

Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability

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In-vitro fertilization (IVF) embryos are selected for transfer on the basis of morphology and rate of development. However, when a number of embryos have similar characteristics, the selection of the best embryos is left to chance. Recently, we proposed a simple, novel method to overcome this problem, based on pre-selection of embryos cleaving early to the two-cell stage. In this study we have adopted the same method to choose embryos fertilized after intracytoplasmic sperm injection (ICSI). Fertilized embryos that had cleaved to the two-cell stage by 27 h post-injection were designated as 'early cleavage' embryos, while those that had not yet reached the two-cell stage were designated as 'no early cleavage'. In all cases, the early cleavage embryos were transferred when available. Early cleavage was observed in 54 (61.4%) of the 88 cycles assessed. There were significantly ($P = 0.04$) more clinical pregnancies in the early cleavage group, 14/54 (25.9%), compared with the no early cleavage group 2/34 (3.2%). No differences between the groups were found when comparing key parameters (age, stimulation protocol and semen characteristics) of the couples. Using the ICSI technique, we have shown that early cleavage to the two-cell stage is not influenced by the timing of fertilization, and is more likely due to intrinsic factors within the oocyte or embryo that promote embryo cleavage after fertilization.

Key words: early cleavage/embryo quality/intracytoplasmic sperm injection/viability

Introduction

One of the major factors that can influence the success of an in-vitro fertilization (IVF) cycle is the selection of the best embryos for transfer. Routinely, the embryos selected for transfer are chosen on the basis of their morphology and rate of development in culture. This line of thinking stems from the idea that faster developing embryos in culture have a greater capacity to establish pregnancies. In one study, Cummins *et al.*

(1986) established an embryo quality and embryo development rating and found that good ratings for both resulted in a greater chance of establishing clinical pregnancies. Other studies have also demonstrated an advantage in transferring embryos on the basis of morphological and developmental assessment (Edwards *et al.*, 1984; Hill *et al.*, 1989; Steer *et al.*, 1992). In the report by Edwards *et al.* (1984), it was clearly shown that embryos cleaving more rapidly had a greater chance of implanting. For example, embryos that had cleaved to the eight-cell stage prior to 55 h after insemination gave rise to a pregnancy rate of >31% when transferred while embryos reaching the eight-cell stage 56 h after insemination or later gave rise to a pregnancy rate of <17%. A similar pattern in pregnancy rates was also evident when the earliest cleaving two-cell embryos were transferred compared to zygotes. In contrast to the above methods, the measurement of several metabolic parameters of embryos by using non-invasive procedures has also been proposed (Leese, 1987); however these methods unfortunately require additional expertise and cost to IVF centres preventing their widespread use.

Whereas the selection of embryos at the time of transfer on the basis of cell number and quality is of significant benefit (Cummins *et al.*, 1986), it does not take into account variability in the time of cleavage. In fact, as discussed by Bavister (1995), difficulties arise in assigning precise cleavage times to embryos due to differences in the timing of sperm penetration and in the duration of the cell cycle in different embryos. Subsequently, if for example a group of four-cell embryos is observed at the time of transfer, it is not possible to distinguish which has just cleaved to the four-cell stage or which has been at the four-cell stage for several hours. Hence, selection of the more advanced embryo is left to chance. The importance of establishing a precise timing of development was demonstrated in the hamster by McKiernan and Bavister (1994) and Gonzales *et al.* (1995). In their studies hamster embryos that had reached the eight-cell stage in a normal time frame had a greater ability to develop to the blastocyst stage and a higher viability when transferred, compared to embryos that had not reached the eight-cell stage at the same timepoint.

In a recent article (Shoukir *et al.*, 1997), we proposed a novel method to evaluate human embryo viability based on the timing of the first cell division. The earliest time the human zygote can reach the two-cell stage after routine IVF ranges between 20 and 27 h post-insemination (Trounson *et al.*, 1982; Balakier *et al.*, 1993; Capmany *et al.*, 1996). By pre-selecting embryos that had undergone early cleavage to the two-cell stage by 25 h post-insemination and then transferring these embryos the following day, we found that patients with early cleaving embryos had more than twice the pregnancy rate and

Table I. The total and mean number (\pm SD) of oocytes, two-pronucleate (2PN) oocytes, embryos observed on day 2, embryos transferred, implantation rate and pregnancies in patients who had at least one embryo that had undergone early cleavage to the two-cell stage by 27 h after ICSI, compared to those who had none

Parameter	No early cleavage	Early cleavage	P-value
No. of cycles	34	54	–
No. of oocytes collected (mean \pm SD)	296 (8.7 \pm 5.0)	489 (9.0 \pm 5.3)	0.8
No. of oocytes injected (mean \pm SD)	238 (7.0 \pm 4.2)	440 (8.2 \pm 4.8)	0.2
No. of 2 PN (mean \pm SD)	137 (4.0 \pm 2.2)	263 (4.9 \pm 2.8)	0.1
Fertilization rate (%)	(57.6)	(59.8)	
Early two cells (% 2-cell/2 PN) (mean \pm SD)	0	122 (46.4) (2.2 \pm 1.6)	–
No. of embryos on day 2 (% day 2 embryos/2 PN) (mean \pm SD)	96 (70.1) (2.8 \pm 1.9)	231 (87.8) (4.3 \pm 2.4)	0.004
No. of embryos transferred (mean \pm SD)	93 (2.7 \pm 0.8)	150 (2.8 \pm 0.7)	0.8
Implantation rate (%) (fetal heart beat/ embryo transferred)	3/93 (3.2)	21/150 (14.0)	0.01
No. of clinical pregnancies (%)	2 (5.9)	14 (25.9)	0.04

three times the implantation rate (Shoukir *et al.*, 1997). In this paper, we have therefore investigated whether the same improvement in pregnancy rate is observed when early cleavage to the two-cell stage is used as an indicator to select embryos for transfer after intracytoplasmic sperm injection (ICSI). The results obtained after ICSI are of particular interest, as the timing of fertilization is more defined than after routine IVF, hence the cleavage rates of embryos are less likely to be determined by the time of fertilization.

Materials and methods

The study was performed on patients entering the IVF programme of the Clinic of Infertility and Gynaecological Endocrinology, Department of Obstetrics and Gynaecology, University Hospital of Geneva, Geneva, Switzerland between October 1994 and March 1997. In all, 88 cycles were assessed where the patient underwent ICSI treatment with embryo transfer. Patients that failed to achieve fertilization were not included. The stimulation protocol adopted by our group has been previously described (Sakkas *et al.*, 1994).

Collected oocytes (day 0) were maintained in our standard culture medium Whittingham's T6 (Quinn *et al.*, 1982) supplemented with 10% maternal human serum. Oocyte retrieval took place between 0830 and 1030 h. ICSI was performed between 1300 and 1500 h (~1400 h) as previously described (Sakkas *et al.*, 1996). The following morning (day 1), the oocytes were washed in 20 μ l culture drops under oil (light white mineral oil; Sigma Pharmaceuticals, Buchs, Switzerland) in Petri dishes and the presence of two pronuclei assessed. On the same day (day 1), at 1700 h (27 h post-injection), the embryos were examined to see if cleavage to the two-cell stage had occurred. These embryos were designated as 'early cleavage' embryos, while those that had not yet cleaved were designated as 'no early cleavage'. Both groups were maintained in separate culture drops. The following day (day 2) the embryos were transferred between 1000 and 1500 h. Routinely, a maximum of three embryos was transferred to the patient. When there were only one or two early cleavage embryos, the embryo transfer group was completed by

adding the best one or two embryos from the no early cleavage group. In nine cycles, four embryos were transferred due to advanced maternal age, repeated failure in previous cycles or poor quality embryos.

On the day of transfer all embryos were assessed for the number of cells per embryo, to ascertain their cleavage rate, and given a quality score based on the presence of fragments and clarity of the cytoplasm of the blastomeres, similar to that previously described by Cummins *et al.* (1986). The ratings given to embryos for cell number were 1 for one cell, 2 for two cells, 3 for three cells, 4 for four cells, 6 for between four and eight cells and 8 for more than eight cells. The ratings given to embryos for quality were the same as those used by Cummins *et al.* (1986), except that the values of 0 for poorest and 3 for best embryo were given.

A pregnancy test was conducted 14 days after transfer. Patients that exhibited three consecutive human chorionic gonadotrophin (HCG) values >5 mIU/ml and where the fetus or fetuses displayed a heart beat by ultrasound examination 4–5 weeks after transfer were considered to have achieved a clinical pregnancy.

The statistical evaluations used were analysis of variance followed by Scheffé's *F*-test for comparisons of mean values (Tables I, IV and V) and χ^2 analysis with continuity correction for comparison of pregnancy rates (Table I and II) (Statview 4.5, Abacus Concepts Inc., Ca, USA). Statistical significance was set at $P < 0.05$.

Results

Early cleavage to the two-cell stage was observed in 54/88 (61.4%) of the cycles. This is a greater percentage than when IVF embryos were checked in our laboratory (Shoukir *et al.*, 1997) at the same time point, and is explained by the fact that ICSI was performed earlier than when oocytes were inseminated in routine IVF. Table I shows the overall results, including the implantation rate and clinical pregnancy outcome, in patients who had at least one early cleavage embryo compared to those that had none. There was no significant difference in the number of oocytes injected or fertilization

Table II. Clinical pregnancy rates according to the number of embryos transferred in patients who had early and no early cleaving embryos after ICSI

No. of embryos transferred	No early cleavage (%)	Early cleavage (%)
1	0/3 (0)	0/4 (0)
2	0/7 (0)	1/9 (11.1)
3	1/20 (5.0)	13/36 (36.1)*
4	1/4 (25.0)	0/5 (0)

*Denotes significantly different $\chi^2 = 5.1$, $P = 0.02$ when compared to patients with no early cleavage embryos.

rate between both groups. The early cleavage patient group however had significantly more embryos per patient on day 2 compared with the no early cleavage group. Patients with early cleaving embryos displayed an implantation rate four times higher than patients without early cleaving embryos. The pregnancy rate in the early cleavage group was also more than four times higher than the no early cleavage group. No cases of ectopic pregnancies were observed in either group, while there were an additional four biochemical pregnancies in the early cleavage group, where three positive HCG values were measured but no positive confirmation of the pregnancy was obtained by ultrasound.

The clinical pregnancy rate according to the number of embryos transferred is represented in Table II. When three embryos were transferred, significantly more pregnancies were obtained in the early cleavage group compared to the no early cleavage group. No comparison was made when one, two or four embryos were transferred due to the low number of cycles. Table III shows the implantation rate and the incidence of single, twin and triplet pregnancies when the cohort of the three embryos transferred contained either: one, two or three early cleavage embryos or all no early cleavage embryos. When all three embryos transferred had undergone early cleavage, the majority of pregnancies obtained were multiple.

When a number of key parameters in the couples were examined, we failed to find any differences between the groups linked to the male or female partner that influenced the occurrence of early cleavage. The mean age (\pm SD) of the females was 33.8 ± 3.9 and 34.5 ± 4.9 in the no early and early cleavage group respectively. When the progress of stimulation, as indicated by oestradiol concentration on the day of HCG and oocyte retrieval and the number of ampoules of Pergonal (Serono, Aubonne, Switzerland) used during stimulation was examined no statistically significant differences were evident between the two groups (data not shown). More importantly, as the majority of patients being treated were male factor patients, we examined whether semen parameters influenced the differences between the groups. There were no significant differences concerning the mean age and the semen characteristics of the male partner between the groups, and semen characteristics did not influence the number of early cleaving embryos observed (Table IV). In addition, no significant trends in pregnancy rates in relation to the different sperm parameters (sperm concentration, motility and morphology) in patients who had early cleaving embryos could be observed.

To examine whether the cohort of embryos transferred to the patients with and without early cleaving embryos differed, we calculated the mean score of the number of cells and mean quality per embryo. The mean number of cells per embryo and the mean quality of cells per embryo on day 2 for those transferred are presented in Table V. The results show that the mean cell numbers of the embryos transferred and the quality rating was significantly higher for early cleavage embryos. When taking this observation into consideration, it could be argued that the early cleaving embryos may have been selected for transfer anyway. However, in 25 of the cycles with early cleaving embryos the patient had more than three embryos available for transfer hence the embryos of the highest cell number and best quality had to be distinguished. In eight of the 25 (32%) cycles, three embryos could be selected at the time of transfer according to cell number and quality. In the remaining 17 cycles (68%), a selection had to be made between two or more embryos of the same cell number and quality to complete the cohort of embryos selected for transfer. It is in these cases that a prior selection of the early cleaving embryos helps to distinguish which of the embryos is more advanced.

Discussion

The kinetics of fertilization and cleavage to the two-cell stage after ICSI have been previously documented in a study by Nagy *et al.* (1994). In their study, cleavage to the two-cell stage was initially observed 20 h after ICSI, whereby 10 out of the 93 normally fertilized oocytes (11%) had cleaved to the two-cell stage. In this study, fertilized oocytes were examined at 27 h after ICSI and at this stage a total of 122/400 (30.5%) had cleaved to the two-cell stage. A comparative study examining the number of fertilized oocytes cleaving to the two-cell stage after sub-zonal insemination reported that between 25.5 and 27 h post-treatment just over 30% of the embryos had cleaved (van Wissen *et al.*, 1995).

Early cleavage was observed in 54 of the 88 cycles (61.4%) examined. Subsequently, in the 54 cycles where early cleavage had taken place a significantly higher number of embryos implanted and pregnancies were established. The question as to why the selection of early cleavage embryos leads to a higher pregnancy rate may be related to a number of factors. Firstly, the early cleaving embryos may simply be fertilized earlier. Although this argument can be used in the case of routine IVF, the direct injection of spermatozoa into the cytoplasm using ICSI ensures a shorter time frame of fertilization. Hence by showing that early cleavage embryos have a greater viability after both routine IVF (Shoukir *et al.*, 1997) and ICSI, it appears that the ability of an embryo to undergo early cleavage is not related to the timing of fertilization. A second parameter that can also influence fertilization is related to the maturity of the oocytes; less mature oocytes are usually fertilized later than the mature ones, leading to a delayed cleavage (Balakier *et al.*, 1993). The removal of the cumulus complex prior to performing ICSI, however, overcomes the presence of immature oocytes. The elimination of these factors influencing the early cleavage of embryos points to the presence of an unknown factor(s) within the oocyte which favours or

Table III. Pregnancy in relation to the number of early cleavage and no early cleavage embryos transferred in patients receiving three embryos at transfer

No. early and no early cleavage embryos transferred		Pregnancy			Implantation rate ^a (%)	Total ^b (%)
No early cleavage	Early cleavage	Single	Twins	Triplets		
Patients with no early cleaving embryos						
3	0	1	0	0	1/60 (1.7)	1/20 (5.0)
Patients with early cleaving embryos						
2	1	1	0	0	1/27 (3.7)	1/9 (11.1)
1	2	4	1	0	6/36 (16.7)	5/12 (41.7)
0	3	3	2	2	13/45 (28.9)	7/15 (46.7)

^aNumber of fetal heart beats per embryo transferred.

^bNumber of clinical pregnancies per transfer.

Table IV. Semen parameters (mean ± SD) for cycles with and without early cleavage to the two-cell stage

No. of early cleaving embryos	No. of cycles	Age	Semen parameters		
			Sperm concentration ×10 ⁶	Motility ^a (%)	Morphology ^b (% normal)
Patients with no early cleaving embryos					
0	34	37.0 ± 6.8	13.8 ± 22.8	29.8 ± 21.9	13.0 ± 13.0
Patients with early cleaving embryos					
1	22		15.8 ± 17.5	35.9 ± 22.9	15.2 ± 13.2
2	14		23.5 ± 17.5	29.4 ± 19.7	15.3 ± 14.0
≥3	18		9.0 ± 8.4	37.7 ± 18.6	11.5 ± 9.3
Total	54	38.3 ± 6.1	15.5 ± 25.3	34.8 ± 20.6	13.9 ± 12.1

^aMotility includes all spermatozoa with forward progression.

^bSeven cycles with no early cleavage and six with early cleavage could not be assessed for morphology due to low sperm numbers.

Table V. Mean number of cells per embryo and mean quality per embryo of embryos transferred in patients with and without early cleavage to the two-cell stage (see Materials and methods for a description of the scoring system)

	Embryos of no early cleavage patients		Embryos of early cleavage patients	
	No early cleavage	All embryos	No early cleavage	Early cleavage
Number of embryos transferred	93	150	47	103
Mean number of cells ± SD	2.7 ± 1.4 ^a	3.6 ± 1.1 ^a	3.1 ± 1.3 ^b	3.8 ± 1.0 ^b
Mean quality of cells ± SD	1.8 ± 1.1 ^c	2.5 ± 0.7 ^c	2.2 ± 0.9 ^d	2.7 ± 0.5 ^d

Like letters a-a; b-b; c-c; d-d are significantly ($P < 0.01$) different.

promotes the early division of embryos after fertilization. This unknown factor subsequently improves the viability of embryos. A number of candidates could be proposed to explain early cleavage. One of these is the expression of human leukocyte antigen (HLA)-G, as in human pre-implantation embryos it has been demonstrated that a correlation exists between HLA-G mRNA expression and improved cleavage rate (Jurisicova *et al.*, 1996). It would therefore be of interest to ascertain whether early cleavage is observed repetitively in certain patients, indicating a genetic influence over this phenomenon. A further candidate can be linked to the metabolic fitness of oocytes from different patients. For example, Van

Blerkom *et al.* (1995) proposed that grossly normal human embryos may not progress to implantation due to a chronically low ATP content that is first established in the oocyte. A further advantage to embryos that have undergone early cleavage is that they will also be less likely to arrive at a critical minimal level of maternal mRNA levels (Bacharova and de Leon, 1980; Clegg and Piko, 1983) prior to activation of the embryonic genome. Human embryos that have experienced retarded fertilization or have a slower cleavage rate may deplete their vital maternal mRNA stores and their viability would be compromised prior to reaching the four- to eight-cell stage when the embryonic genome is activated (Braude

et al., 1988). As the manifestation of the above mentioned challenges to embryo development would likely lead to a poor morphology of the developing embryo it was of interest to note that embryos that had undergone early cleavage had a significantly higher quality compared to those that had cleaved later.

The early cleavage of embryos was also investigated in relation to the sperm parameters of the male. It has previously been shown that a strong paternal effect is associated with the development of human embryos to the blastocyst stage (Janny and Ménéz, 1994). These authors found evidence that frozen and abnormal spermatozoa gave rise to embryos with a significantly lower cleavage rate and potential to form blastocysts. Prior to this study, a number of other authors also suggested that semen quality and embryo development may be related (Ron El *et al.*, 1991; Chan *et al.*, 1993; Parinaud *et al.*, 1993). When examining the influence of sperm parameters on the number of early cleavage embryos and the outcome of pregnancy, we found no difference between males with varying sperm parameters. The previous studies that have indicated a paternal effect have however been based on using routine IVF insemination procedures where cleavage rates and embryo quality may be influenced by the timing of fertilization. In this study however, the timing of fertilization is more definite, because of the use of ICSI, hence the influence of a poor sperm parameter on fertilization is largely removed. Indeed a number of studies have already shown that sperm parameters have little influence on fertilization rates and embryo quality after ICSI (Van Steirteghem *et al.*, 1993; Payne *et al.*, 1994; Nagy *et al.*, 1995; Tournaye *et al.*, 1995).

A final point of consideration arises from certain studies that provide evidence that male embryos cleave faster than female embryos (Tsunoda *et al.*, 1985; Burgoyne, 1993; Peippo and Bredbacka, 1995). In the human, it has also been proposed that sex selection is inadvertently performed in IVF programmes because the faster cleaving and higher quality embryos are selected for transfer (Tarin *et al.*, 1995). The technique of embryo selection used in this study would therefore predictably give rise to more males. In this study, and our previous study (Shoukir *et al.*, 1997), there do not appear to be any tendencies to a selection of males; however, the number of pregnancies is too limited to come to any conclusion.

In this and our previous study (Shoukir *et al.*, 1997), we have shown that the assessment of early cleavage to the two-cell stage can be used as an indicator of embryo viability and is subsequently a strong prognostic factor of the likelihood of pregnancy. The simple action of selecting early cleaving two-cell embryos alleviates the problem of guessing which are the more advanced embryos at the time of transfer. Using the ICSI technique, we have also shown that the early cleavage of embryos to the two-cell stage is not entirely influenced by the timing of fertilization, and is therefore more likely due to intrinsic factors within the oocyte or embryo that promote embryo cleavage after fertilization.

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