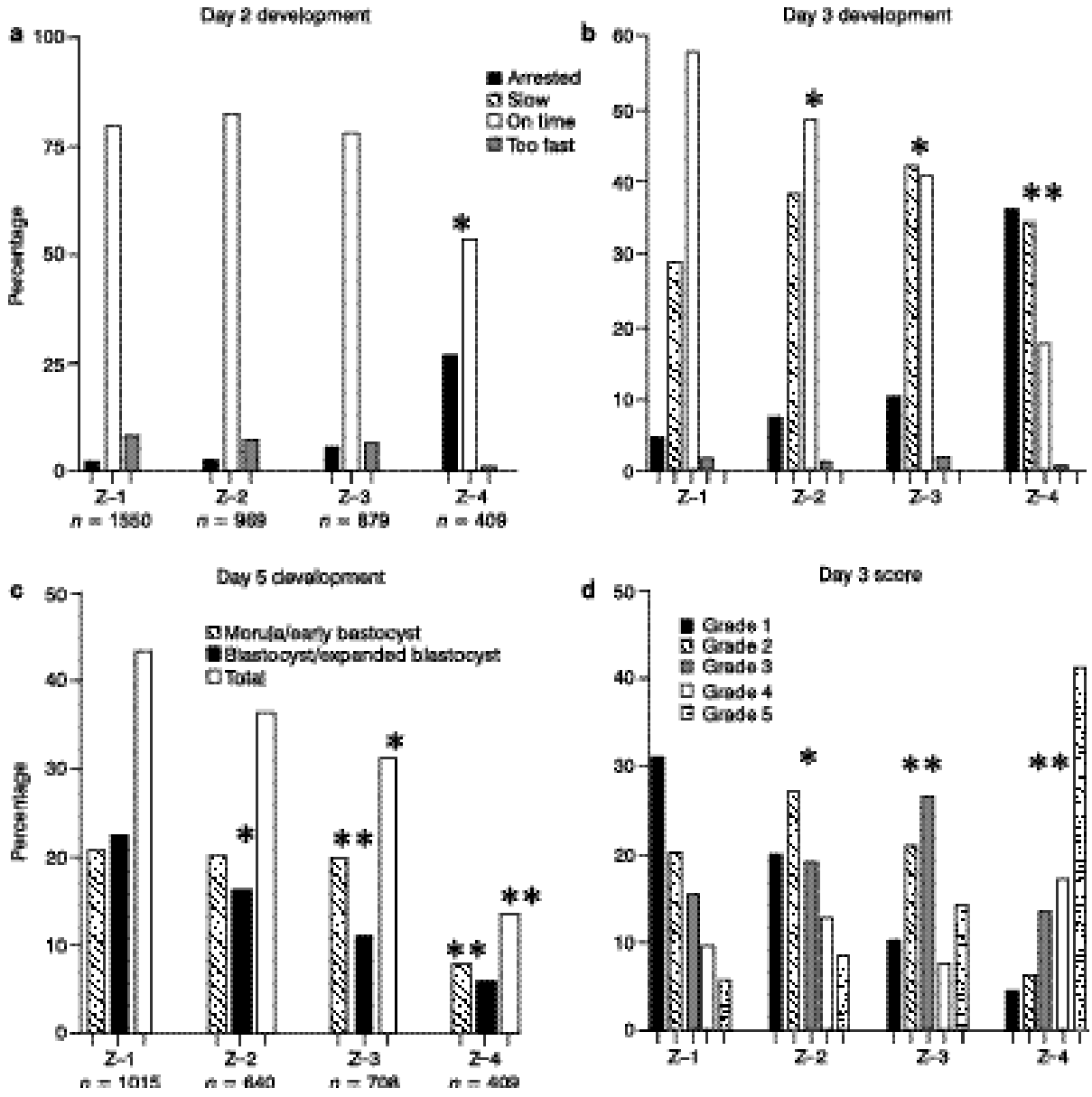

Figure 5. Development according to the Z-score. **a.** Day 2 development: arrested = 1 cell, on time = 2–4 cells, too fast = >4 cells. **b.** Day 3 development: arrested = <4 cells; slow = 4–6 cells; on time = 7/8 cells; too fast = >8 cells. **c.** Day 5 development. **d.** Day 3 score. *n* = number of embryos in each group. Asterisks indicate significant differences: * $P < 0.5$; ** $P < 0.1$.

On day 3, there were more slower-growing embryos (less 8-cell) and more arrested embryos in the group with no halo ($P < 0.5$). The day 3 morphology score, which is related to the cell number and fragmentation patterns, showed a significant difference between those embryos with halos and those without halos. Nearly half of the embryos with a halo (45%) had grade 1 or 2 morphology, which means they had little fragmentation. Embryos without a halo had only 34% grade 1 and 2 with 42% with grade 3 and 4, implying a high degree of fragmentation, coupled with slower embryos. On day 5, there was a highly significant difference in the overall rate of blastocyst formation. Of the 2022 embryos with halos placed in extended culture, 44.2% reached the blastocyst stage and of the 543 without halos, only 26.1% formed blastocysts ($P < 0.01$) (**Figure 6b**).

There was also a significant difference between the overall numbers of embryos in each zygote score (**Figure 6d**). Pronuclear oocytes that presented with a halo had a Z-score pattern that was similar to the overall data. Oocytes that had no halo had significantly more Z3 pronuclear embryos and fewer Z1 and Z2 embryos ($P < 0.1$).

Overall, oocytes with a halo had better NPB orientation, faster development and morphological scores on day 3 and more blastocyst development on day 5 than oocytes that had no halo.

Nuclei orientation and development

At the time of fertilization check, the orientation of the nuclei relevant to the polar bodies was noted. When this was

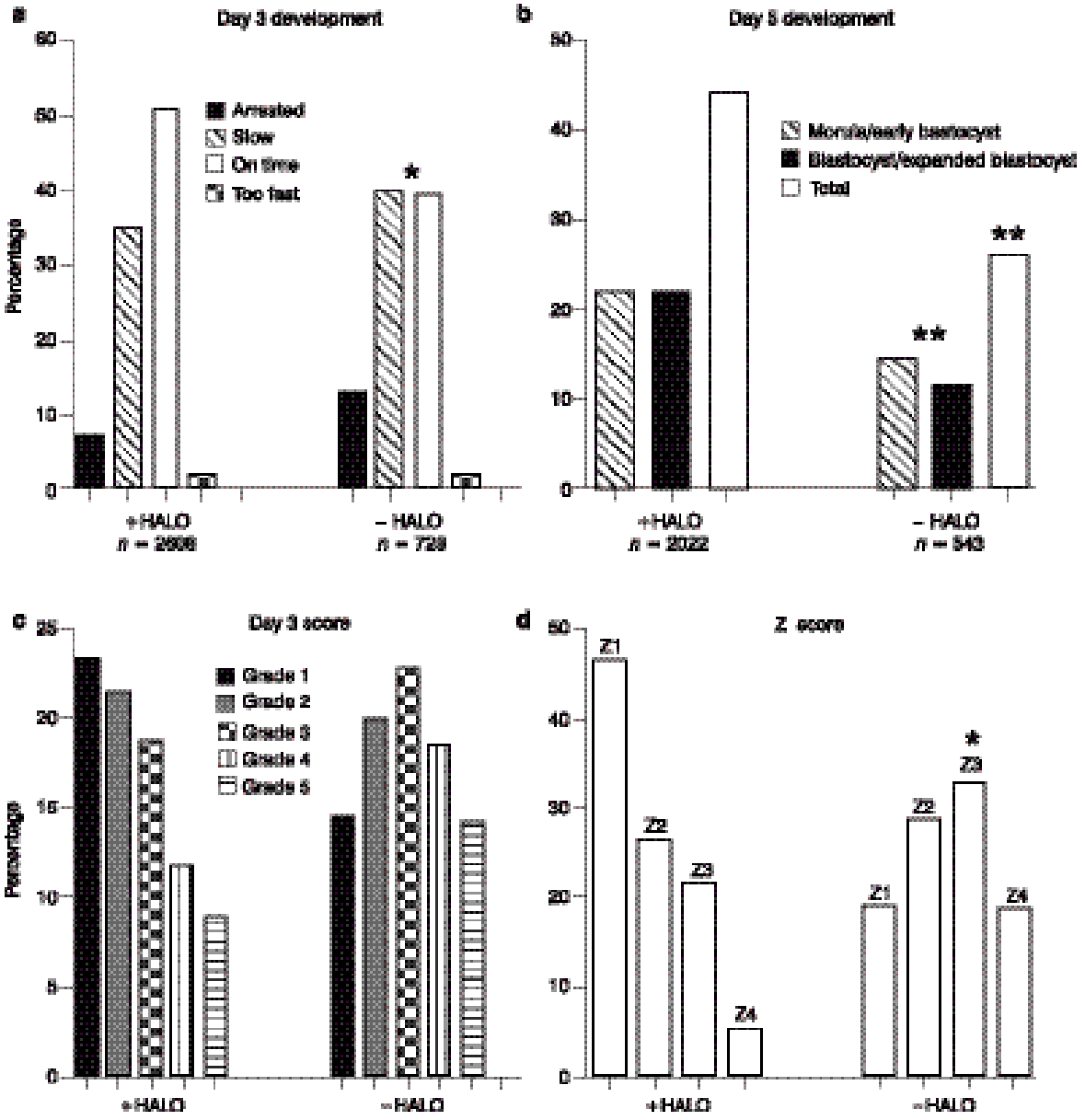

Figure 6. Development according to the cytoplasmic halo. **a.** Day 3 development; arrested = <4 cells; slow = 4–6 cells; on time = 7–8 cells; too fast = >8 cells. **b.** Day 5 development. **c.** Day 3 score. **d.** Z score: percentage of each Z score with and without a halo. *n* = number of embryos in each group. Asterisks indicate significant differences: * $P < 0.5$; ** $P < 0.1$.

correlated with day 2, 3 or 5 development or day 3 morphology score, no differences were found. On day 3, 48.7 and 50.8% of embryos from group 1 and 2 respectively were at the 8-cell stage; 42.0 and 46.2% had grade 1 and 2 morphology and 36.8 and 37.2% had reached the blastocyst stage on day 5. There was also no difference in the spread of zygote score between those with a right angle or lined-up orientation. These percentages were the same as those for the group as a whole.

Discussion

The data presented in this paper indicate that the morphology of the pronuclear oocyte has a direct effect on continued in-vitro development, which could be translated into the effects recorded after transfer, namely increased implantation rates (Scott and Smith, 1998; Tesarik and Greco, 1999; Ludwig *et al.*, 2000; Scott *et al.*, 2000; Wittemer *et al.*, 2000; Balaban *et al.*, 2001; Tesarik *et al.*, 2002; Zollner *et al.*, 2002).

NPB

The Z-score, or the state of the NPB had no effect on the first and second cell cycle, with no differences seen between the groups. Since the oocyte is ovulated with all the machinery in place for these initial cell cycles, it is unlikely that nuclear events will have much of an effect. However, once the embryonic genome is switched on, at about the 4–8-cell stage, nuclear function becomes vital. As the oocyte matures the nucleoli, or protein machines of the cell, switch off and disappear (Crozet *et al.*, 1986), only reforming at about the 4-cell stage (Laurincik *et al.*, 2000), which coincides with activation of the embryonic genome and resumption of protein

synthesis (Goessens, 1984; Flechon and Kopecny, 1998; Hyttel *et al.*, 2000). This is when the embryo begins to grow. The nucleoli that reform do so from the NPB, which are the central point of pronuclear scoring.

In mitotic cells, asynchrony in numbers and patterns of NPB between nuclei leads to abnormal development. Since many cell cycle control proteins are located in the nucleolus (Pedersen, 1998), asynchrony in the numbers of NPB may cause abnormal cell cycles, which may be the foundation of cancer cells. Most cancer cells studied to date have abnormal and asynchronous NPB in daughter mitotic cells. When this is applied to early embryos, those with asynchrony between the nuclei may result in embryos with disrupted cell cycles. From the current data, it can be argued that this is true. Embryos with asynchrony in NPB, Z3, have slower development, more fragmentation and abnormal cleavage and finally fewer embryos continuing development to the blastocyst stage. When transferred, they result in fewer implantations (Scott and Smith, 1998; Tesarik and Greco, 1999; Ludwig *et al.*, 2000; Scott *et al.*, 2000; Wittemer *et al.*, 2000; Balaban *et al.*, 2001; Tesarik *et al.*, 2002; Zollner *et al.*, 2002).

As the pronuclei form after fertilization, there is polarized distribution of the chromatin into the furrow between the nuclei (Van Blerkom *et al.*, 1995a). The NOR are attached to the chromatin and should thus polarize or align with it. If there is correct chromatin polarization, the NOR and thus the NPB will appear polarized. Non-alignment or polarization of chromatin leads to failure of development (Van Blerkom *et al.*, 1995a). It is suggested that the lack of polarization of the NPB in the Z3 embryos is a morphological indication of a lack of chromatin polarization, which will lead to abnormal development as recorded in this data. Slower development of the Z2 embryos, which have synchrony but are delayed in the alignment of the chromatin into the furrow, or onto the mitotic plate, can also be attributed to this.

Asynchrony in the coalescence of the NPB as they progress through the first mitotic cycle to the second, where nucleolar function resumes, could also lead to disrupted growth. Generally cell cycle events are controlled by checkpoints, or surveillance systems, that either block or allow cell cycle transitions (Hartwell and Weinert, 1989). Incomplete, incorrect or damaged transitions or events are stopped by these mechanisms (Nasmyth, 1996). Some of these surveillance mechanisms operate to ensure that chromosome duplication and completion occur correctly, and that chromosome alignment on the spindle is both timely and spatially correct (Steuerwald *et al.*, 2001). If incorrect alignment is detected, a signal is transduced/initiated, which stops the system until the correct alignment is attained. Further, the mitogenic and cell-cycle control proteins involved in some growth regulatory functions that are located in the nucleolus (Pedersen, 1998) could also be moderated if there are differences between the nuclei and thus reformation of the full nucleolus. This could account for the slower development of the Z3 embryos and finally their poor implantation potential.

During nuclear transfer experiments, utilizing somatic cells transferred into oocytes, extensive re-modelling of the somatic nucleus is required. There also needs to be re-modelling of the nucleolus, which are deconstructed and return to NPB (Kanka *et al.*, 1999). As the construct begins to grow, the nucleolus will need to re-form for RNA synthesis, as it does in early embryos. Many of the problems that occur with nuclear transfer embryos utilizing somatic cells, derive from the advanced, incomplete or incorrect reformation of the nucleolus from the NPB. These can also include the lack of complete deconstruction, leading to asynchrony between the stage of reformation of the nucleolus and the embryo cleavage stage. This leads to abnormal development and finally, failure to implant. This could be what is happening in Z3 embryos, which may have an inability to reform the nucleolus due to the asynchrony and lack of polarization of the NPB in the two nuclei.

An aspect of cell ageing that finally leads to cell death is complete fragmentation of the nucleoli, which presents as many small scattered and unequal sized NPB in the mitotic daughter cells (Guarente, 1997). This pattern can often be seen in the NPB of oocytes, being referred to as Z3–3 and Z3–4 in the current scoring scheme. Although this not true ageing, the fragmentation of the NPB could indicate that the embryo is on a path of ultimate cell death. This again is evidenced by the poor development of Z3 embryos with fragmented NPB.

Synchrony between the nuclei and polarization of the NPB is a necessary event in any mitotic cell. Asynchrony results in abnormal development (cancer) and lack of polarization, coalescence and reformation of functional nucleoli has profound effects on the ability of cells to grow and function. Control of the cell cycle is also partially dictated by the nucleolus, as are certain aspects of ageing. Clearly, the lack of synchrony and or polarization of NPB in the two pronuclei of embryos will have profound effects on development. These data show that a first indication of this is decreased in-vitro development of the embryos can be the NPB in the nuclei.

Cytoplasmic halo

The poor development of pronuclear oocytes lacking a cytoplasmic halo was significant. Although only 22% of the embryos had no halo, the developmental consequences were marked with slow development, poor morphology, increased fragmentation and limited blastocyst formation. The halo effect has been variously attributed to cytoplasmic rotation or movement (Payne *et al.*, 1997) or to the differential distribution of mitochondria (Muggleton-Harris and Brown, 1988; Barnett *et al.*, 1996). Human, hamster and mouse pronuclear oocytes do have different distributions of mitochondria, which alter with time and are related to the need for energy distribution around the embryo (Van Blerkom *et al.*, 2000, 2002).

Prior to fertilization, mitochondria are not polarized, and are located in a ring around the nucleus where they remain until cleavage. A subset of active, proton-pumping mitochondria move to the pericortical area of the oocyte, spreading in a band from the polar body to the opposite pole, but with none actually at the pole opposite the polar body. At fertilization the spermatozoon triggers an increase in intracellular calcium concentrations, which begins the transition from oocyte to embryo (Carroll *et al.*, 1996; Carroll, 2001). With fertilization,

the cortical granules are released and a series of calcium oscillations begin and which continue for many hours. These waves are both time and spatially coordinated. The waves start at the point of sperm entry and this is followed by a second wave, both moving to the opposite side of the oocyte. After this the cytoplasm moves or rotates, and this is in the same direction as the wave. With each wave, there is more cytoplasmic movement, rotation or displacement, away from the point of sperm entry. These waves stop when the pronuclei are formed (Tang *et al.*, 2000).

The calcium release affects mitochondria and ATP generation. Calcium is a key second messenger for the dehydrogenases (pyruvate, oxoglutarate, isocitrate) and as concentrations of calcium in and around mitochondria rise, mitochondrial metabolism is stimulated. Mitochondria have a low affinity for calcium, but their distribution to the prepericortical area, into areas of high calcium concentrations generated by the calcium waves, will expose them to the required high concentrations which will meet overload threshold levels. As they take up calcium they generate ATP (Van Blerkom and Runner, 1984; Scott, 2002; Van Blerkom *et al.*, 2002).

The halo in pronuclear embryos could be the cytoplasm movement related to the calcium waves and the redistribution of active mitochondria. Since the lack of, or disruption of, calcium waves will result in no or abnormal transitions from oocyte to embryo through incomplete or incorrect completion of fertilization, a lack of cytoplasmic movement seen through a lack of the halo could also result in decreased, abnormal and arrested development. The data presented here does show that a lack of halo results in slow and delayed development and decreased embryo morphology.

During development of all species there is cytoplasmic rotation and movement for polarity establishment. Polarity in early embryos is an essential aspect of development from low order animals through human embryos (Edwards and Beard, 1997; Gardner, 2001; Scott, 2001). Disruptions of polarity will lead to non-viable development. At the first mitotic cleavage, products laid down in the oocyte during development will be distributed to the 2-cells. If the cytoplasmic rotation and movement has been disrupted there could conceivably be unequal or incorrect distribution of certain gene products and or mitochondria to the cells, which will be perpetuated through development (Antczak and Van Blerkom, 1997; Van Blerkom *et al.*, 2002). If the halo effect indicates that cytoplasmic rotation is occurring, helping to establish these polarity axes, lack of a halo could indicate that this has not occurred resulting in incorrect or unequal divisions and arrested and non-viable embryos.

The morphology of day 3 embryos with and without a halo was also significantly different. Embryos without a halo had lower day 3 grades, which correlates with increased fragmentation. Although the exact causes of fragmentation have not been established, it has been variously attributed to apoptosis (Alikani *et al.*, 2000), ATP deficiencies in the embryo (Van Blerkom *et al.*, 2001), blastomer loss through apoptosis, due to chromosomal abnormalities (Pellestor *et al.*, 1994), or merely anucleate blebbing, associated with cleavage, which is reabsorbed (Hardarson *et al.*, 2002). Whatever the mechanism or underlying cause, fragmentation has been associated with decreased implantation.

In the case of increased fragmentation in embryos without halos, it could conceivably be related to disruptions in the distribution of mitochondria and therefore energy or ATP generation, as suggested by Van Blerkom (Van Blerkom *et al.*, 2001). At the 2- to 4-cell stage, this will not show up, as the data indicate, since the embryo is relying on maternal signal for most of its household activities. Subsequently, the embryonic genome takes over and the effects will be manifested, as seen with decreased development and increased fragmentation.

At fertilization, the cytoplasmic movement could also be responsible for the rotation of the male pronucleus onto the female one (Van Blerkom *et al.*, 1995a), which is essential for putting the centromere and chromatin into the furrow between the nuclei. Since the centromere in the human is sperm derived, it needs to be in the furrow for duplication and to move the chromatin to the opposite sides of the oocyte during the first mitotic cleavage. Failure to rotate will lead to abnormal separation of chromatin and non-viable embryos. Again, if the halo is indicating the cytoplasmic movement, it is a key factor in indicating that this rotation is occurring.

Finally, there were a statistically increased number of Z3 pronuclear oocytes in the group without a halo. The origin or causes of the different types of Z3 oocytes may be diverse. Some have been attributed to the spermatozoon, with asynchronous condensation of the nucleoli or the source of the spermatozoon (Tesarik and Kopecny, 1990; Tesarik *et al.*, 2002). Another reason for Z3 development may be at the oocyte level and due to events that occur within the follicle. It has been shown that lack of adequate blood flow to the follicle results in an oocyte with decreased competence (Nargund *et al.*, 1996; Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Huey *et al.*, 1999). Without sufficient blood flow or oxygenation the follicles will switch from oxidative phosphorylation to glycolysis for ATP production, resulting in decreased amounts of ATP available to the oocyte (Kaelin, 2002; Maxwell and Ratcliff, 2002). This could have profound effects on the maturation events, RNA synthesis and the final NPB status of the oocyte. It may cause early fragmentation of the NPB or abnormal or delayed coalescence, resulting in the Z3 morphology. It has also been shown that transferred embryos from cohorts in which the non-transferred remainder or the follicular fluid had lowered ATP content had reduced developmental competence (Van Blerkom *et al.*, 1995b). Oocytes that are metabolically depressed may not have enough active mitochondria to sequester to the pericortical region, resulting in the lack of halo. These two events could be linked and caused by the lack of blood flow to the follicle and manifested at the morphological level as Z3 pronuclear oocytes lacking a halo.

Overall, the lack of a visible halo results in decreased development and increased fragmentation. That there are also an increased number of Z3 oocytes without halos could point to an ovarian origin and specifically to lack of sufficient ATP production to either initiate NPB progression or mitochondrial activity.

Nuclear orientation

The nuclear orientation had no effect on the overall development of the embryos, and was not related to day 3 morphology or to the Z-score. Since the nuclei are probably very mobile within the embryo due to the cytoplasmic streaming, and rotation occurs fairly rapidly, this is not surprising. Further, nuclear membrane breakdown occurs a few hours prior to initiation of the first cleavage division so any rotation after this will not be easily seen.

Conclusions

To date, there has been no definitive method of selecting a viable human embryo from a cohort resulting from an assisted reproduction attempt. Pronuclear oocyte morphology has been correlated with implantation and pregnancy potential (Scott and Smith, 1998; Tesarik and Greco, 1999; Ludwig *et al.*, 2000; Scott *et al.*, 2000; Wittemer *et al.*, 2000; Balaban *et al.*, 2001; De Placido *et al.*, 2002; Rienzi *et al.*, 2002; Tesarik *et al.*, 2002; Zollner *et al.*, 2002). Day 3 development (Racowsky *et al.*, 2000; Shapiro *et al.*, 2000), and day 3 morphology (Puissant *et al.*, 1987; Steer *et al.*, 1992; Tan *et al.*, 1992; Roux *et al.*, 1995; Rijnders and Jansen, 1998) have both been linked to implantation potential. The fragmentation patterns of day 3 embryos (Antczak and Van Blerkom, 1999; Alikani *et al.*, 2000; Desai *et al.*, 2000) have shown correlations with implantation, as has the evenness of cell division (Roux *et al.*, 1995; Hardarson *et al.*, 2001). Finally blastocyst development and morphology are correlated with implantation potential (Dokras *et al.*, 1993; Gardner and Lane, 1997; Gardner *et al.*, 1998; Scholtes and Zeilmaker, 1998; Gianaroli *et al.*, 2000; Huisman *et al.*, 2000; Milki *et al.*, 2000; Scott *et al.*, 2000; Balaban *et al.*, 2001). The data presented here show that the pronuclear morphology of a fertilized oocyte has a significant effect on the in-vitro developmental ability of the embryo, which therefore affects the day 3 development and morphological score, the fragmentation of the embryo and its ability to grow to the blastocyst stage. The pronuclear morphology is probably directly related to the gametes, and is therefore the earliest point at which the health and normality of these can be judged. Thus to realize the goal of single embryo transfers, scoring of the embryos should begin at the earliest point the newly formed embryo can be scored, the pronuclear stage, which directly affects all subsequent development.

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