Comparative analysis of pregnancy rates after the transfer of early dividing embryos versus slower dividing embryos

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BACKGROUND: We compared retrospectively the pregnancy outcome in two subgroups of ICSI patients, using early division (26 h post injection) to the 2-cell stage as a criterion for embryo quality and viability (ability to produce a pregnancy). METHODS AND RESULTS: In the early dividing embryo (EDE) group, at least one of the transferred embryos was early dividing. In the late dividing embryo (LDE) group, no early dividing embryo was transferred. Additionally, tubal and uterine transfer in the two groups was also evaluated. Clinical pregnancy rates in the EDE group were significantly increased when compared with that in the LDE group (41.3 versus 20.0%). This was also true for ongoing pregnancy rates (33.3 versus 16.3%). The tubal transfer route showed increased (but not significant) ongoing pregnancy rates when compared with uterine transfer in both EDE (38.5 versus 25.0%) and LDE (22.7 versus 8.3%) groups respectively. In uterine transfer cycles, however, clinical pregnancy rates for EDE were significantly increased compared to LDE (37.5 and 11.1% respectively). The baby rate (number of live babies/embryos transferred) was also significantly increased in the EDE group and the tubal transfer group. Statistical analysis of pregnancy outcome, adjusted for the total number of embryos transferred (expressed as percentage risk difference ± %RD), resulted significantly in favour of EDE compared to LDE (RD = 18%, P = 0.02). When adjusted for the combined factors: total number of embryos transferred, EDE and LDE, the pregnancy outcome result was significantly in favour of tubal transfer compared to uterine transfer (RD = 15%, P = 0.05). Pregnancy results of the LDE group only were significantly better in the tube compared to the uterus (RD = 19%, P = 0.04) but not significantly so for the EDE group (RD = 10%, P = 0.4). CONCLUSION: Early division is associated with embryo quality and a very easy and successful embryo transfer selection method. Our results also suggest that when EDE are available, both tubal and uterine embryo transfer can be considered. When only LDE are available, however, tubal transfer should be the preferred transfer route.

Key words: 2-cell stage embryos/early dividing embryos/ICSI/pregnancy/tubal embryo transfer

Introduction

The question by Edwards and Beard (1999): ‘do any other species (but the human) display such an immense loss of reproductive potential?’ summarizes the disappointment of scientists and patients when embryo transfer success rarely exceeds 30%. The identification of the embryo destined for implantation can improve the success and many embryo selection methods have been proposed and tried in an effort to identify the embryo with the greatest ability to implant, whilst transferring fewer embryos so that multiple pregnancies are avoided (Boiso, 2002).

Embryo selection is traditionally done using embryo morphology as a guide (Gerris et al., 1999, 2000; Van Royen et al., 1999). Other methods of selection include pronuclear morphology (Payne et al., 1997; Edwards and Beard, 1997, 1999; Scott and Smith, 1998; Tesarik and Greco, 1999; Ludwig et al., 2000; Scott et al., 2000, 2003; Tesarik et al., 2000; Salumets et al., 2002), blastocyst culture (Gardner et al., 1998, 2002; Tsirigotis, 1998; Behr, 1999; Edwards and Beard, 1999; Jones and Trounson, 1999; Sakkas, 1999; Kolibianakis and Devroey, 2002; Blake et al., 2003), oocyte and pronuclei polarity and cleavage symmetry (Edwards and Beard, 1997, 1999; Payne et al., 1997, 1998; Scott and Smith, 1998; Behr, 1999; Tesarik and Greco, 1999; Ludwig et al., 2001; Boiso, 2002).

Another method, using ‘early division’ to the 2-cell stage, 25–27 h post insemination/injection, also showed significantly improved pregnancy rates when such embryos were selected and transferred (Shoukir et al., 1997; Sakkas et al., 1998, 2001; Bos-Mikich et al., 2001; Lundin et al., 2001; Petersen et al., 2001; Fenwick et al., 2002; Salumets et al., 2003). Edwards and Beard (1999) also commented on the importance of the first cleavage and suggested that early selection criteria up to and including the first division may have advantages.

Human Reproduction vol. 19 no. 5 © European Society of Human Reproduction and Embryology 2004; all rights reserved

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In this retrospective study, we evaluated the effect of early dividing (EDE) and late dividing (LDE) embryo transfers on pregnancy outcome in ICSI patients and in addition the effect of transfer route, i.e. tubal or uterine, in such transfers. This method of selection is very simple and assures that embryos are not subjected to long periods of exposure outside the incubator.

Materials and methods

Patients
This retrospective study included patients eligible for our ICSI programme. A total of 143 ICSI cycles was evaluated (detailed embryo quality evaluation was available in 138 of the patients).

Female partners of the couples included in the study had patent Fallopian tubes and were <38 years old. Only transfer cycles where at least two embryos were available for transfer were included in the study.

All categories (idiopathic, male factor, azoospermic) of male partners were included in the study.

Media
Medicult sperm preparation medium, HEPES-buffered (Harrilabs, South Africa), was used for semen preparation, enzymatic embryo denuding and sperm injection drops. Medicult universal IVF medium (Harrilabs, South Africa), was used for semen preparation, enzymatic embryo denuding and sperm injection drops. Medicult paraffin oil (Harrilabs, South Africa) was used to cover medium drops and Medicult PVP (Harrilabs, South Africa) used for sperm immobilization.

Stimulation protocol
Ovarian stimulation was carried out by the administration of GnRH agonist (Synarel®; Montosano South Africa, Searle) in a long protocol, followed by hMG [Pergonal®; Serono, South Africa (Pty) Ltd] and/or pure FSH (Metrodin®; Serono) from cycle day 3. Patients were followed up by performing estradiol determinations as well as serial ultrasonographical measurement of all the follicles. Ovulation was induced by the administration of hCG (Profasi®; Serono) as soon as the leading follicle reached 18 mm in diameter.

Semen preparation
Where possible, motile sperm were isolated by a standard swim-up technique. In cases of oligo- and asthenozoospermia, gradient centrifugation was the method of choice. Ejaculated, testicular biopsy, cryopreserved ejaculated and cryopreserved testicular biopsy semen specimens were all included in the study.

Follicle aspiration and oocyte handling
Follicle aspiration was done under conscious sedation (Dormicum; Roche Products (Pty) Ltd, South Africa; or Diprovan; Zeneca Pharmaceuticals, South Africa). Oocytes were recovered by transvaginal ultrasound-guided follicle aspiration 34–36 h after hCG administration.

Retrieved oocytes were incubated with their cumulus mass for ≥3 h in a small Petri dish with lid (37°C, 5% CO₂) until denuding.

The cumulus mass was removed 3–5 h post retrieval using 40 IU/ml hyaluronidase (Sigma) and denuding mouth pipettes. Denuded oocytes were rinsed three times in 1 ml medium. Metaphase II oocytes were identified and incubated in 50 μl drops covered with paraffin oil until injection (37°C, 5% CO₂). ICSI was performed as soon as possible after denuding, i.e. ≥3 h but ≤5 h post retrieval.

ICSI
A standard method was followed. Prerequisites for successful injection were immobilization of the sperm cell in polyvinylpyrrolidone (PVP) and mild cytoplasmic aspiration. Oocytes were washed in fresh medium drops after injection and then incubated individually in 50 μl drops of medium (under oil, 37°C, 5% CO₂). Oocytes were transferred to fresh medium daily. Embryos destined for transfer at the 8-cell stage were cultured in Medicult M3 medium from day 2 onwards.

Evaluation of fertilization, early division, cleavage and embryo quality
Each oocyte was evaluated individually at the following times: ~18 h post injection for 2PN and two polar bodies; 25–27 h (~26 h) post injection for 2-cell division (early division); ~45 h post injection for 4-cell division; ~72 h post injection for 6–8-cell division.

Embryo grading (modified from Veeck, 1991)
Embryos were regarded as ‘good quality’ embryos when they were at the 4-cell stage at 48 h post injection or at the 6–8-cell stage, 72 h post injection with equal sized blastomeres and minor or no cytoplasmic fragmentation.

Embryo transfer and pregnancy evaluation
Embryos were transferred either at the 4-cell or 6–8-cell stage (day 2 or day 3) according to the workload in the laboratory. Two transfer methods were followed: tubal (laparoscopical) transfer or uterine (transvaginal) transfer using the Wallace catheter (Simms, UK). Two or more (but less than five) embryos were transferred per patient. The study was not randomized and patients were given the choice for either tubal or uterine transfer after counselling and explanation of the details of each method. Embryos that were not transferred and that reached the blastocyst stage on day 5 post injection were cryopreserved.

For transfer, early dividing embryos (EDE; 2-cell stage 26 h post injection) were always the first choice. The rest of embryos were chosen using embryo morphology grading.

Pregnancies were reported as positive when βhCG serum levels were >10 mIU/ml, 10 days post transfer and increased to four times that value on day 14 after transfer. Clinical pregnancy rates (CPR) included spontaneous abortions as well as biochemical, ectopic and ongoing pregnancies. The ongoing pregnancy rate (OPR) (due to the retrospective nature of the study) consisted of patients with live births. Early pregnancy loss cycles included patients with spontaneous abortions as well as biochemical and ectopic pregnancies. The number of fetal sacks was not available for all patients (implantation rates were therefore not available), but we report live babies/number of embryos transferred (‘live baby rate’).

Outcomes measured
Patients were divided into two groups to evaluate the pregnancy outcome. EDE group: cycles where at least one of the transferred embryos showed early division at 26 h post injection. LDE group: cycles where none of the transferred embryos showed early division at 26 h post injection. In each group, two subgroups were investigated: a tubal transfer group and a uterine transfer group.

Two main outcomes were considered: clinical pregnancy rate (CPR) and ongoing pregnancy rate (OPR). The associations of these pregnancy rates with early cleavage and placement of embryos (tubal or uterine) were adjusted for the number of embryos transferred and the embryo quality. Early pregnancy loss and live baby rate were also evaluated.
Statistical analysis

The association between good quality embryos (GQE) and EDE, adjusted for female age, was done using a generalized estimation equation (GEE) model (Lee and Chia (1993)). This model adjusts for the clustering effect of embryos within women and estimates the odds ratio of GQE based on the factor EDE adjusted for the age effect of the women.

Fisher's exact test (two-sided) was used to calculate the difference between pregnancy rates and live baby rate in the different groups (not adjusted for number of embryos transferred and embryo quality).

Each transferred embryo was classified according to quality (good/poor) and division (early/late) and placement (tubal/uterine). Differences in pregnancy rates were then analysed using the Mantel–Haenzsel (MH) method. Results were expressed graphically as the percentage risk difference (%RD) and were tested for significant differences ($P < 0.05$) (Metaview—Cochran Collaboration).

Results

The day of transfer (day 2 or 3) had no effect on the pregnancy results in this study. The percentage of embryo transfers performed on day 2 and day 3 in the tubal and uterine transfer groups were similar (data not shown). Day 2 and 3 transfers resulted in similar pregnancy rates (29.9 and 32.8% respectively) and the average number of embryos transferred was not different (3.1 and 3.4 respectively). Pregnancy rates in the early dividing group were also the same for day 2 and day 3 transfers (42.4 and 43.3% respectively).

Early cleavage was observed in 44% (63/143) of the cycles included in the study. Almost 15% [14.5% (110/759)] of the total number of embryos and 23.8% (110/462) of the transferred embryos were early dividing embryos.

The mean female age (31.7; 33.3 years), fertilization rates (71.4; 70.4%) and number of embryos transferred (3.3; 3.2) were very similar for EDE and LDE respectively.

Good quality embryos (GQE) were significantly associated with early division ($P < 0.0001$) as well as female age ($P = 0.004$). The adjusted odds ratio for EDE was 2.85 (95% confidence interval: 1.67–4.83) meaning that the odds of having a GQE given that it was an early divider was almost three times that of late dividing embryos.

The pregnancy outcomes are presented in Figures 1 and 2. These figures were not adjusted for the number of embryos transferred and give only an indication of the effect of EDE and placement.

The inclusion of at least one EDE in the cohort of embryos transferred resulted in increased pregnancy rates (clinical and ongoing) and live baby rates for both tubal and uterine transfers. 
Tubal transfer also resulted in increased clinical PR, ongoing PR and live baby rates (Figures 1 and 2). Statistical analysis, however, showed significant differences only in the following cases: in clinical PR, EDE versus LDE [41.3% (26/63) and 20.0% (16/80) respectively, \( P = 0.0092 \)]; ongoing PR, EDE versus LDE [33.3% (21/63) and 16.3% (13/80) respectively, \( P = 0.0286 \)] (Figure 1); and live baby rate, EDE versus LDE [15.5% (32/206) and 6.6% (9/177) respectively, \( P = 0.0020 \)] (Figure 2). In tubal transfer cycles for live baby rate, EDE versus LDE [18.8% (9/22) and 9.7% (1/13) respectively, \( P = 0.0218 \)] (Figure 2); and in uterine transfer cycles for clinical PR, EDE versus LDE [9.7% (13/134) and 3.4% (4/122) respectively, \( P = 0.0394 \)] (Figure 2).

Since it is well known that the number of embryos transferred has an influence on pregnancy rates, this factor has been included in the results shown in Figures 3–6 (Mantel–Haenszel analysis).

Figures 3 and 4 represent the effect of EDE, adjusted for the number of embryos transferred, on pregnancy rates. EDE showed a significant ‘risk’ for a clinical pregnancy (RD = 18%, \( P = 0.02 \)) (Figure 3). Figure 4 shows a similar trend in ongoing pregnancy rates (RD = 13%, \( P = 0.06 \)) in favour of EDE transfer.

Figure 5 represents clinical pregnancy results for tubal and uterine transfer, adjusted for both the number of embryos transferred as well as EDE or LDE transferred. The overall effect was significantly in favour of tubal transfer (RD = 15%, \( P = 0.05 \)) (Figure 5). Analysis of the LDE group showed a significantly favourable result in combination with tubal

<table>
<thead>
<tr>
<th>Total number of embryos transferred</th>
<th>EDE n/N</th>
<th>LDE n/N</th>
<th>RD (95% CI Fixed)</th>
<th>Weight %</th>
<th>RD (95% CI Fixed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2/8</td>
<td>1/12</td>
<td></td>
<td>14.1</td>
<td>0.17 (0.17, 0.51)</td>
</tr>
<tr>
<td>3</td>
<td>15/33</td>
<td>10/40</td>
<td></td>
<td>53.2</td>
<td>0.20 (-0.01, 0.42)</td>
</tr>
<tr>
<td>4</td>
<td>6/17</td>
<td>4/21</td>
<td></td>
<td>27.6</td>
<td>0.16 (-0.12, 0.44)</td>
</tr>
<tr>
<td>5</td>
<td>1/3</td>
<td>1/4</td>
<td></td>
<td>5.0</td>
<td>0.08 (-0.60, 0.76)</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>24/61</td>
<td>16/77</td>
<td></td>
<td>100.0</td>
<td>0.18 (0.03, 0.33)</td>
</tr>
<tr>
<td>Test for heterogeneity chi-square</td>
<td>0.15</td>
<td>df = 3</td>
<td>( p = 0.99 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect z = 2.35</td>
<td></td>
<td>( p = 0.02 )</td>
<td></td>
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**Figure 3.** The effect of EDE versus LDE transfer in clinical pregnancy outcome adjusted for the number of embryos transferred (expressed as percentage risk difference: %RD). \( n = \) number pregnant; \( N = \) transfers; LDE = no early dividing embryos transferred; EDE = at least one early dividing embryo transferred.

<table>
<thead>
<tr>
<th>Total number of embryos transferred</th>
<th>EDE n/N</th>
<th>LDE n/N</th>
<th>RD (95% CI Fixed)</th>
<th>Weight %</th>
<th>RD (95% CI Fixed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0/8</td>
<td>1/12</td>
<td></td>
<td>14.1</td>
<td>-0.08 (-0.31, 0.15)</td>
</tr>
<tr>
<td>3</td>
<td>12/33</td>
<td>8/40</td>
<td></td>
<td>53.2</td>
<td>0.16 (-0.04, 0.37)</td>
</tr>
<tr>
<td>4</td>
<td>5/17</td>
<td>2/21</td>
<td></td>
<td>27.6</td>
<td>0.20 (-0.05, 0.48)</td>
</tr>
<tr>
<td>5</td>
<td>0/3</td>
<td>0/4</td>
<td></td>
<td>5.0</td>
<td>0.00 (-0.42, 0.42)</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>17/61</td>
<td>11/77</td>
<td></td>
<td>100.0</td>
<td>0.13 (-0.01, 0.27)</td>
</tr>
<tr>
<td>Test for heterogeneity chi-square</td>
<td>4.10</td>
<td>df = 3</td>
<td>( p = 0.25 )</td>
<td></td>
<td></td>
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<tr>
<td>Test for overall effect z = 1.86</td>
<td></td>
<td>( p = 0.06 )</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Figure 4.** The effect of EDE versus LDE transfer in ongoing pregnancy outcome adjusted for the number of embryos transferred (expressed as percentage risk difference: %RD). \( n = \) number pregnant; \( N = \) transfers; LDE = no early dividing embryos transferred; EDE = at least one early dividing embryo transferred.
transfer (RD = 19%, $P = 0.04$) (Figure 5). Tubal transfer also appeared favourable for EDE, but did not reach significance (RD = 10%, $P = 0.4$).

Figure 6 shows the ongoing pregnancy results. The overall effect was also significantly in favour of tubal transfer (RD = 15%, $P = 0.03$) (Figure 6). Although tubal transfer appeared favourable for both LDE and EDE transfers, the results did not reach significance. The LDE group showed a marginal effect (RD = 15%, $P = 0.07$) (Figure 6) in favour of tubal transfer.

The selection of early dividing embryos (EDE) for embryo transfer resulted in a high percentage of triplet and twin gestations [14.3% (3/21) and 23.8% (5/21) per ongoing pregnancy respectively]. Singleton gestations were 61.9% (13/21). For LDE transfer no triplet gestations were found. Twin gestation rate per ongoing pregnancy was 38.5% (5/13) and the singleton pregnancy rate 61.5% (8/13), similar to EDE outcomes.

**Discussion**

The ultimate goal of assisted reproduction is to achieve a singleton, ongoing pregnancy. The need to characterize embryos with optimal implantation potential is obvious and all the different embryo development stages (from 1-cell to blastocyst) have been proposed as markers for embryo quality and viability.

In our study, the first cell division after ICSI fertilization was used to indicate embryo viability. We performed a retrospective, comparative study to investigate the effect of at least one EDE versus only LDE embryo transfer on pregnancy outcome. This approach was first reported by Shoukir *et al.* (1997) and Sakkas *et al.* (1998). Several studies that are more recent have also used this approach and all showed the value of early division as a marker for embryo viability (Bos-Mikich *et al.*, 2001; Lundin *et al.*, 2001; Petersen *et al.*, 2001; Sakkas *et al.*, 2001; Fenwick *et al.*, 2002; Salumets *et al.*, 2003).

The results from our own study were similar to these reported studies. The clinical and ongoing pregnancy rates in the cases where EDE were transferred were significantly higher than when only LDE were available for transfer (Figure 1). This result was also true for live baby rate with significantly more live babies/embryos transferred, delivered in the EDE group (Figure 2). Statistical evaluation, also taking into account the total number of embryos transferred, also showed EDE to be significantly favourable (Figures 3 and 4).

Our study differed from most other published studies with respect to the method of embryo transfer. We were interested in the comparison of two transfer methods, i.e. tubal and uterine transfer in the respective EDE and LDE groups. The uterus and
Fallopian tube have different nutritional environments (Gardner et al., 1996, 1998, 2002) and cleavage stage embryos (day 2 and 3) are possibly better adapted for development in the Fallopian tube. Although the uterus seems to be a suitable environment for embryos (Scott and Smith, 1998), many studies showed the increased success of tubal transfer of early stage embryos over that of uterine transfer (Yovich et al., 1988; Hammitt et al., 1990; Frederick et al., 1994; Van Voorhis et al., 1995; Boldt et al., 1996; Tournaye et al., 1996; Bulletti et al., 1996; Kumar et al., 1997; Levran et al., 1998; Castelbaum et al., 1998). Recently it was also shown that uterine contractility at the time of cleavage stage embryo transfer could influence uterine transfer outcome adversely (Fachin et al., 2001). Our results consequently indicated an increased pregnancy as well as live baby rate trend after tubal transfer compared to uterine transfer. This increase was statistically significant only for the live baby rate in the late dividing embryo (LDE) group. More refined statistical evaluation also showed that tubal transfer was significantly favourable for LDE (Figures 5 and 6). Tubal transfer of LDE might therefore ‘rescue’ these probably less viable embryos. Clinical pregnancy and live baby rate results from uterine transfers showed that LDE performed significantly worse when compared to EDE. These results confirm the possible advantage of the tubal environment for LDE. The possible mechanism for this ‘rescue’ can only be speculated on and is probably associated with the influence of different nutrient environments in the Fallopian tube and the uterus as well as asynchronization of the endometrium (Gardner et al., 2002). It was also interesting to note that in our study the fetal loss rate was increased in the uterine transfer group compared to tubal transfer group, possibly emphasizing the more suitable environment of the Fallopian tube for cleavage stage embryos.

![Table](https://academic.oup.com/humrep/article-abstract/19/5/1155/602230)

**Figure 6.** The effect of tubal versus uterine transfer in ongoing pregnancy outcome adjusted for the number of embryos transferred as well as EDE and LDE transferred (expressed as percentage risk difference: %RD). n = number pregnant; N = transfers; LDE = no early dividing embryos transferred. EDE = at least one early dividing embryo transferred.
early selection criteria indicate viable blastocysts. This would avoid long-term culture needed for blastocyst transfer (Boiso, 2002; Salumets et al., 2003). A multi-step embryo scoring system approach was also suggested by Van Royen et al. (1999), Scott et al. (2000) and Sakkas et al. (2001).

The EDE method is, however, non-invasive and very simple, and was shown to correlate well with other methods. Sakkas et al. (1998, 2001) concluded that early cleaving embryos give rise to better quality embryos due to an intrinsic, unknown factor within the oocyte. Positive correlations of EDE and good embryo morphology have also been also reported (Lundin et al., 2001; Fenwick et al., 2002; Salumets et al., 2003). Some reasons for the superiority of EDE and its correlation to good quality embryos and blastocyst development have been discussed (Scott and Smith, 1998; Fenwick et al., 2002; Salumets et al., 2003). Some of the factors mentioned are correct spatial arrangement in oocytes, differences of individual sperm to stimulate calcium transients or the oocyte’s ability to respond to these stimuli, oocyte maturity, DNA replication (shorter S-phase) and chromosomal abnormalities.

The results from our study also showed that embryo quality (morphology) of EDE was significantly better than that of LDE, indicating an indirect way of selecting the best quality embryos. Similarly to the results from Fenwick et al. (2002), preliminary results from our clinic (embryos not transferred in GIFT, ICSI and IVF cycles) also indicated a correlation between EDE and the ability of blastocyst formation in specified blastocyst medium. Fifty-two per cent (52%) of EDE developed into blastocysts compared to 21.8% (P < 0.0001) for LDE (unpublished data), indicating that an earlier embryo stage can possibly be implemented for the selection of viable embryos.

From our study therefore, early cleavage seems to be a very easy and successful selection method for viable embryos and should be included in the scoring system to choose the best embryo to transfer. Tubal transfer was the other factor that contributed significantly to increased pregnancy outcomes, specifically for LDE transfer.

Acknowledgements

Sincere thanks to the personnel of the Reproductive Biology Unit, Tygerberg Hospital and the theatre personnel of the Tygerberg Hospital. The Harry and Doris Crossley Research Fund, University of Stellenbosch, Tygerberg, South Africa supported this research.

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Submitted on November 19, 2003; accepted on January 21, 2004