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The morphology of human pronuclear embryos is positively related to blastocyst development and implantation*

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Human embryos are selected for transfer using morphology at the cleaving and blastocyst stages. Zygote morphology has been related to implantation and pregnancy. The aim of this study was to relate pronuclear morphology to blastocyst development. Zygotes were scored according to distribution and size of nucleoli within each nucleus. Zygotes displaying equality between the nuclei had 49.5% blastocyst formation and those with unequal sizes, numbers or distribution of nucleoli had 28% blastocyst formation. Cleaving embryos that were selected initially by zygote morphology and secondarily by morphology on day 3 had increased implantation (IR) and pregnancy rates (PR; 31 and 57%), compared with those selected by morphology alone (19 and 33% respectively; *P* < 0.01). There was a significant difference between zygote-scored and non-scored cycles on day 3 (PR: 57 versus 33%; IR: 31 versus 19%) and on day 5 (PR: 73 versus 58%; IR; 52 versus 39%). Zygote scoring can maintain pregnancy rates for both day 3 and day 5 transfers, increase implantation rates and reduce the numbers of embryos required to achieve a pregnancy.

Key words: blastocyst/nucleoli/pronuclear morphology/zygote scoring

Introduction

The efficiency of IVF and embryo transfer in the human is low, with <30% of embryos that are transferred ever achieving their full developmental potential (Edwards and Beard, 1999). Since the implantation rates have remained relatively low, the practice of replacing multiple embryos (*n* = 2–6) in order to increase the likelihood of pregnancy, is common practice. This has led to an unacceptable level of high-order multiple pregnancies. To overcome this problem, some countries have restricted the number of embryos that can be replaced, in some instances limiting this number to two. Although this will reduce the level of multiple pregnancies, it also reduces the pregnancy rate.

Initially, most IVF centres replaced embryos on the second day of culture, at the 2–4-cell stage. By allowing development for an additional day, permitting more critical assessment of the embryos after further cleavage divisions, an increased implantation rate was achieved (Dawson *et al*., 1995). This system has been widely adopted, with a concomitant increase in implantation rates. For both day 2 and day 3 embryo transfers, embryo selection is based on the key morphological features of cleaving embryos that have been previously related to increased implantation (Pruissant *et al*., 1987; Steer *et al*., 1992; Tan *et al*., 1992).

The morphology of early human zygotes at the 1-cell stage has also been used successfully in an IVF programme as a means of embryo selection for both day 1 pronuclear transfers (Scott and Smith, 1998) and at the 8-cell stage on day 3 (Tesarik and Greco, 1999). The zygote grading system used by Scott and Smith (Scott and Smith, 1998) was based on empirical observations correlated with pregnancy and on previously published observations of zygote morphology (Van Blerkom, 1990; Wright *et al*., 1990). The basis of the grading system was a combination of pronuclear size, nucleoli number and distribution, and cytoplasmic appearance. In addition, changes in cytoplasmic appearance and progression to first cleavage division were considered. This grading system was used prospectively to increase the incidence of implantation when utilizing day 1 embryo transfers and to select embryos for cryopreservation. Pregnancy rates (PR) were equivalent when day 1 and day 3 transfers were compared.

Tesarik and Greco reported a modified grading system in which the nucleoli size, number and distribution, were utilized in a single-observation scoring (Tesarik and Greco, 1999). Embryos were then replaced on the third day of culture where the embryo morphology was used as the primary selection criterion. In a retrospective analysis of their data, Tesarik and Greco found a strong association between implantation and the equality of nucleoli within each nucleus of the pronuclear embryos from which the resulting transferred cleaving embryos arose. The advantages of the Tesarik zygote-grading system over the Scott system are the single observation and fewer parameters for consideration.

With the introduction of extended culture and blastocyst transfer, reported pregnancy and implantation rates have

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increased (Gardner *et al*., 1998). This has led to the ability to select one or two blastocysts for transfer without reducing the chances of pregnancy while minimizing the incidence of high-order multiple pregnancies. However, only 40–50% of all zygotes placed in extended culture are reported to reach the blastocyst stage and, of these, 30–40% implant, meaning that even extended culture with blastocyst transfer is inefficient. Since there is clearly a relationship between zygote morphology and the ability to implant, screening embryos at the zygote stage and again at the blastocyst stage could increase the implantation rate, enabling single blastocyst transfers. A second level of embryo screening could also help in selecting patients for extended culture.

This paper describes the use of a simple, single-observation zygote scoring system modified from an earlier version (Scott and Smith, 1998) over 20 consecutive months (January 1998 to August 1999) in an IVF programme in which extended culture and blastocyst transfers account for ~50% of embryo transfers. The zygote score was related to the ability to grow to the blastocyst stage and to implantation and fetal development.

Materials and methods

Patients

All patients were beneficiaries of the Department of Defense of the United States. There was no exclusion for any form of infertility presentation except that the age limit for the women was <43 years at the start of treatment. Couples presenting with male factor infertility, including those requiring sperm aspiration, were treated by intracytoplasmic sperm injection (ICSI).

Ovarian stimulation and oocyte retrieval

A microdose gonadotrophin-releasing hormone (GnRH) agonist flare protocol (Leondires *et al*., 1999) was used routinely in all patients, except those at risk of severe ovarian hyperstimulation. All patients were started on a low-dose oral contraceptive pill (ethinyl oestradiol 0.30 mg/norgestrel 0.3 mg; Wyeth Pharmaceuticals, Philadelphia, PA, USA) at least 1 month prior to their treatment cycle. This enabled timing of ovarian stimulation for formation of batches of patients since the unit runs in series. On the designated baseline day of treatment, use of oral contraceptive pill was discontinued. On the third day after stopping the pill, the microdose GnRH agonist flare was started using Leuprolide (Lupron; TAP Pharmaceuticals, Deerfield, IL, USA) 40 μg s.c. twice daily. On the third day of the microdose flare, administration of gonadotrophins was initiated and this was designated as day 1 of stimulation (Fertinex, 150 IU twice daily; Serono, Norwell, MA, USA). Women aged ⩾35 years and those with previously documented poor response received an additional 75 IU human menopausal gonadotrophin (HMG, Humegon; Organon, West Orange, NJ, USA). Oocyte retrievals were carried out 35 h after administration of human chorionic gonadotrophin (HCG, 10 000 IU i.m.). Progesterone in oil (50 mg i.m. twice daily), was begun on the day of oocyte retrieval and continued for another 6 weeks in those achieving pregnancy.

Oocyte collection, insemination and embryo culture

All oocyte and embryo handling was performed under oil and all procedures carried out in humidified, heated, gassed isolettes (Hoffman Surgical Equipment Co, Conshohocken, PA, USA). All cultures utilized small drops (100 μl for insemination; 10 μl for embryo culture) under an oil overlay (M-3500; Sigma Chemical Company, St Louis, MO, USA) in Falcon 3002 Tissue culture dishes. Embryo transfers were performed with Wallace Catheters (SimsPortex, Hythe, Kent, UK) under ultrasound guidance in the same medium in which the embryos had been growing, on days 3 or 5. No HEPES-buffered medium was used for handling or washing oocytes or embryos, except for the drops in which ICSI was performed. The medium for sperm washing, insemination, embryo culture and extended culture changed for each series in a process of continuous quality improvement and systems development. Sperm preparation, insemination and early embryo culture was in P1 medium (Irvine Scientific, Irvine, CA, USA) and extended culture in Irvine blastocyst medium in the first series. In series 2, sperm preparation and insemination was in human tubal fluid (HTF) medium (In Vitro Care; San Diego, CA, USA), early embryo culture in S1 or G1 medium (Scandinavian IVF, Uppsala, Sweden) and extended culture in S2 or G2 medium (Scandinavian IVF) or the Irvine P1/Blastocysts medium system. Irvine medium and HTF were supplemented with 5 mg/ml human serum albumin (In Vitro Care).

Embryos were cultured in individual 5 μl drops in series 1, and in 5–7 μl drops in groups of one to four, according to zygote score in series 2. No more than four embryos were placed in each drop.

Statistical analysis

Relationships between zygote morphology, day 3 morphology, blastocyst formation and implantation and pregnancy rates were sought. Differences between pregnancy and implantation rates were compared using Fisher's Exact test and χ^2 analysis.

Zygote scoring

The IVF programme initially involved day 3 embryo transfers with embryo selection based primarily on cleaving embryo morphology. Three or more embryos were replaced in a large number of instances, which led to an unacceptable number of high-order multiple pregnancies. To help reduce this, a blastocyst culture programme was initiated, since the transfer of only two blastocysts has been shown to give equal or better pregnancy rates than three day 3 embryos (Gardner *et al*., 1998). Initially, all day 3 embryos not used for embryo transfer were transferred to extended culture medium and cryopreserved on day 5 as blastocysts. The zygote scoring system (Scott and Smith, 1998) was adopted, but due to the volume of work and time constraints, sequential observations were not performed. The grading system was further refined, resulting in five main groups.

Initial zygote scoring system

There were five pronuclear categories based on both the number and distribution of nucleoli in the pronuclei. They were graded 1–5 according to nuclear size, nuclear alignment, nucleoli alignment and distribution and the position of the nuclei within the zygote (see Figure 1). Grade 1 had equal numbers of nucleoli aligned at the pronuclear junction. The absolute number was not counted but was between three and seven. Grade 2 had equal numbers of nucleoli of equal sizes in the same nuclei but with one nucleus having alignment at the pronuclear junction and the other with scattered nucleoli. Grade 3 zygotes had equal numbers and sizes of nucleoli (between three and seven) which were equally scattered in the two nuclei. Grade 4 zygotes had unequal numbers (a difference of more than one nucleolus) and/or sizes of nucleoli. Grade 5 zygotes were those with pronuclei that were not aligned, were of grossly different sizes or were not located in the central part of the zygote.

Revised zygote scoring system: Z-score

The zygote scoring system was further revised after a review of the results from the first series, taking into account the zygote grade, day

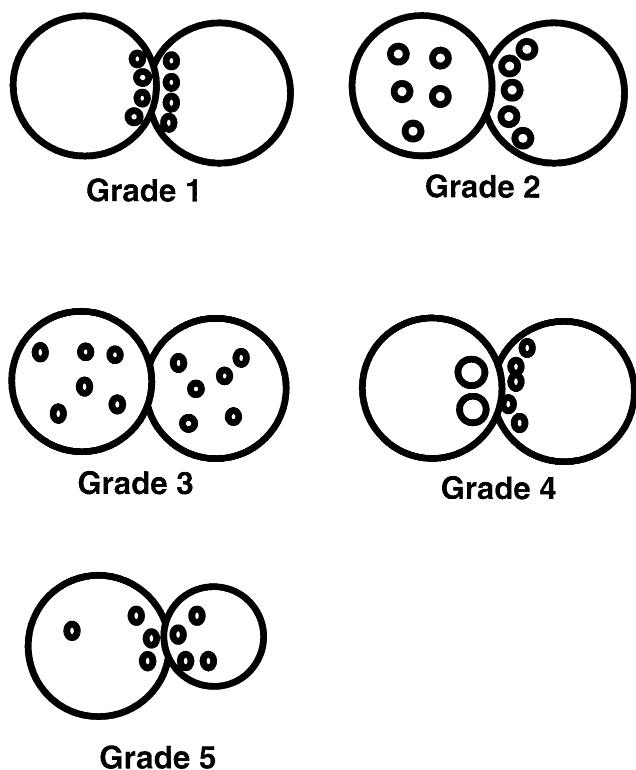

Figure 1. Diagrammatic representation of zygote grades using the initial grading system in series 1.

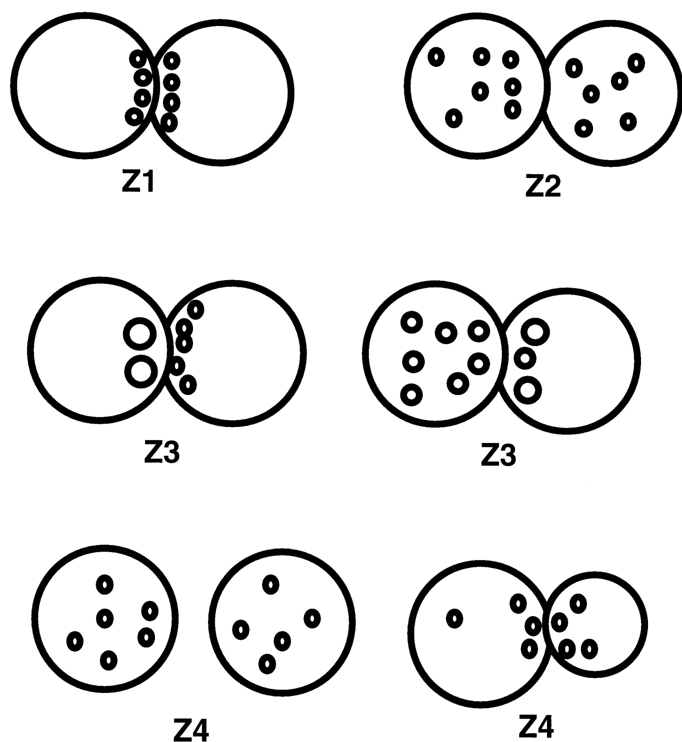

Figure 2. Diagrammatic representation of revised zygote scoring system for series 2.

3 morphology and ability to grow to the blastocyst stage. Grades 2 and 4 zygotes were combined as Z3 zygote score. The more desirable morphologies were grades 1 and 3 and were renamed Z1 and Z2 (see Figures 2 and 3). The revised system took account of nuclear size and alignment, nucleoli number and distribution. The nucleoli needed to be aligned, or be beginning to align, at the pronuclear junction, between three and seven per nucleus with no more than one nucleolus difference between the nuclei and equal in size. Although the size of the nucleoli was not measured, zygotes with either very small pinpoint nucleoli or very large ones were designated as Z3. The new system was adopted for ease of use and speed of scoring and to simplify further the scoring system for practical daily use by a large number of technicians. The goal was for clinical application in embryo selection and to reduce the numbers of embryos transferred.

Day 3 scoring

On day 3, embryos were scored as grade 1–5 according to certain morphological criteria. Grade 1 = 8-cells, <10% fragmentation, good cell–cell contact, no multinucleated blastomeres; grade 2 = 8-cell, 10–20% fragmentation or lacking good cell–cell contact, no multinucleated blastomeres; grade 3 = 6–7-cells or 8-cells with 20% fragmentation or uneven blastomere size, no multinucleated blastomeres; grade 4 = >8-cells or 4–6-cells or 8-cells with >20% fragmentation or uneven blastomere size or multinucleated blastomeres; grade 5 = <4-cells or grossly fragmented or with half of the blastomeres 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 blastocoele 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 and organized inner cell mass; >60 cells.

In series 1, embryos were selected for transfer based on day 3 morphology only, with high grade embryos being selected before low-grade embryos. In series 2, embryos that had accurate Z-scoring were selected initially by Z-score and then by embryo morphology. If there were adequate Z1 and Z2 embryos, these were used as the group out of which day 3 or day 5 embryos were selected. However, if there were no good grade day 3 or 5 embryos that had arisen from Z1 or Z2 zygotes, embryos were selected by morphology alone. Poor grade embryos arising from Z1 and Z2 zygotes were not transferred in preference to embryos with good grade day 3 or 5 morphologies. Those that had no accurate Z-scoring were selected by morphological criteria only on either day 3 or day 5.

Results

During the 20 month period (January 1998 to August 1999), 755 cycles were initiated for IVF/ICSI leading to 613 oocyte retrievals and 597 embryo transfers resulting in 317 clinical pregnancies confirmed by ultrasound (42.0% of cycles initiated; 51.7% of oocyte retrievals; 53.1% of embryo transfers). There was no difference between IVF and ICSI cycles in the results for zygote score, blastocyst development, or pregnancy rates and thus all the data were combined. The main differences in outcome were related to age, with older patients not performing as well as younger ones. However, age had no effect on the distribution of zygote scores or the development of blastocysts from zygotes, thus these data were not broken down for age. The age (mean ± SD) of the group was 34.9 ± 3.2 years (range 23–43). In scored cycles, zygotes were scored independently of

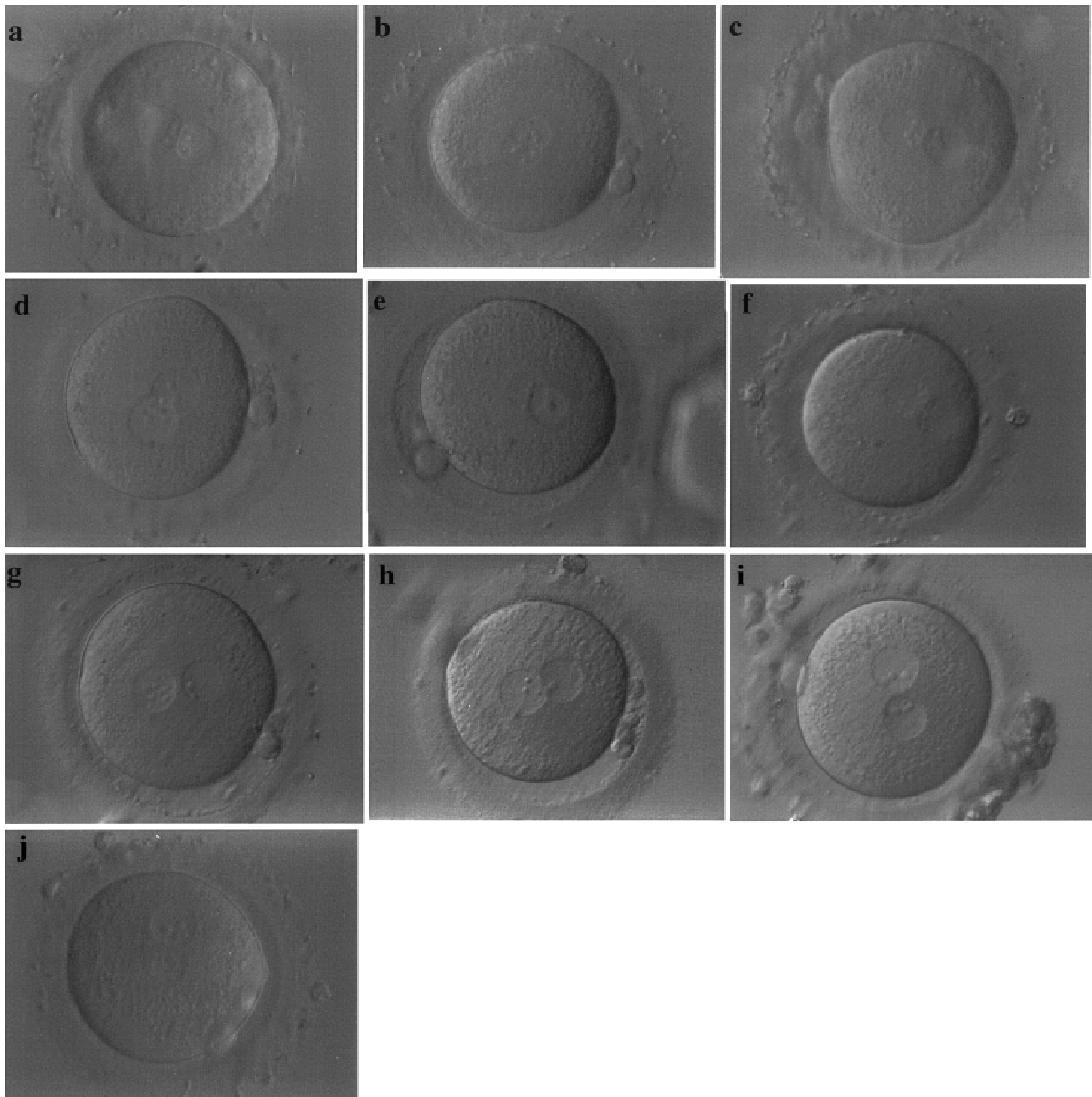

Figure 3. Zygote morphologies described for revised zygote scoring systems: (**a**, **c**) Z1, aligned, equal; (**b**, **c**) Z2, aligning, equal size and number; (**d**) Z3, pinpoint nuclei, scattered; (**f**, **g**, **h**) Z3, unequal alignment, numbers and size; (**i**) Z1, fast growing; (**j**) Z4, unequal sized pronuclei.

how many there were, or the age of the patient. In series 1, a full blastocyst transfer programme was not in place. In series 2, women who had sufficient zygotes (see below) had extended culture and day 5 transfers, regardless of age or zygote score. Not all cycles had zygote scoring recorded according to criteria. This was due to technician training, ambiguous scoring or no scoring at all. Only cycles with clear accurate zygote scoring were used to examine the relationship between Z-score and day 3 morphology, blastocyst development and pregnancy rate after embryo transfer.

Over this time period, three different media were used. A detailed analysis of the results from the different media showed a patient, rather than a system, variation (data not shown, unpublished). There was no difference in the spread of zygote scores or the subsequent development of these zygotes when they were fertilized in either HTF or P1 medium. The number of high-scoring zygotes that developed to the blastocyst stage in the different media systems was not different so the data were not broken down by media.

Series 1

There were 170 oocyte retrievals performed with 153 day 3 embryo transfers and 12 day 5 embryo transfers (total 165); five women had no embryo transfer due to failed fertilization. The pregnancy data are presented in Table I. The zygotes were scored using the initial scoring system described above, with embryo selection for day 3 transfer based on morphology, regardless of zygote score. All zygotes in this series were scored, embryos were cultured in individual drops, one observer scored all zygotes and embryos and the relationship between zygote grade and day 3 morphology was documented (Figure 4). When patients elected to have blastocyst cryopreservation, embryos not used in transfer on day 3 were placed in extended culture and the number of blastocysts developing

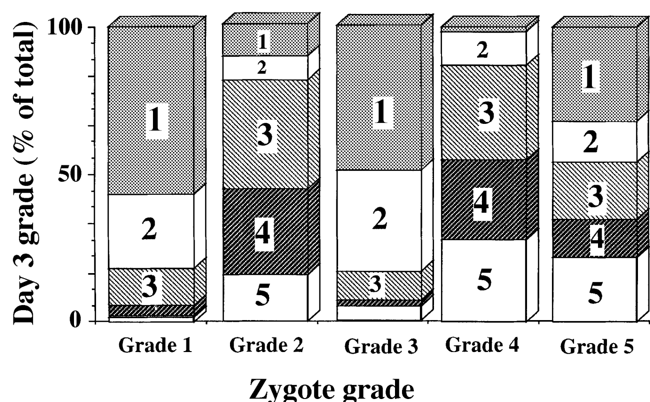

Figure 4. Relationship between zygote grade using the initial scoring system and day 3 embryo morphology in series 1. (See text for details of the zygote and embryo scoring system grades.)

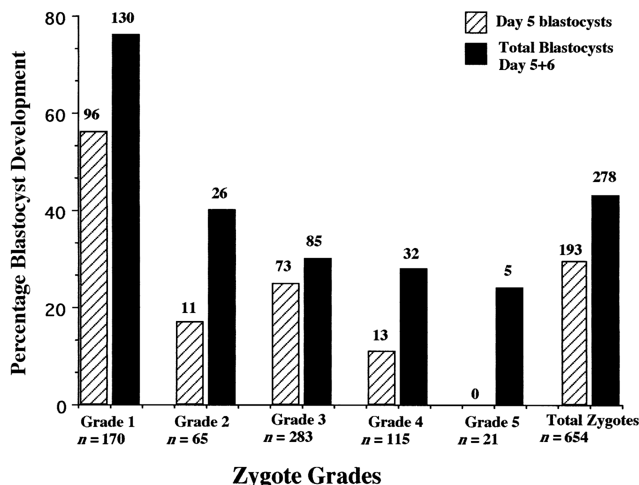

Figure 5. Blastocyst development on day 5 and days 5 and 6 from 654 graded zygotes placed in extended culture in series 1.

Table I. Series 1: cycle, transfer and pregnancy data

	n (%)	Clinical pregnancy/cycle (%)
Cycles initiated	201	38.8
Cycles retrieved	170	45.8
Cycles transferred	165[a]	47.3
Clinical pregnancies	78	
Ongoing/delivered	68	33.8 per cycle initiated
Spontaneous abortions	10 (12.8)	
Mean no. of embryos transferred	3.48	
	Day 3 transfer	Day 5 transfer
No. of embryo transfers	153	12
Clinical pregnancies (%/transfer)	70 (45.8)	8 (66.6)
Ongoing/delivered (%/transfer)	60 (39.2)	8 (66.6)
Spontaneous abortions	10 (14.3)	0
Mean no. of embryos transferred	3.52	2.0[b]
Implantation rate	(19.7)	52.4[b]

[a]Five patients showed failed fertilization.
[b]Significant difference between groups ($P < 0.01$).

from each zygote grade on days 5 and 6 was recorded. Blastocysts were cryopreserved at this stage. Not all day 3 embryos were placed in extended culture since some were frozen on day 3; many grade 5 embryos were arrested and not placed in extended culture and some women elected to have no cryopreservation. Their embryos were discarded according to laboratory protocols.

The highest percentage of high-grade day 3 embryos arose from grade 1 (Figure 3a,c) and grade 3 zygotes (Figure 3b,d). Although it was not possible to say with certainty which embryos implanted, all women who became pregnant after a day 3 embryo transfer had at least one embryo transferred which arose from a grade 1 zygote.

During this series, only 12 women had day 5 transfers carried out. All were aged <35 years, had more than eight good grade embryos on day 3 (regardless of zygote score) and elected to have a day 5 transfer to avoid multiple gestation. Although the blastocysts were not selected for transfer based on zygote score, those that were transferred arose from grade 1 zygotes.

A total of 1314 zygotes were scored. Of these, 390 (30%) were grade 1; 213 (16%) were grade 2; 425 (32%) were grade 3; 208 (16%) were grade 4; and 78 (6%) were grade 5. Day 3 embryo transfers were performed with 490 of these embryos and a total of 654 were placed in extended culture.

Of the 654 embryos placed in extended culture, 193 (30%) developed to blastocysts by the morning of day 5 of culture (at 112–114 h post-insemination) and a total of 278 blastocysts formed when totalling day 5 + day 6 blastocysts (43%) on the morning of day 6 (136–138 h post-HCG) (Figure 5). The 170 grade 1 zygotes placed in extended culture resulted in a higher percentage of blastocysts than all the other grades and a higher percentage by day 5. Of the 130 blastocysts formed from grade 1 zygotes, 96 were blastocyst by day 5 (56%) of zygotes placed in culture. There were no differences between the development of grades 2, 3 and 4 zygotes to the blastocyst stage when day 5 + day 6 blastocysts were totalled. However, grade 3 zygotes had more blastocysts on day 5 (73; 25%) than grade 2 (11; 17%) or grade 4 zygotes (13; 11%), implying that they were developing faster. Grade 5 zygotes formed blastocysts but they were not cryopreserved. This was based on observations that if the nucleoli were of grossly different sizes there was an increased risk of the embryo being aneuploid (Munné *et al.*, 1997; Munné and Cohen, 1998).

The conclusions drawn from this first series were that there was a relationship between day 3 morphology and zygote grade and between the ability to grow to the blastocyst stage and zygote grade. Grade 1 and 3 zygotes resulted in a higher percentage of good grade day 3 embryos and day 5 blastocysts than the other grades. Pregnancy was related to the transfer of grade 1 zygotes, whether on days 3 or 5.

Series 2

Based on the data from series 1, the revised zygote scoring system (Figure 2) was used in series 2. In this series, 554 cycles were initiated with 443 oocyte retrievals performed (80%). There were 432 embryo transfers, six patients with failed fertilization and five with elective cryopreservation of all embryos. In this series, day 5 transfers were used more frequently with 204 (47%) day 5 transfers and 228 day 3 transfers (53%). The data for series 2 are shown in Table II.

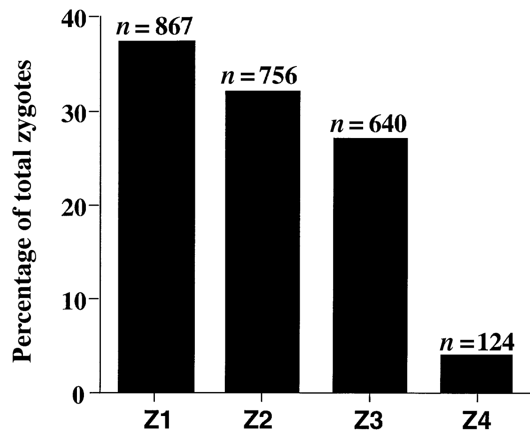

Figure 6. Distribution of Z-scores for 2387 zygotes in series 2.

Table II. Series 2: cycle, transfer and pregnancy data

	n (%)	Clinical pregnancy/cycle (%)
Cycles initiated	554	43.1
Cycles retrieved	443	53.9
Cycles transferred	432[a]	55.3
Clinical pregnancies	239	
Ongoing/delivered	218	39.4 per cycle start
Spontaneous abortions	21 (8.7)	
Mean no. of embryos transferred	2.73	
	Day 3 transfer	Day 5 transfer
No. of embryo transfers	228	204
Clinical pregnancies (%/transfer)	98 (42.9)	141 (69)[b]
Ongoing/delivered (%/transfer)	87 (38.2)	131 (64.2)[b]
Spontaneous abortions	11 (11)	10 (7)
Mean no. of embryos transferred	3.2	2.1[b]
Implantation rate	21.4	45.1[b]

[a]Patients (n = 11) had no transfer; five had all embryos frozen; six had failed fertilization.
[b]$P < 0.01$.

There was a reduction in the mean number of embryos transferred in this series compared with series 1 (2.73 versus 3.48, $P < 0.05$), due to the increased number of day 5 transfers that were performed (47 versus 7% in series 1). The decision to use day 3 or day 5 transfers was based on: (i) total number of zygotes on day 1 (more than six for women aged ⩽35 years and more than eight for women aged >35 years); and (ii) on day 3 embryo morphology. If there were more than four high-grade embryos on day 3 for women aged ⩽35 years or more than six embryos for women >35 years, a day 5 transfer was considered. There was no significant difference in the pregnancy rates for day 3 transfers between the two series (43 versus 45.4%). The day 5 pregnancy rates between series 1 and 2 could not be compared.

In series 2, 211 cycles (48%) had zygote scoring according to criteria. This was done in a non-systematic manner but was done regardless of age or number of oocytes retrieved. When the Z-score was recorded it was used to determine which embryos were selected for transfer on days 3 or 5, based on the observations from series 1.

For the scored cycles, the distribution of zygote score of the 2387 zygotes is shown in Figure 6. There was a fairly equal distribution of Z1, Z2 and Z3 zygotes with only 5% being Z4. The percentage of Z3 zygotes (27%) was approximately equal to that of grade 2 + grade 4 zygotes (32%) from the first series, which is consistent with the development of the revised scoring system.

There were 1284 zygotes placed in extended culture. These were the zygotes that were left after day 3 transfers where day 5 cryopreservation was elected and those from patients in which a day 5 transfer was planned. The development of blastocysts by 96 h of culture (112–114 h post-HCG, time of day 5 embryo transfer) and of day 5 + day 6 (136–138 h post-HCG) from the different Z-scores is shown in Figure 7. There was a higher percentage of day 5 blastocysts from Z1 zygotes placed in extended culture, than any of the other Z-scores ($P < 0.001$; Fisher's exact test) and a greater overall incidence of blastocyst development ($P < 0.001$; Fisher's exact test). The development of blastocysts from Z2 zygotes was greater than development from Z3 zygotes on both day 5 and day 5 + day 6 ($P < 0.05$; Fisher's exact test). This implies that Z1 and Z2 zygotes were cleaving more rapidly than embryos arising from Z3 zygotes.

The pregnancy data for series 2 for transfers in which embryos were selected by morphology (non-scored) or by Z-score (scored) is shown in Table III. For the scored cycles, embryos were selected initially by Z-score and then by embryo morphology. There was no difference in the maternal ages of the groups (mean 34.3 years; range 21–43). Day 3 transfers were used when there were not sufficient embryos to use extended culture (see above).

When day 3 embryos were selected for transfer initially by Z-score (Z1 and Z2 being the desired Z-scores) with embryo morphology as a secondary screen, the implantation and clinical pregnancy rates were significantly increased ($P < 0.01$). All patients who achieved a clinical pregnancy had at least one Z1 or Z2 embryo transferred. This is noteworthy in this group, since patients who had day 3 transfers were those with fewer oocytes retrieved or fewer zygotes. The use of zygote scoring increased the ability to select those embryos with greater potential to implant, regardless of stimulation response.

The data for day 5 transfers also show a difference between scored and non-scored cycles. There was an increase in both implantation and pregnancy rates when Z-score was used as the initial selection criterion ($P < 0.01$). There was also a trend for fewer embryos being transferred in scored versus non-scored cycles. With day 3 transfers, this was based on Z-score and blastocyst development observed in series 1, and with day 5 transfers it resulted from the fact that there were a number of elective single embryo transfers performed. Again this was based on the data relating the Z-score to the blastocyst development noted in series 1.

Figure 8 shows the pregnancy and implantation rates after the transfer of blastocysts arising from different Z-scores. Initially, results of blastocysts originating from Z1 zygotes were used followed by Z2 then Z3. In some instances, there were not two blastocysts on the morning of day 5. In this case (15/204, 7%) three embryos (one blastocyst and two morulae) were transferred. The use of compacted morulae originating

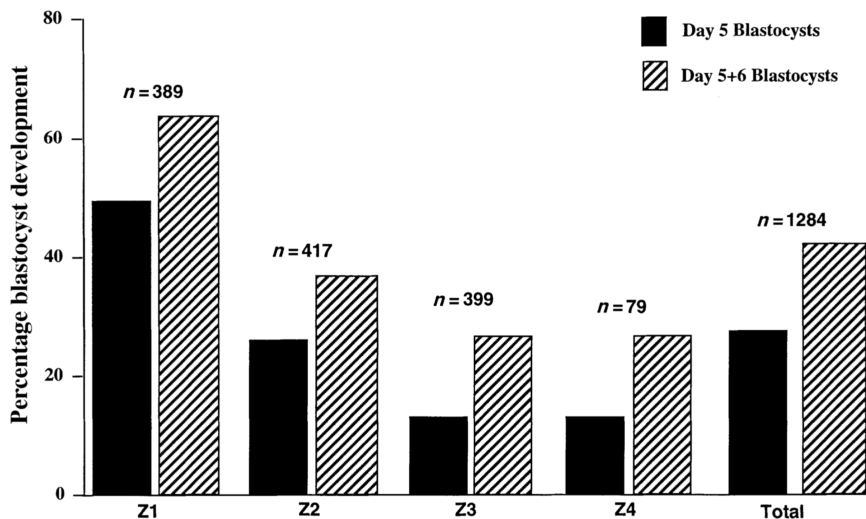

Figure 7. Development of 1284 zygotes from different Z-scores to the blastocyst stage by day 5 [112–114 h after administration of human chorionic gonadotrophin (HCG)] and day 5 + day 6 (136–138 h post-HCG).

Table III. Pregnancy data from the transfer of embryos on days 3 or 5 that were selected with or without zygote scoring in series 2

	Scored	Non-scored	Total
Day 3 transfers			
No. of transfers	94	134	228
Clinical pregnancies	54	44	98
Pregnancy rate/transfer (%)	57[a]	32.8	42.9
Mean no. of embryos transferred	2.8	3.3	3.1
Implantation rate (%)	31[a]	19	21
Day 5 transfers			
No. of transfers	117	87	204
Clinical pregnancies	85	50	135
Pregnancy rate/transfer (%)	72.6[a]	57.5	66.2
Mean no. of embryos transferred	1.9	2.2	2.1
Implantation rate (%)	52.3[a]	38.7	45.1

[a]Significant difference ($P < 0.01$) between scored and non-scored groups.

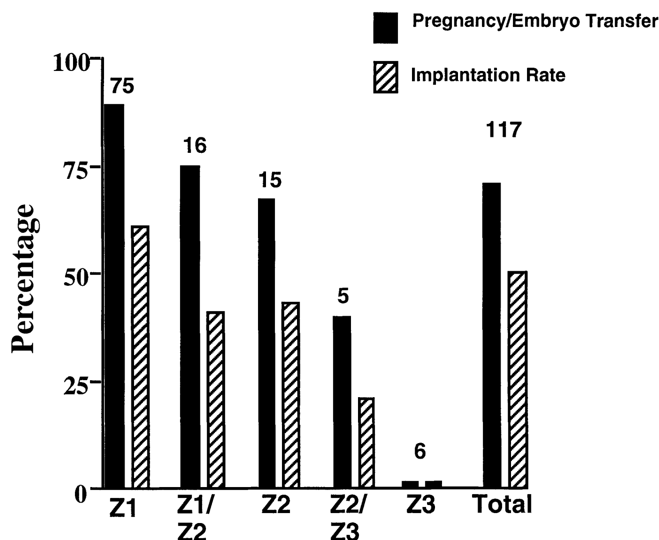

Figure 8. Relationship between Z-score of blastocysts transferred and pregnancy and implantation rates.

from Z1 or Z2 zygotes made no difference to pregnancy rates. The mean number of blastocysts transferred in the Z1 group was <2, due to the elective transfer of single embryos. Again, the confidence to do this was based on the data from series 1 and patient selection based on clinical indices.

When only blastocysts arising from Z1 zygotes were transferred there were increases in implantation and pregnancy rates compared with the other combinations ($P < 0.01$). When one Z1 and Z2 blastocyst were used for embryo transfer, the pregnancy rate was reduced compared to replacing only Z1 blastocysts ($P < 0.05$), and the implantation rate was significantly reduced (61 versus 41%, $P < 0.01$). There was no difference between replacing one Z1 + one Z2 blastocyst or two Z2 blastocysts. The use of blastocysts arising from Z3 zygotes had a negative impact on pregnancy and implantation rates. When the only blastocysts that were available arose from Z3 zygotes there were no pregnancies ($n = 6$).

Taken together, the data show a relationship between zygote morphology, ability to implant and for a successful pregnancy to continue. In addition, it has been shown that the ability of an embryo to grow *in vitro* to a blastocyst that is capable of implanting is directly related to the morphology of the zygote.

Discussion

The data presented in this paper show that there is a relationship between the morphology of the zygote at 16–18 h post-insemination and its ability to continue with development both *in vitro* and *in vivo*. This can be used pro-actively as a means of selecting embryos for either a day 3 or day 5 embryo transfer with resultant increased implantation and pregnancy rates. By using this zygote scoring method, the numbers of embryos required to achieve a pregnancy can be reduced without a reduction of pregnancy rates, which is a desired outcome for the avoidance of high-order multiple pregnancies.

These data further illustrate that not all embryos have the same potential either *in vitro* or *in vivo*. Using active zygote scoring those with higher potential (Z1 and Z2) can be used

for all procedures including embryo transfer extended culture or embryo cryopreservation. Those with lower potential (Z3) may rarely implant even if the morphological features of the cleaving embryos or blastocysts would indicate otherwise. This could help to reduce the numbers of embryos that are cryopreserved and stored indefinitely. If a patient repeatedly produces oocytes that result in poor Z-score embryos, different ovarian stimulation protocols could be sought which might result in oocytes with more potential. Since age, fertilization medium, fertilization route or rate had no effect on the Z-score, it is assumed that this is governed by the oocyte itself.

Oocyte growth begins in the fetus, giving rise to the oogonia (Tsafriri, 1988), some of which transform into oocytes by entering the first meiotic division. There is a large attrition of oogonia during this phase and by birth all the oogonia are at various stages of the first meiotic prophase, have entered at least the diplotene, and most the dictyate stage, of the first meiotic division; after birth all arrest at the dictyate stage (Anderson and Hirshfield, 1992; Eppig *et al*., 1996). During this phase, the somatic cells in the gonad enclose individual oocytes forming the preantral follicles (Sorenson and Wassarman, 1976; Schultz and Wassarman, 1977; Lintern-Moore and Moore, 1979; Eppig *et al*., 1996) which are recruited to enter the growing phase, form Graafian follicles and potentially ovulate. During its growing phase, the oocyte increases in size 300-fold, the granulosa cells proliferate and an antrum forms in the follicle which contains the oocyte surrounded by granulosa cells, forming the antral follicle.

As the oocyte increases in size its metabolism changes and as the germinal vesicle increases in size the nuclear to cytoplasmic ratio alters. The few nucleoli that are present also increase in size, indicative of ribosomal (r)RNA synthesis (Baker and Franchi, 1967) and there is a dramatic increase in the number of mitochondria and in their ultrastructure (Baker and Franchi, 1967; Balakier, 1978). The Golgi apparatus changes in activity and structure, the cortical granules move to the subcorticle region of the growing oocyte (Balakier, 1978) and the number of ribosomes also increases dramatically (Biggers, 1971)

Only fully-grown oocytes are competent to resume meiosis, with nuclear progression from the dictyate stage of the first meiotic prophase to metaphase II of the second meiotic division (first meiotic reduction) forming an unfertilized egg. Oocyte maturation with germinal vesicle breakdown occurs when the flow of meiosis arresting substances to the oocyte via the gap junctions between the oocyte and granulosa cells is decreased (Eppig, 1990). During the growth phase of the oocyte both nuclear and cytoplasmic maturation occurs and these need to be completed in a co-ordinated manner for the successful development of the mature oocyte with subsequent fertilization. Nuclear maturation includes the resumption of the first meiotic division and progression to metaphase II. Cytoplasmic maturation encompasses all the events that prepare an oocyte for successful fertilization such as zona pellucida acquisition, ability to release calcium and cortical granules, mitochondrial changes, protein synthesis during the growing phase, cytoskeletal changes. However, both can proceed without the other, but the resulting oocytes (even if they are fertilized and embryonic development ensues) are generally not viable (Eppig, 1990; Anderson and Hirshfield, 1992; Eppig *et al*., 1994). Some of the aberrant events in zygote development that are observed may be directly related to an asynchrony in these events. The many small, scattered nucleoli may indicate a true asynchronous development of nuclear and cytoplasmic factors. Other fertilization failures could also be related directly to asynchrony in the nuclear and cytoplasmic development.

The oocyte develops in the follicle, which is of vital importance to its final ability to develop fully. During the growth of the follicle the blood supply increases which is presumably to increase the oxygen concentration available to the follicle and oocyte for metabolism through oxidative phosphorylation. It has been documented that oocytes that derive from follicles with poorly developed blood supply are non-viable (Hartshorne, 1989; Gregory *et al*., 1994). A decrease in blood flow has even been associated with both decreased fertilization and increased spindle and chromosome defects (Van Blerkom *et al*., 1997). This hypoxia in the follicle has also been associated with disorganized cytoplasm and lower cystolic pH and ATP content (Van Blerkom and Henry, 1992; Van Blerkom *et al*., 1995). Van Blerkom has shown that oocytes vary dramatically in their ATP content at ovulation, between patients and within a cohort from a single patient. It would be interesting to see if there was any association between nucleoli formation and hypoxia, again underscoring how important the sequence of oocyte formation is.

The Z-score is based on the state of the nuclei as well as the nucleoli within the 1-cell fertilized embryo, the zygote. When an oocyte is activated by the entry of the spermatozoa (by normal fertilization or ICSI) a series of complex events ensues. In human fertilization, the centriole (the microtubule organizing centre), is sperm-derived (Schatten, 1984; Sathananthan *et al*., 1991). The centriole and the microtubules arising from it are responsible for bringing the male and female pronuclei together. If this does not occur normally, development cannot continue. Thus, the first aspect of Z-scoring is the alignment of the pronuclei which, by 16–18 h post-insemination, need to be aligned or appearing to touch. Failure to have progressed to this stage is indicative of failure of one or some fertilization events. These embryos rarely progress well and rarely form blastocysts.

The size of the pronuclei is also important. There is normally a slight difference in the size of the male and female pronuclei. However, large differences in size (Figure 3j) are associated with chromosomal defects such as aneuploidy (Munné and Cohen, 1998; Sadowy *et al*., 1998). Many of these zygotes can give rise to normal appearing day 3 embryos and day 5 blastocysts. If the embryos have not been scored at the zygote stage they could conceivably be used for embryo transfer, which would decrease the chances of positive pregnancy outcome. Thus, at an early stage in the IVF process, embryos likely to have gross chromosomal abnormalities can be eliminated from the pool available for transfer by simply noting the relative sizes of the pronuclei.

The major aspect of Z-scoring relates to the size, number and distribution of nucleoli. Nucleoli form within the nucleus at areas known as the ‘nucleolus organizing regions’. The

nucleolus organizing regions are located on the chromosomes in the nucleus where the genes coding for rRNA are located. The nucleoli are the sites where pre-rRNA is synthesized and are active sites on the chromosomes of the oocyte and sperm pronuclei. They are comprised of a chromatin portion and a ribosomal nuclear protein portion. The number and location of nucleolus organizing regions are species specific but the nucleoli appear to differ according to the cell type, its activity and the stage of development in any one species (Goessens, 1984; King *et al*., 1988). The nucleoli synthesize rRNA which is essential for meiotic competence (Motlik *et al*., 1984a,b). Oocytes in antral follicles have well-defined nucleoli. As the oocyte matures and is ovulated, the rRNA synthesis decreases and the nucleoli become small and scattered (Crozet *et al*., 1986). At fertilization, rRNA synthesis resumes with concomitant changes in the nucleoli, and they reform and grow. There is also evidence that they begin to coalesce as more synthesis occurs (Tesarik and Kopecny, 1989, 1990). Ovulated oocytes and the early embryo rely exclusively on maternal RNA that was synthesized pre-ovulation. As the nucleoli in the decondensing sperm nucleus and the female nucleus form, new rRNA is synthesized, as shown by the appearance of the nucleoli. As the embryonic gene becomes active, the rRNA being synthesized by the new unique embryo can take over, which in the human occurs at about the 4-cell stage (Braude *et al*., 1988). It is, therefore, important for the zygote to initiate the events leading to rRNA synthesis in a timely and co-ordinated sequence.

There is a tendency for nucleoli to fuse (Goessens, 1984; Tesarik and Kopecny, 1989, 1990). This is cell cycle related with more nucleoli being present at the beginning of the G_1 phase. The nucleoli continue to fuse until, at the time of the S phase, one or two per nucleus are left. During a mitotic cycle, daughter cells display synchrony in their nuclei content and fusion. There is also a correlation between ploidy and nucleoli content. Asynchrony in daughter cells is a result of aberrant chromosomal function.

In Z-scoring the state of the nucleoli in this transition to renewed rRNA synthesis is presumably what is being observed. All the Z1 and Z2 zygotes that gave rise to good grade blastocysts that were transferred had between three and seven even-sized nucleoli per nucleus (Figure 3a–d). Zygotes with unequal numbers or sizes and/or very unequal distributions of nucleoli (Z3) had the poorest outcomes (Figure 3e–h). Inequality between the nuclei might be a marker for asynchrony between male and female pronuclei, signalling chromosomal abnormalities or aberrant meiotic events, leading to breakdown of normal development with lack of embryonic implantation.

In a subset of patients ($n = 9$), nucleoli coalescence was very rapid, resulting in two or three very large nucleoli per nucleus by 16–18 h post-insemination (Figure 3i). Embryos resulting from these zygotes compacted early and were generally forming tight junctions with only 8–10 blastomeres on day 3. However, they did not routinely form blastocysts and any that were formed had too few cells and did not implant. Subsequently, when embryos from these zygotes were transferred on day 3 they had a very high implantation rate (15/27 implanted: 55%; seven out of nine women pregnant). An explanation for this phenomenon is that these embryos may be growing very rapidly and depleting metabolic substrates in the culture system. By transferring them back to the uterine environment at an earlier stage they realized their potential. This further lends credence to the need to score embryos at each point of development and not rely exclusively on one time point or parameter for embryo selection.

In another subset of patients ($n = 3$) the zygotes routinely had many very small, scattered nucleoli (Figure 3e). None of them became pregnant after many attempts. This would be consistent with a very early stage of nucleolus development. It could be that oocytes that give rise to these forms of zygotes within the time frame of accepted development are developmentally delayed due to slow nuclear and cytoplasmic maturation. For women who routinely produce zygotes of this form, alterations in the stimulation protocol may be beneficial. Additionally, this approach could also be used when the zygotes display asynchrony. This asynchrony could be due to male factors, with the male pronucleus not decondensing in a timely manner (Tesarik and Kopecny, 1989).

In conclusion, these data show that the morphology of the human zygote at 16–18 h post-fertilization can be used as a means of pre-selecting which embryos will have the highest potential for implantation. The Z1 and Z2 zygotes resulted in significantly higher numbers of good morphology blastocysts on day 5. All pregnancies from day 5 transfers resulted from the transfer of blastocysts that originated from Z1 or Z2 zygotes. This scoring system can be used to select those patients who would benefit from a day 5 transfer in systems where limiting the numbers of embryos transferred at earlier stages is not feasible due to patient demand, lack of regulations and the high cost of performing assisted reproduction. If only certain embryos have potential, selection can begin early, again limiting the numbers of patients being offered day 5 transfers at a time when the culture systems are not optimal.

The knowledge that, as early as the zygote stage, only certain embryos have potential could help limit the numbers of embryos that are cryopreserved. Cryopreserving embryos with little to no developmental potential is of no benefit to the patient. More importantly, it can be used to help reduce the numbers of embryos required at embryo transfer, on any transfer day, to establish a pregnancy and thus reduce the incidence of high-order multiple pregnancies. Overcoming the oocyte problems, which could be leading to these zygote morphologies, will require an understanding of the events that trigger the maturational events and those that control the nuclear and cytoplasmic maturation. If women are routinely producing oocytes that result in zygotes of poor morphology, different approaches to ovulation induction may be required. It could also be argued that these women might benefit from the in-vitro maturation of their oocytes, where ovarian influences can be removed.

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