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The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection

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The transfer of blastocysts has been associated with a very high implantation rate. However, not all embryos achieve the blastocyst stage. Our study was set up to demonstrate whether embryo morphology on day 3 predicts subsequent blastocyst formation. A prospective study was carried out in 48 patients with a mean of 2.9 failed in-vitro fertilization (IVF) attempts. In this new cycle, the morphology of the embryos on day 3 was noted. After pre-selection of the embryos which would have been transferred on day 3, all embryos were cultured individually and allowed to develop further until transfer on day 5. The clinical pregnancy rate per transfer was 46%, and the overall implantation rate was 24%. When only blastocysts were transferred the pregnancy rate was 53% with an implantation rate of 30%. Thirty-nine per cent of all embryos reached the blastocyst stage on day 5; 47% of class 1 and 2 embryos (good quality) in contrast to 21% of class 3 and 4 embryos (poor quality). Respectively 45% of class 1 and 2 embryos and 69% of class 3 and 4 embryos arrested in development or degenerated. Only 51% of the embryos that were transferred on day 5 had been pre-selected for transfer on day 3. In conclusion, it appears that the predictive value of embryo morphology on day 3 for subsequent blastocyst formation is limited.

Key words: human blastocysts/implantation rate/IVF/predictive value embryo morphology/pregnancy rate

Introduction

Multiple pregnancies, especially triplets and more, are a significant drawback of in-vitro fertilization (IVF) treatments. This situation, of course, can be prevented by the transfer of only one embryo. However, as the 'take-home baby rate' per treatment cycle in IVF still does not approach natural fecundity, many centres try to improve their rates by transferring more embryos, accepting this risk. These embryos are generally transferred at the 4- to 8-cell stage. They are often selected from a cohort based upon their morphological appearance but as implantation rates are still far from optimal, one may justifiably question the predictive value of current accepted morphological criteria for the ability of these embryos to implant. Unquestionably the morphology of embryos 2 or 3 days after insemination has some predictive value for implantation potential; however this value may be limited by the fact that they are still in part depending on the maternal genome. As has been published, the embryonic genome is fully activated after the 8-cell stage (Braude *et al*., 1988). Some genetic damage to the embryo may not be observed before the full transition from maternal to embryonic genome. It could, therefore, be advantageous to delay embryo transfer until after this transition, i.e. until the blastocyst stage. The blastocyst stage is normally reached on day 5 after insemination, and transfer of blastocysts 5 days after insemination has been associated with a very high implantation rate (Scholtes and Zeilmaker, 1996). In addition, not all embryos reach the blastocyst stage at this time, which makes selection of embryos easier. In addition, transfer of a limited number of embryos on day 5 reduces the risk of large multiple pregnancies. One can confidently transfer fewer embryos as a result of the higher implantation rate, with still a good chance of the occurrence of a singleton pregnancy.

Since it has been reported that the overall pregnancy rates are similar after transfer on either day 3 or day 5 (Scholtes and Zeilmaker, 1996), it could be that the benefit obtained by a better selection method does not outweigh the disadvantage of exposing embryos for longer to artificial culture conditions. Furthermore, if the number of embryos available is small, there is no advantage in a long culture period. The key question is whether embryo morphology on day 3 is predictive of further development to the blastocyst stage.

In order to address this question we established the present study in which patients with supernumerary good quality embryos and failed IVF–embryo transfer in the past could take advantage of postponing the transfer to day 5, giving the embryos the opportunity to develop into blastocysts. The results of this study have previously been presented in part (Rijnders and Jansen, 1996; Rijnders *et al.*, 1997).

Materials and methods

Patients and treatment

A prospective study was carried out between May and December 1995 and included 48 patients. Patients were only included when at least five embryos were available in the present cycle. Most of them had experienced a number of previous unsuccessful cycles with transfer despite the presence of a sufficient number of good quality embryos. Ovarian stimulation was performed using human menopausal gonadotrophin (HMG) or follicle-stimulating hormone (FSH), with or without gonadotrophin-releasing hormone (GnRH) agonists, using either the short or long protocol. Human chorionic gonadotrophin (HCG, 10 000 IU) was administered when the dominant follicle

was >17 mm mean diameter in the case of HMG or FSH alone, and >20 mm GnRH agonists were used. Transvaginal oocyte retrieval was performed 35 h after HCG administration. Depending on the number of follicles, either HCG (1500 IU, 3–4 times) or progesterone suppositories (300 mg daily for 10 days) were given as luteal support. All embryo transfers were performed on day 5, preferably with blastocysts.

Culture conditions

Oocytes were collected and cultured in drops (160–200 µl) of equilibrated culture medium under mineral oil. The culture medium was a mixture of Earle's and Ham's F-10 without hypoxanthine and thymidine and with 8.7% v/v pasteurized plasma protein solution (GPO; Netherlands Red Cross Blood Bank, Amsterdam, The Netherlands) containing all plasma proteins obtained from screened and pasteurized donor blood. The culture medium contained 5.499 g NaCl; 0.336 g KCl; 0.173 g $MgSO_4.7H_2O$; 2.096 g $NaHCO_3$; 0.135 g $NaH_2PO_4.2H_2O$; 0.846 g glucose; 0.020 g Na-pyruvate (all from Merck, Darmstadt, Germany); 0.577 g Ca-L-lactate (Fluka, Buchs, Switzerland); 1.731 g Ham's F-10 powder (Life Technologies, Paisley, Scotland) and 1.25 ml of penicillin/streptomycin solution (5000 IU/ml penicillin and 5000 µg/ml streptomycin; ICN Biomedicals Inc.) per litre Analar water (BDH Laboratory Supplies, Poole, UK). To each litre of this Earle's and Ham's F10 mixture, 95 ml of the pasteurized plasma protein solution was added. Culture medium with the same composition was used throughout the whole culture period. Oocytes, sperm suspensions and embryos were cultured under strictly controlled incubation conditions (37.0°C; 5% CO_2) without the use of co-culture. Insemination or intracytoplasmic sperm injection (ICSI) was carried out 38–42 h post HCG injection. Fertilization was confirmed by the presence of two pronuclei 16–18 h after insemination or ICSI. All embryos were cultured until transfer on day 5.

Embryo pre-selection and selection

For 41 patients, all embryos were moved into fresh separate culture drops (80–100 µl) on day 3, enabling evaluation of individual embryo morphology and developmental stage. At this time an initial assessment of embryo quality was performed and a provisional selection was made as to which embryos would be most eligible for transfer if performed on that day (day 3). After this pre-selection, all embryos were allowed to develop further until transfer on day 5. The morphological quality of the embryos on day 3 was assessed according to the number of blastomeres and percentage of fragments. Embryos with no fragments were classified as class 1 embryos, those with less than 20% of the volume of the embryo consisting of fragments were classified as class 2. Those with 20–50% fragmentation were classified as class 3 and those with more than 50% as class 4. Since it has been reported that implantation rates of embryos without fragments and with less than 20% fragmentation are comparable (Scholtes and Zeilmaker, 1996), for the analysis of our results, class 1 and 2 embryos were combined, as were class 3 and 4 embryos.

On day 5 the morphology of all embryos was re-evaluated. At this time 2 or 3 embryos, preferably blastocysts, were transferred. If these were not available, non-cavitating embryos were transferred.

The number of embryos transferred was dependent on the age of the woman, the previous history and the embryo quality.

Pregnancy and implantation

A urine pregnancy test (sensitivity: >50 IU/l is positive) was performed at least 15 days after follicle puncture and at least 7 days after the last HCG injection of 1500 IU for luteal support. This was consistent with the definition of a clinical pregnancy given by Jones *et al.* (1983). The clinical implantation rate (IR) was defined as the number of gestational sacs per number of embryos transferred and vital implantation rate as the number of gestational sacs per embryo transferred exhibiting fetal heart beat.

Statistical analysis

Data are expressed as mean ± SD, and/or median. Where appropriate, data are analysed with Student's *t*-test or χ^2 test.

Results

Patient characteristics

A total of 48 patients were included in this study. The mean age of the patients was 33.7 ± 4.3 years. The mean duration of infertility was 4.9 ± 2.9 years. The patients had a mean of 2.9 ± 2.4 previous transfers not resulting in pregnancy (in total 293 embryos replaced in 139 transfers; mean 2.1 embryos per embryo transfer).

IVF/ICSI results

From these 48 patients, 809 oocytes were recovered (mean 16.9 ± 9.9), resulting in 564 embryos on day 3 (mean 11.8 ± 5.7), with a fertilization and cleavage rate of 70%. All treatments were followed by embryo transfer on day 5. A total of 108 embryos were transferred, with a mean of 2.25 embryos per transfer. The clinical pregnancy rate per embryo transfer was 45.8% (Table I). Twenty-six of the 108 embryos implanted (gestational sac on ultrasound; clinical implantation rate of 24.1%) of which 25 exhibited cardiac activity (vital implantation rate of 23.1%).

Pregnancy and implantation rate

When only blastocysts (B) or expanded blastocysts (EB) were transferred, a clinical pregnancy rate of 52.5% per transfer was observed, equating to an implantation rate of 30.1% per blastocyst (Table II). Mixed transfers, i.e. blastocysts combined with non-cavitating embryos [morula/compaction stage (M/C) or less], resulted in a 20% pregnancy rate per transfer. No pregnancies were observed following transfers of only non-cavitating embryos on day 5.

Blastocyst formation and predictive value of embryo morphology on day 3

Embryos that were cultured individually (n = 498 from 41 patients) were assessed for further development. On day 3, 69% (n = 342) of these embryos were class 1 or 2 embryos and 31% (n =156) class 3 or 4. Only 47% (n = 162) of the class 1 and 2 embryos reached the blastocyst stage; 21% (n = 32) of the class 3 and 4 embryos developed to the blastocyst stage ($P < 0.01$; Table III). It appeared, therefore, that embryo morphology on day 3 had a limited predictive value. In total, 39% of all embryos developed into blastocysts on day 5; 84% of the blastocysts originated from class 1 and 2 embryos and 16% from class 3 and 4.

From the 92 embryos that were earmarked for transfer on day 3, only 49 actually reached the blastocyst stage (53.3%), 15 reached the morula/compaction stage (16.3%) and 28 embryos showed developmental arrest or degeneration (30.4%; Table IV).

Table I. In-vitro fertilization (IVF) outcome after embryo transfer on day 5 after insemination

Embryo transfers	Clinical pregnancies	Pregnancy rate/ embryo transfer (%)	Embryos for embryo transfer	Gestational sacs	Clinical IR (%)	Heart beat	Vital IR (%)
48	22	45.8	108	26	24.1	25	23.1

IR = implantation rate.

Table II. Pregnancy and implantation rates (PR and IR) in relation to day 5 embryo morphology

Transfer of:	Embryo transfer	Embryos transferred	Clinical pregnancies	PR/embryo transfer (%)	Gestational sacs	Clinical IR (%)
Only B/EB	40	83	21	52.5	25	30.1
Mixed: ≤M/C+B/EB	5	16	1	20	1	6.3
Only ≤M/C	3	9	0	0	0	0
Total	48	108	22	45.8	26	24.1

B/EB = blastocyst and/or expanded blastocyst.
M/C = morula and/or compaction.

Table III. Blastocyst formation in relation to day 3 embryo morphology

Embryos	Total	Degenerated (%)	Arrested (%)	M/C (%)	B/EB (%)
Class 1 + 2	342	24 (7)	130 (38)	26 (8)	162 (47)
Class 3 + 4	156	10 (6)	98 (63)	16 (10)	32 (21)
Total	498	34 (7)	228 (46)	42 (8)	194 (39)

B/EB = blastocyst and/or expanded blastocyst.
M/C = morula and/or compaction.

Table IV. Development of pre-selected day 3 embryos

Embryos	Total	Degenerated	Arrested	M/C	B/EB
Class 1 + 2	86	1	25	11	49
Class 3 + 4	6	0	2	4	0
Total (%)	92	1 (1.1)	27 (29.3)	15 (16.3)	49 (53.3)

B/EB = blastocyst and/or expanded blastocyst.
M/C = morula and/or compaction.

In 20% of the transfers on day 5, all embryos had been pre-selected on day 3, whereas 56% of the transfers were only in part in agreement with pre-selection. Moreover, 24% of the day 5 transfers showed no agreement with the prior selection. Only 51% of all embryos which were actually transferred on day 5 had been pre-selected for transfer on day 3.

Virtual implantation rate

If the pre-selected embryos had been transferred on day 3, it is possible to calculate the virtual implantation rate and compare this with the actual implantation rate obtained on day 5 (Table I). The implantation rate of an (expanded) blastocyst is 30% (see Table II). The IR for non-cavitating embryos (morula/compaction stage and arrested embryos) was estimated to be 3% (Scholtes and Zeilmaker, 1996), and for degenerated embryos to be 0%.

Using these implantation rates and the actual development of the pre-selected embryos, the virtual implantation rate of these embryos would have been only 17.4%. In contrast, in our study, we have obtained a clinical implantation rate on day 5 of 24.1% (NS).

Discussion

This study was designed to demonstrate the predictive value, if any, of embryo morphology at a relatively early stage for blastocyst formation and subsequent ability to implant. From our data it is clear that this value is limited: approximately half the class 1 and 2 embryos reached the blastocyst stage, whilst as many as one in five class 3 to 4 embryos will arrive at this stage. We further confirmed the finding that blastocysts have a high implantation rate. It is clear, however, that when day 3 embryo morphology is used as the only selection criterion for transfer, often the best embryos are not chosen. Although some paternally derived genes are already activated between the 3- and 4-cell stage (Taylor *et al.*, 1997), it is generally accepted that full embryonic genome activation is completed after the 8-cell stage. Therefore, some patients may indeed benefit from postponing the embryo transfer until after this period (i.e. blastocyst).

Culturing embryos for a longer period of time could also provide more information on embryo quality and implantation potential as long as culture conditions are not the limiting factor. Van Os *et al.* (1989) already showed that pregnancy rates are similar when transferring embryos on either day 2 or day 3. Later, Huisman *et al. (*1994) showed that after 4 days of culture, embryos could be selected that had a high implantation rate; conversely, earlier publications regarding

the transfer of day 5 embryos showed a lower implantation rate than standard transfer (Bolton *et al*., 1991).

Ménézo *et al.* (1992) and Olivennes *et al.* (1994), co-culturing embryos with Vero cells, human granulosa cells or Fallopian tubal cells, reported high implantation rates after blastocyst transfer. Desai used modified α-minimum essential culture medium (α-MEM) for murine embryos and found a higher implantation rate in the presence of co-culture. To date they have only cultured human spare embryos to blastocysts that were not replaced (Desai *et al*., 1997). Since co-culture may impose additional unknown risks, such as contamination with micro-organisms, viral RNA or prions, these efforts did not meet with universal approval. Scholtes and Zeilmaker (1996) did not use co-cultures and reported an overall implantation rate of 23% on day 5, and an implantation rate as high as 35% when only blastocysts were transferred. In our study, similar results were achieved: an overall implantation rate of 24.1% and 30.1% per blastocyst transferred.

Recently, Gardner *et al.* (1998) reported very high rates of blastocyst formation with the use of two different sequential media. However, in addition to the fact that this study only contains eight patients in the day 5 group, it introduces a second variable next to extending the culture period. Doubts have been expressed as to the long term effect of prolonging the culture period and changing the composition of the culture medium in relation to transfer of blastocysts (Rieger, 1997). It seems self-evident that follow-up of the offspring for a sufficient time after birth should take place. To minimize the effects of several variables, in our study the same culture medium was used throughout the entire culture period, with one medium refreshment at day 3.

A recent case report of a pregnancy after transfer of a zona free blastocyst opens interesting new possibilities (Fong *et al*., 1997). This approach has been further investigated by Jones *et al*. (1998) in 35 patients, where they found similar rates to those in our study, in which zonae were left intact. We feel that at present it is premature to judge on the merits and risks of zona removal.

The patients in our study generally had supernumerary, good quality embryos in the past, but failed to become pregnant after several attempts. These patients were a group selected because of a negative outcome, who may benefit from transfer of blastocysts as a result of improved embryo selection.

It appears that blastocyst formation on day 5 offers more information about the implantation potential compared to embryo morphology on day 3. A total of 49% of the embryos pre-selected on day 3 were not transferred on day 5, partly because there were better embryos present on day 5, that had not been pre-selected, but mostly because these embryos arrested in their development or degenerated, despite their good quality on day 3 (31% of the selected but non replaced embryos reached the blastocyst stage, 16% the morula/compaction stage and 53% stopped dividing or degenerated).

Although only 47% of the class 1 and 2 embryos developed into blastocysts, most of the blastocysts on day 5 developed from class 1 and 2 embryos (84%), i.e. from embryos which could have been selected as well on day 3. If the pre-selected embryos had actually been transferred on day 3, their implantation rate would have been only 17.4% (virtual implantation rate), compared to the 24.1% actual implantation rate on day 5. Thus by extending the culture period from 3 to 5 days, the implantation rate could theoretically be improved by 39%. Although the differences are not significant (a power analysis revealed the need for 250 embryos in total), the findings may be clinically relevant as the implantation rate of arrested or degenerated embryos can be considered to be close to zero. It is clear that it cannot be known what the fate would have been of an embryo that degenerated or arrested development between day 3 and 5 in culture if it had been replaced on day 3, but we find it hard to conceive that such an embryo would have developed normally when other embryos from the same cohort under the same culture conditions and during the same time formed morphologically good blastocysts.

The drawback of our procedure may be the possible decrease in the number of pregnancies resulting from the transfer of cryopreserved and thawed blastocysts due to a lower number of embryos suitable for cryopreservation (as compared to day 3) and, in our hands, a decreased survival rate of the blastocyst after thawing. The blastomeres have formed tight junctions at this stage, hence enabling cellular signalling mechanisms, and these more advanced embryos might be more sensitive to the destructive forces of cryopreservation. As yet we have not been able to approach the results of the Ménézo group concerning cryopreservation of co-cultured blastocysts (Kaufman *et al*., 1995); however more work needs to be done regarding this particular aspect.

In conclusion, it appears that the predictive value of embryo morphology before the full activation of the embryonic genome (day 3 embryos) is limited. Hence a distinct group of patients may benefit from prolonging the embryo culture period. Those patients producing embryos on day 3, which are hard to select because of the presence of a large number of embryos of roughly similar morphology, may have several good quality blastocysts by day 5 and as a result, have a greater chance to become pregnant despite previous failures using less advanced embryos. However it is clear that the composition of the culture medium and the culture conditions may play a pivotal role in these considerations.

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