

Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures

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BACKGROUND: The reduction of multiple pregnancies by using elective single embryo transfers (eSET) requires critical and careful selection of the embryo for transfer. The current study was undertaken to assess whether early cleavage could be used as a marker of embryo competence in eSET procedures. **METHODS:** The study included analysis of 178 eSET procedures. All embryos were checked for early cleavage at 25–27 h post insemination or ICSI. The embryos that possessed two cells at 25–27 h post insemination or ICSI were designated as ‘early cleavage’ (EC) embryos and those that had not yet cleaved were classified as ‘no early cleavage’ (NEC) embryos. Selection of the embryo for transfer was based on embryo morphology and growth rate on day 2 and not early cleavage. Clinical parameters were compared between 72 EC and 106 NEC single embryo transfers. **RESULTS:** A significantly higher clinical pregnancy rate was observed after transfer of EC (50%) than NEC (26.4%) embryos. **CONCLUSIONS:** The current study provides compelling evidence that EC embryos possess significantly higher developmental competence than NEC embryos.

Key words: early cleavage/embryo viability/ICSI/IVF/single embryo transfer

Introduction

The increase in the proportion of elective single embryo transfers (eSET), driven by the need to reduce the incidence of multiple pregnancies, is a phenomenon seen throughout Europe (Gerris *et al.*, 1999; Vilska *et al.*, 1999; Martikainen *et al.*, 2001; Tiitinen *et al.*, 2001). The successful eSET programme should, however, meticulously address all issues of embryo selection. Plenty of data have been gathered showing that the gross embryo morphology (Hill *et al.*, 1989; Erenus *et al.*, 1991; Scott *et al.*, 1991; Staessen *et al.*, 1992; Shulman *et al.*, 1993; Giorgetti *et al.*, 1995; Alikani *et al.*, 1999) and developmental rate (Cummins *et al.*, 1986; Claman *et al.*, 1987; Staessen *et al.*, 1992; Giorgetti *et al.*, 1995; Ziebe *et al.*, 1997) are the most reliable markers of embryo viability. The correlation between blastomere uniformity and positive outcome of IVF and ICSI procedures is also well established (Giorgetti *et al.*, 1995; Hardarson *et al.*, 2001). In addition, some other morphological features such as variation in zona thickness (Cohen *et al.*, 1989) and the presence of multinucleated blastomeres (Pelinck *et al.*, 1998) have been shown to affect implantation rate. According to some authors, the selection of viable embryos could even be performed at the zygote stage (Scott and Smith, 1998; Tesarik and Greco, 1999). Several studies have also suggested that early cleavage of zygotes occurring by 25–27 h after insemination or ICSI could be used to predict the implantation potential of embryos

(Edwards *et al.*, 1984; Shoukir *et al.*, 1997; Sakkas *et al.*, 1998, 2001). The current study was undertaken to assess the importance of this developmental marker in determining the viability of embryos in eSET procedures.

Materials and methods

The study was performed at two infertility clinics in Helsinki: Infertility Clinic of the Family Federation of Finland and Helsinki University Central Hospital. A total of 178 eSET, performed between January, 2002 and June, 2002, were included in this study. From the analysed cycles, 133 were IVF and 45 ICSI procedures. Patients were deemed eligible for eSET if they were ≤ 37 years old, and were in their first or second IVF or ICSI treatment. Other indications for eSET were: patient's wish to avoid multiple pregnancies, previous successful IVF or ICSI treatment and risk of ovarian hyperstimulation syndrome. By definition, eSET procedures included only those IVF or ICSI cycles where at least two embryos were available from which one was selected for transfer. In our practice, eSET is performed only when a good quality embryo possessing mononucleated blastomeres and $< 20\%$ of fragmentation could be selected for transfer (Vilska *et al.*, 1999). Currently, in our clinics, more than half of the all IVF or ICSI procedures fulfil these criteria for eSET.

Ovarian stimulation regimen, oocyte collection, IVF and ICSI procedures and embryo transfer were similar at both clinics and have been reported elsewhere (Vilska *et al.*, 1999; Tiitinen *et al.*, 2001). Briefly, the patients underwent pituitary down-regulation with GnRH agonist (Synarela; Syntex Nordica AB, Sweden) commenced in the

Table I. Comparison of clinical parameters in IVF/ICSI cycles where early cleavage (EC) and no early cleavage (NEC) embryos were transferred

Parameter	EC	NEC	P-value
No. of cycles	72	106	–
Age (years, mean \pm SD)	33.3 \pm 3.8	33.5 \pm 4.3	NS
No. of IVF cycles (%)	50 (69.4)	83 (78.3)	NS
No. of ICSI cycles (%)	22 (30.6)	23 (21.7)	NS
No. of oocytes per patient (mean \pm SD)	13.3 \pm 7.6	11.8 \pm 7.5	NS
No. of embryos per patient (mean \pm SD)	8.3 \pm 4.9	7.4 \pm 4.7	NS
No. of blastomeres in transferred embryos (mean \pm SD)	4.0 \pm 0.4	3.7 \pm 0.9	0.003
Degree of fragmentation in transferred embryos (%; mean \pm SD)	8.0 \pm 7.9	10.3 \pm 9.7	NS
No. of evenly cleaved transferred embryos (%)	40 (55.6)	38 (36.2)	0.011
No. of positive hCG measurements (%)	40 (55.6)	30 (28.3)	< 0.0001
No. of preclinical abortions (%)	4 (10)	2 (6.7)	NS
No. of clinical pregnancies (%)	36 (50)	28 (26.4)	0.001

NS = not significant.

mid-luteal phase of the previous menstrual cycle. When suppression was achieved, ovarian stimulation was performed using recombinant FSH (Gonal-F; Serono, Switzerland; or Puregon; Organon; The Netherlands). When two or more follicles reached the size of ≥ 17 mm in diameter, hCG (Pregnyl; Organon, or Profasi; Serono) was administered. Transvaginal oocyte retrieval was performed after 36 h.

Culture media for oocytes and embryos were either IVF medium (MediCult, Denmark) or Sydney IVF Fertilisation and Cleavage Medium (Cook IVF, Australia). The spermatozoa were prepared using both density gradient centrifugation (PureSperm; NidaCon, Sweden) and swim-up. Insemination or microinjection was carried out 4–6 h after oocyte retrieval. The oocytes were checked for the presence of pronuclei and polar bodies 16–18 h after insemination. The zygotes were examined again 25–27 h after insemination or ICSI to see whether they had undergone the first mitotic cleavage. Embryos that possessed two cells at 25–27 h after insemination were designated as ‘early cleavage’ (EC) embryos and those that had not yet cleaved were classified as ‘no early cleavage’ (NEC) embryos.

The embryo quality was evaluated 44–46 h after insemination or ICSI, considering the blastomere number, the degree of fragmentation, the uniformity of blastomeres and the presence of multinucleated blastomeres. The degree of fragmentation was expressed as a percentage of the perivitelline space occupied by anucleate cytoplasmic fragments. Embryos were considered evenly cleaved when the difference in size between blastomeres was $\leq 10\%$. Only embryos with mononucleated blastomeres were accepted for eSET. The embryo selection for transfer on day 2 was not influenced by early cleavage, but instead was based on embryo morphology and growth rate (as measured by blastomere numbers).

After embryo transfer, micronized vaginal progesterone (Lugesteron; Leiras, Finland) was used for luteal support. A positive serum hCG test (>10 mIU/ml) performed 2 weeks after embryo transfer confirmed pregnancy. The clinical pregnancy was documented by the presence of a positive fetal heart activity on transvaginal sonography at the sixth or seventh week of pregnancy. All eSET procedures with transient elevation of hCG level but without recognized clinical pregnancy were classified as preclinical abortions.

Different clinical parameters were compared between EC and NEC embryo transfers. The effects of different factors on the clinical pregnancy rate were also estimated. In both of these calculations, χ^2 -test and independent-samples Student’s *t*-test were used. The independence of different factors influencing the clinical pregnancy rate was tested applying generalized linear analysis using SAS system (Release 8.1.) procedure GENMOD, binominal distribution and standard logit link. The significance level was considered at $P < 0.05$.

Table II. The influences of cleavage stage and early cleavage on the clinical pregnancy rate of IVF/ICSI cycles

Cleavage stage	EC		NEC		P-value
	No. of transfers	No. of clinical pregnancies (%)	No. of transfers	No. of clinical pregnancies (%)	
2 cells	1	1 (100)	19	1 (5.3)	–
4 cells	67	34 (50.7)	80	26 (32.5)	0.025
≥ 5 cells	4	1 (25)	7	1 (14.3)	–

Table III. The effects of the regularity of blastomere divisions and the early cleavage of embryos on the clinical pregnancy rate of IVF/ICSI cycles

	No. of transfers	No. of clinical pregnancies (%)
Even cleavage		
EC	40	22 (55) ^a
NEC	38	14 (36.8)
Uneven cleavage		
EC	32	14 (43.8) ^b
NEC	67	14 (20.9)

^a $P < 0.0001$ when compared with unevenly cleaved NEC embryo transfers.

^b $P = 0.018$ when compared with unevenly cleaved NEC embryo transfers.

EC = early cleavage; NEC = no early cleavage.

Results

The analysis included 178 eSET procedures with 362 (26.3%) EC and 1017 (73.7%) NEC embryos. The mean age of patients was 33.4 years (range 23–42 years) and the overall clinical pregnancy rate was 36% (64/178). (Note: 85% of all patients met the inclusion criteria of ≤ 37 years.) The transfer of EC embryos resulted in significantly ($P = 0.001$) higher clinical pregnancy rate when compared to NEC embryo transfers (50% versus 26.4%) (Table I). The groups were similar in respect of the age of patients, the proportion of IVF and ICSI procedures, the number of oocytes and embryos and the rate of preclinical abortions. The mean degree of fragmentation was found to be similar in EC and NEC transferred embryos, but EC embryos possessed significantly more blastomeres (4.0 versus 3.7; $P = 0.003$), and also exhibited evenly sized blastomeres more frequently (55.6 versus 36.2%; $P = 0.011$) than NEC embryos.

Table IV. The analysis of factors predicting the clinical pregnancy rate of IVF/ICSI cycles

Parameter	Clinical pregnancy		P-value
	Yes	No	
No. of cycles	64	114	
Age (years, mean \pm SD)	33.2 \pm 3.8	33.5 \pm 4.2	NS
No. of IVF cycles (%)	46 (71.9)	87 (76.3)	NS
No. of ICSI cycles (%)	18 (28.1)	27 (23.7)	NS
No. of oocytes per patient (mean \pm SD)	12.5 \pm 6.3	12.4 \pm 8.2	NS
No. of embryos per patient (mean \pm SD)	8.0 \pm 4.3	7.6 \pm 5.0	NS
No. of blastomeres in transferred embryos (mean \pm SD)	4.0 \pm 0.4	3.8 \pm 0.8	NS
Degree of fragmentation in transferred embryos (%; mean \pm SD)	8.6 \pm 7.6	9.8 \pm 9.8	NS
No. of evenly cleaved transferred embryos (%)	36 (56.3)	42 (37.2)	0.014
No. of EC embryos (%)	36 (56.3)	36 (31.6)	0.001
No. of NEC embryos (%)	28 (43.8)	78 (68.4)	0.001

EC = early cleavage; NEC = no early cleavage; NS = not significant.

In the majority of IVF and ICSI procedures, a 4-cell embryo was transferred (82.6%; 147/178) (Table II). After 4-cell stage embryo transfers, markedly ($P = 0.025$) better clinical pregnancy rate was achieved with EC (50.7%) than with NEC embryos (32.5%).

The effects of the regularity of blastomere divisions and the early cleavage of embryos on the clinical pregnancy rate are summarized in Table III. The significantly ($P < 0.0001$) higher clinical pregnancy rate was observed after transfer of evenly cleaved EC embryos (55%) than unevenly cleaved NEC embryos (20.9%). Also, markedly ($P = 0.018$) better clinical pregnancy rate was seen following transfer of unevenly cleaved EC embryos (43.8%) than unevenly cleaved NEC embryos (20.9%).

The analysis of all factors predicting the clinical outcome of IVF and ICSI procedures (Table IV) demonstrated a clear positive correlation ($P = 0.001$) between the early cleavage of transferred embryos and the establishment of the pregnancy, as more EC embryos were transferred to the pregnant than to non-pregnant women (56.3 versus 31.6%). Also, the proportion of evenly cleaved transferred embryos was higher among pregnant than non-pregnant women (56.3 versus 37.2%; $P = 0.014$). Other clinical parameters, as the mean age of patients, the number of oocytes and embryos per patient, the number of blastomeres and the degree of fragmentation in transferred embryos did not differ between pregnant and non-pregnant women. Further analysis, applying generalized linear analysis using SAS system procedure GENMOD (Release 8.1.), binominal distribution and standard logit link, indicated that the timing of the first cleavage as well as the regularity of blastomeres determine independently the developmental potential of embryos (P -value for interaction 0.79).

The analysis of all embryos ($n = 1379$) demonstrated that embryo quality on day 2 is highly related to early cleavage of embryos. The proportion of embryos with $<20\%$ fragmentation and equal blastomeres was markedly higher ($P = 0.044$) among EC (50.5%) than NEC (44.2%) embryos. EC embryos also possessed significantly more blastomeres on day 2 as the proportion of EC embryos with ≥ 4 blastomeres (94.1%) exceeded significantly ($P < 0.0001$) that of NEC embryos (51%). In addition, the incidence of multinucleated blastomeres

(MNB) was lower ($P = 0.005$) in EC (18.9%) than in NEC (29.6%) embryos.

Discussion

In the current study, the effect of the early cleavage on the viability of transferred embryos was evaluated in a selected group of good prognosis patients undergoing eSET aiming to prevent multiple pregnancies. The clinical pregnancy rate of 36% for 178 eSET matched appropriately with average clinical pregnancy rate calculated for all eSET procedures performed in our clinics during the last 3 years (Vilksa *et al.*, 1999; Tiitinen *et al.*, 2001). The common feature for all study patients was that a single good quality embryo was transferred 2 days after oocyte retrieval. Though the selection of embryo was unrelated to early cleavage, a significantly better clinical pregnancy rate was observed after transfer of EC (50%) than NEC (26.4%) embryos. To exclude the possibility that the higher clinical pregnancy rate achieved with EC embryos could be caused by the fact that these embryos contained more blastomeres at the time of embryo transfer than NEC embryos, we subsequently analysed only the 4-cell stage embryo transfers. Again, a better clinical pregnancy rate was achieved with EC (50.7%) than with NEC (32.5%) embryos.

As only good quality embryo transfers were included in the current study, the comparison with previous studies, which also covered the analysis of transfers of poor quality embryos, has its limitations. However, the results of our study correspond with prior reports (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001) and support the view that EC embryos possess significantly higher developmental competence than NEC embryos. In the study by Shoukir *et al.* (1997), the pregnancy rate for EC embryo transfers (33%) was more than double that of NEC embryo transfers (15%) in IVF procedures (Shoukir *et al.*, 1997). An even bigger difference between EC and NEC embryos was found in ICSI procedures as patients who received EC embryos exhibited almost 4-fold higher clinical pregnancy rate (26%) than those having NEC embryos transferred (6%) (Sakkas *et al.*, 1998). In most of these earlier studies, however, EC embryos had been preferentially transferred. When not enough EC embryos were available, the

embryo transfer group was completed with NEC embryos, making it impossible to ascertain which of the transferred embryos led to the pregnancy. Transferring only a single embryo enabled us to conclusively demonstrate the positive effect of early cleavage on embryo implantation.

The analysis of possible factors predicting the clinical pregnancy rate after eSET revealed that both early cleavage and regularity of blastomere divisions are important determinants of embryo competence. These findings are in agreement with studies (Giorgetti *et al.*, 1995; Hardarson *et al.*, 2001) showing the relationship between even cleavage of embryos and better capacity for implantation. However, further analysis of our data indicated that these two factors influence independently the embryo viability. A possible reason why we did not observe the effect of age of patients and routine embryo parameters (the number of blastomeres and the degree of fragmentation) on the success rate of eSET was that single embryo was predominantly transferred to younger patients (85% of all patients were ≤ 37 years old) having good quality embryos.

The results of this study have also led us to revise our embryo selection criteria and currently EC embryo is preferentially selected for transfer in eSET procedures when at least two good quality embryos are available. The question whether to transfer one or two embryos is, however, more complex and cannot be solved solely based on embryo quality, albeit two-embryo transfer could be considered more seriously when no good quality EC embryo is available.

It has been demonstrated that EC embryos have better morphology (Lundin *et al.*, 2001) and a higher number of blastomeres (Sakkas *et al.*, 2001) 2 days after insemination or ICSI. In addition, a better blastocyst formation rate has been demonstrated for EC than NEC embryos (Fenwick *et al.*, 2002). Our findings confirm and complement the previous studies, as, in addition to better quality on day 2, EC embryos had also lower incidence of MNB when compared with NEC embryos. The reasons for better quality and viability of EC embryos remain largely unknown. The previous evaluation of the timing of the first cleavage may allow an embryologist to discern on the day of embryo transfer whether a 4-cell embryo had just cleaved to the 4-cell stage or had reached the 4-cell stage hours earlier (Sakkas *et al.*, 2001). In addition, early cleavage of zygotes may depend on the quality of oocytes (Lundin *et al.*, 2001), but the paternal effect cannot be ruled out (Salumets *et al.*, 2002). However, the semen characteristics have not been shown to have any effect on early cleavage of either IVF or ICSI zygotes (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998). Therefore, more detailed studies are needed to specify these putative biological mechanisms underlying this phenomenon. Future studies should also concentrate on the incidence of chromosomal abnormalities in EC and NEC embryos.

The reduction of multiple pregnancies by using eSET requires critical and careful selection of the embryo for transfer. Traditionally, cleavage stage embryos are selected for transfer 2 or 3 days after insemination considering simultaneously their morphological appearance and growth rate (Puissant *et al.*, 1987; Steer *et al.*, 1992). According to some authors, the selection of cleavage stage embryos for transfer

could be substantially improved by evaluation of pronuclear morphology (Scott and Smith, 1998; Tesarik and Greco, 1999). However, in the recent study (Salumets *et al.*, 2001), we failed to show any beneficial effect of the evaluation of pronuclear morphology on clinical pregnancy outcome following eSET procedures.

Postponing the transfer until embryos have reached the blastocyst stage has also been used as a possible way to select the most viable embryos for transfer. Although high pregnancy (50–70%) and implantation (30–50%) rates have been reported for blastocysts transfers (Gardner *et al.*, 1998; Marek *et al.*, 1999; Langley *et al.*, 2001) the clinical evidence on the preference of blastocyst transfers to cleavage stage embryo transfers is still controversial. Several prospective studies have shown the superiority of blastocyst transfers over day 2/3 embryo transfers (Gardner *et al.*, 1998; Van Der Auwera *et al.*, 2002), while others have been unable to confirm these findings (Scholtes and Zeilmaker, 1996; Coskun *et al.*, 2000; Huisman *et al.*, 2000; Lundqvist *et al.*, 2002). In a recent study (Rienzi *et al.*, 2002), day 3 embryo transfers with combined evaluation at pronuclear and cleavage stages demonstrated similar results to blastocyst transfers. The clinical pregnancy rate for EC embryos (50%) found in the current study is also comparable to that usually reported for blastocysts. Though the basis of our embryo selection differed from that of Rienzi *et al.* (2002) (Rienzi *et al.*, 2002), both of these studies suggest that embryo selection could be successfully accomplished on days 2/3, making the extended culture and blastocyst transfer redundant.

In conclusion, the current study provides compelling evidence that early cleavage is a strong indicator of embryo competence. However, larger prospective studies are needed to confirm the usefulness of early cleavage in embryo selection for eSET.

Acknowledgements

We thank all members of our IVF teams for their assistance during this study. Tonu Möls is acknowledged for his help in statistical analysis.

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Submitted on September 26, 2002; resubmitted on November 27, 2002; accepted on January 8, 2003