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A prospective, randomized study comparing day 2 and day 3 embryo transfer in human IVF

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It is believed that delayed transfer of embryos after IVF allows for a better selection of good quality embryos. Hence, the number of embryos and all other prognostic factors being equal, transfer of day 3 embryos should be associated with higher implantation and pregnancy rates than transfer of day 2 embryos. To investigate this hypothesis, a prospective randomized study was carried out to compare implantation and pregnancy rates between day 2 and day 3 transfers. The relationship between the embryo quality score of day 2 and day 3 embryos and their respective implantation rates was also analysed. In a 2 year period all patients undergoing infertility treatment and in whom at least seven normally fertilized oocytes were obtained were included in the study. A minimization procedure was performed taking into account the patient's age and the method of fertilization (IVF or intracytoplasmic sperm injection). By using a uniform policy of embryo transfer, the number of embryos transferred was similar in both groups. The outcome parameters were embryo quality, implantation and pregnancy rates. No difference was observed in implantation and pregnancy rates between transfers on day 2 versus day 3 (23.8 versus 23.8% and 47.9 versus 46.8% respectively). The incidence of embryos of moderate to poor quality was higher in embryos cultured for 3 days compared with those cultured for 2 days. It is concluded that the outcomes of embryo transfer in terms of implantation and pregnancy rates are comparable for day 2 and day 3 embryos, although the overall embryo quality score decreases when embryos are kept in culture till day 3.

Key words: culture/embryo quality/embryo transfer/implantation/pregnancy

Introduction

Since the start of IVF, embryos have been transferred 2 days after IVF at the 2- to 4-cell stage. This policy was adopted because the uterus, for lack of suitable culture media able to sustain embryonic development for several days, was supposed to provide the best environment for the survival of the embryo. The timing of the arrival of the embryo in the uterus, however, is premature compared with the situation *in vivo*, where the embryo enters the uterus at the morula stage 4–5 days after ovulation (Harper *et al*., 1994). Transfer of embryos to the uterus on day 3 after oocyte retrieval may be closer to the physiological time of uterine entry than transfer on day 2. Moreover, delaying embryo transfer would allow the selection of the most vital embryos for transfer. It has been observed, indeed, that a large proportion of human embryos which arrest *in vitro* do so between the 4- and 8-cell stage (Bolton *et al*., 1989).

Several studies comparing embryo transfer on day 2 versus day 3 after oocyte retrieval have been performed but the conclusions are conflicting. One study (Van Os *et al*., 1989) randomized day 2 and day 3 embryo transfers based on the day of the week on which oocyte retrieval took place and found no difference in pregnancy rates. Another study (Dawson *et al*., 1995) concluded from a retrospective study that pregnancy rates were similar between day 2 and day 3 transfers but that the implantation rate in the day 3 group was higher. In a retrospective study (Carillo *et al*., 1998) the pregnancy and implantation rates were found to be increased after transfer on day 3. From a prospective randomized study (Ertzeid *et al*., 1999), it was concluded that extending the embryo culture period from 2 to 3 days had no effect on implantation and live birth rates. As the topic of preferential transfer on day 2 or 3 remains controversial, it was thought that it would be useful to perform a prospective, randomized study to compare implantation and pregnancy rates between day 2 and day 3 transfers.

Materials and methods

Patient population

Over a 2 year period, all patients (n = 746) undergoing infertility treatment by routine IVF or intracytoplasmic sperm injection (ICSI) and who had seven or more normally fertilized oocytes were included in this study. All patients were treated using a short protocol: gonadotrophin-releasing hormone (GnRH) analogue (Decapeptyl; Ipsen, Destelbergen, Belgium) followed by ovarian stimulation with human menopausal gonadotrophin (HMG, Humegon; Organon, Oss,

The Netherlands). An injection of 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl; Organon) was given to induce final oocyte maturation. At 35 h after HCG the injection, a transvaginal ultrasound-guided oocyte retrieval was performed.

Randomization and minimization for age and the procedure of fertilization (IVF or ICSI) were performed by a computer program. ICSI was performed in ~84% of the cases where IVF was considered as unsuitable (previously failed IVF) or in cases of oligoasthenoteratozoospermia. The setting is a university-based referral centre for male infertility.

Embryo culture and protein supplement

Earle's balanced salt solution was prepared weekly using reagents from Sigma (Bornem, Belgium): Earle's balanced salt solution (E6132), sodium pyruvate (P4562; 1 mmol/l), sodium bicarbonate (S5761; 25 mmol/l), penicillin (P4687; 0.021 mmol/l) and water (W1503). The osmolality of the medium was adjusted to 285 mOsm/kg H_2O.

For embryo culture this medium was supplemented with human serum albumin (HSA; Belgian Red Cross) at a concentration of 0.4% (w/v). The serum was guaranteed to be prepared from blood that was non-reactive for HBsAg (hepatitis B surface antigen) or human immunodeficiency virus (HIV).

IVF/embryo transfer procedure

Freshly ejaculated semen was washed in Earle's medium supplemented with HSA 0.4% (w/v) by centrifugation at 1600 rpm for 5 min after a 30 min liquefaction period. The pellet was further processed by the side migration technique for ICSI as described previously (Dozortsev *et al*., 1996). For IVF, the sperm sample was put on a two-layer Percoll gradient (45/90%) and the pellet of the lower fraction was used for insemination of oocytes. For routine IVF, oocytes were cultured in medium droplets under oil (M8410; Sigma) together with ~100 000 sperm cells/ml at 37°C in a 5% CO_2 atmosphere. For ICSI, the cells of the cumulus and corona radiata were removed by incubation of the cumulus–oocyte complexes for <1 min in HEPES-buffered Earle's medium containing 80 IU/ml hyaluronidase (H3757; Sigma) followed by gentle aspiration of the cumulus–oocyte complexes in and out of a hand-drawn glass pipette with an inner diameter of 150 µm. Denuded oocytes were rinsed several times in culture medium. Until the moment of injection, the oocytes were kept in 25 µl drops of Earle's medium supplemented with HSA in a Petri dish under mineral oil (Sigma, M8410) and stored in an incubator containing 5% CO_2 in air at 37°C. Microinjection was carried out on the heated stage of an inverted microscope (Axiovert 135; Zeiss, Zaventem, Belgium). The details of the microinjection procedure have been described previously (Dozortsev *et al*., 1996). The presence of two pronuclei and two polar bodies was assessed 16–18 h after IVF or ICSI. Patients with a yield of at least seven normally fertilized oocytes were randomized for transfer on either day 2 or day 3 after oocyte retrieval. Stratification was based on type of infertility treatment (IVF or ICSI) and on age of the patient.

In the day 2 transfer group, embryonic development was assessed under the inverted microscope 42–44 h after IVF or ICSI. In the day 3 transfer group, embryos were first evaluated 42–44 h after IVF or ICSI and then for a second time 24 h after the first evaluation. Embryos were classified based on morphological criteria as described previously (Laverge *et al.*, 1997). Briefly, embryos without anucleated fragments and with equally-sized blastomeres were graded as type I. Embryos with some anucleated fragments (<10%) and/or with unequally-sized blastomeres were graded as type I–II. Embryos with unequally-sized blastomeres with either ⩽20%, up to 50% or >50% anucleated fragments were classified as type II, II–III, and III respectively. Embryos of grade I and I–II were classified as excellent quality embryos, embryos of grade II as good quality embryos and embryos of grade II–III and III as moderate to poor quality embryos. Embryos with at least one blastomere with more than one nucleus were classified as multinucleated embryos and were considered as not suitable for transfer or cryopreservation. As a rule, two embryos were transferred in all patients aged <38 years if two embryos of excellent or good quality were available. Transfer of a maximum of three embryos was allowed in patients with two previous unsuccessful IVF cycles, patients aged >38 years or when no embryos of good quality were available. Supernumerary embryos up to type II were cryopreserved with a dimethyl sulphoxide (DMSO) slow-freezing protocol.

Pregnancy was defined as positive if the βHCG measured in venous blood was >20 mIU/ml. Clinical pregnancy was defined as a positive pregnancy test followed by the presence of a fetal sac on transvaginal ultrasound 4 weeks after transfer. Biochemical pregnancy was defined as a positive pregnancy test not followed by the presence of a fetal sac.

Statistical analysis

Statistical analysis was carried out using a χ^2-test or an unpaired *t*-test where appropriate. $P < 0.05$ was considered to be statistically significant. The anticipated difference in pregnancy rate per cycle between day 2 and day 3 transfers was 10%. It was calculated that at least 370 cycles were needed in each group to prove a difference of 10% with a power of 80%.

Results

Patient profile

Since minimization was based on female age, the mean patient's age was similar in both groups of patients who received their transfer either on day 2 or 3 after oocyte retrieval. Patients selected for this study (at least seven normally fertilized oocytes) represented 35% of the total number of patients treated in our centre.

Embryo quality

A summary of the results on embryo quality is shown in Table I. Overall significantly less embryos were of excellent or good quality after culture for 3 rather than 2 days (10.3 and 40.1% versus 12.9 and 44.2% respectively). The shift to poorer embryo quality on day 3 was found both after ICSI (Table II) and IVF (Table III).

Implantation and pregnancy rates

The better morphological appearance of embryos after day 2 culture and embryo transfer on day 2 was not associated with overall higher pregnancy and implantation rates compared with day 3 culture (47.9 and 23.8% versus 46.8 and 23.8% respectively; Table I). There was no significant difference in pregnancy and implantation rates between day 2 and day 3 transfers when fertilization was by ICSI (Table II). When fertilization was by IVF a statistically higher implantation rate was found in the day 2 versus day 3 group (26.8 versus 16.3%, $P = 0.04$; Table III).

Outcome of pregnancies

The incidence of biochemical and ectopic pregnancies and of miscarriages did not differ significantly for pregnancies established following day 2 or day 3 embryo transfer (Table IV).

Table I. Embryo quality, pregnancy and implantation rates in IVF and intracytoplasmic sperm injection (ICSI) cycles with embryo transfer on day 2 versus day 3

	Day 2	Day 3	*P* value
No. of cycles	374	372	
Mean no. of COC	16.82 ± 6.34	17.52 ± 6.62	NS
Mean no. of fertilized oocytes	11.95 ± 4.10	12.51 ± 4.64	NS
Fertilization rate per COC (%)	71.0	71.4	NS
Embryology			
Quality based on degree of fragmentation			
No. (%) of excellent quality embryos	576 (12.9)	480 (10.3)	<0.0001[a]
No. (%) of good quality embryos	1973 (44.2)	1864 (40.1)	<0.0001[b]
No. (%) of moderate to poor quality embryos	1179 (26.4)	1472 (31.6)	<0.0001[c]
No. (%) of embryos with MNB	740 (16.5)	836 (18.0)	NS[d]
Mean no. of embryos transferred	2.48 ± 0.82	2.49 ± 0.87	NS
Mean embryo score per transfer	8.08 ± 2.27	7.90 ± 2.54	NS
Pregnancy and implantation rate			
Pregnancy rate per cycle (%)	179/374 (47.9)	174/372 (46.8)	NS
Clinical pregnancy rate per cycle (%)	166/374 (44.4)	164/372 (44.1)	NS
Implantation rate per transferred embryo (%)	220/926 (23.8)	220/925 (23.8)	NS

MNB = multinucleated blastomeres; COC = cumulus–oocyte complexes; NS = not significant.
[a,b,c,d]χ^2 following 4×2 contingency table analysis ($P < 0.0001$).

Table II. Embryo quality, pregnancy and implantation rates in intracytoplasmic sperm injection (ICSI) cycles with embryo transfer on day 2 compared with day 3

	Day 2	Day 3	*P* value
No. of cycles	313	313	
Mean no. of COC	16.87 ± 6.56	17.52 ± 6.69	NS
Mean no. of fertilized oocytes	11.92 ± 4.13	12.50 ± 4.65	NS
Fertilization rate per COC (%)	70.6	71.4	NS
Embryology			
Quality based on degree of fragmentation			
No. (%) of excellent quality embryos	422 (11.3)	378 (9.7)	<0.02[a]
No. (%) of good quality embryos	1645 (44.2)	1531 (39.1)	<0.0001[b]
No. (%) of moderate to poor quality embryos	1031 (27.6)	1258 (32.2)	<0.0001[c]
No. (%) of embryos with MNB	632 (16.9)	746 (19.0)	<0.02[d]
Mean no. of embryos transferred	2.50 ± 0.85	2.50 ± 0.86	NS
Mean embryo score per transfer	8.05 ± 2.30	7.90 ± 2.60	NS
Pregnancy and implantation rate			
Pregnancy rate per cycle (%)	151/313 (48.2)	154/313 (49.2)	NS
Clinical pregnancy rate per cycle (%)	140/313 (44.7)	147/313 (47.0)	NS
Implantation rate per transferred embryo (%)	182/784 (23.2)	197/783 (25.2)	NS

MNB = multinucleated blastomeres; COC = cumulus–oocyte complexes; NS = not significant.
[a,b,c,d]χ^2 following 4×2 contingency table analysis ($P < 0.0001$).

Discussion

This prospective study compares day 2 and day 3 embryo transfers with true randomization and of sufficient size to prove or refute a clinically relevant difference in pregnancy rate between both procedures. A minimization procedure was used on a group of patients with at least seven normally fertilized oocytes taking into account two important prognostic factors, i.e. the age of the patient and the procedure of fertilization (IVF or ICSI). The number of embryos transferred was controlled for by a uniform policy of embryo transfer. In the present study, pregnancy and embryo implantation rates were comparable in day 2 and day 3 transfer groups (47.9 and 23.8 % in the day 2 group versus 46.9 and 23.8% in the day 3 group). A statistically lower percentage of excellent and good quality embryos was obtained after culture until day 3 versus day 2 (10.3 and 40.1% versus 12.9 and 44.2%; $P < 0.0001$). A possible explanation for the comparable pregnancy rates despite the difference in embryo quality is that it is still possible to select good quality embryos for transfer due to the fact that at least seven normally fertilized oocytes were required for a patient to be included in the study. Contrary to our hypothesis, delay of embryo transfer by 1 day, at least under these circumstances, does not improve pregnancy rate. The higher implantation rate for day 2 versus day 3 embryos in the subgroup of patients with routine IVF is not readily explainable. However, due to the small sample size in this IVF only group, no conclusions can be drawn.

A number of studies addressing the same issue have already been published and have come to the same or opposite conclusions. In a prospective, randomized study for patients undergoing IVF (Van Os *et al.*, 1989), no significant difference in pregnancy rates between day 2 (n = 434 cycles) and day

Table III. Embryo quality, pregnancy and implantation rates in IVF cycles with embryo transfer on day 2 versus day 3

	Day 2	Day 3	*P* value
No. of cycles	61	59	
Mean no. of cumulus oocyte complexes (COC)	16.57 ± 5.16	17.51 ± 6.27	NS
Mean no. of fertilized oocytes	12.10 ± 3.96	12.53 ± 4.64	NS
Fertilization rate per COC (%)	73.0	71.5	NS
Embryology			
Quality based on degree of fragmentation			
No. (%) of excellent quality embryos	154 (20.8)	102 (13.8)	<0.001[a]
No. (%) of good quality embryos	328 (44.4)	333 (45.1)	NS
No. (%) of moderate to poor quality embryos	148 (20.1)	214 (29.0)	<0.0001[c]
No. (%) of embryos with MNB	108 (14.7)	90 (12.1)	NS[d]
Mean no. of embryos transferred	2.33 ± 0.60	2.41 ± 0.75	NS
Mean embryo score per transfer	8.20 ± 2.13	7.93 ± 2.15	NS
Pregnancy and implantation rate			
Pregnancy rate (%)	28/61 (46.0)	20/59 (33.9)	NS
Clinical pregnancy rate (%)	26/61 (42.6)	17/59 (28.8)	NS
Implantation rate (%)	38/142 (26.8)	23/142 (16.3)	0.04

MNB = multinucleated blastomeres; COC = cumulus–oocyte complexes; NS = not significant.
[a,b,c,d]χ^2 following 4×2 contingency table analysis ($P < 0.0001$).

Table IV. Outcome of the IVF treatment

	Day 2			Day 3		
	Total	ICSI	IVF	Total	ICSI	IVF
Deliveries	142	118	24	135	120	15
Biochemical pregnancy	13	11	2	10	7	3
Ectopic pregnancy	1	1	0	2	2	0
Miscarriage						
of a singleton	16	16	0	18	17	1
of a twin	0	0	0	4	3	1
Partial miscarriage	6	5	1	4	4	0
Embryo reduction	1	0	1	1	1	0
No. of singleton pregnancies	114	99	15	111	100	11
No. of twin pregnancies	50	40	10	50	44	6
No. of triple pregnancies	2[a]	1	1	3[a]	3	0

[a]All triplets resulted from a transfer of three embryos.

3 transfer ($n = 324$ cycles) was found. These authors also reported that the mean embryo quality (morphological criteria) was slightly better in the day 2 group, although no figures about embryo quality were mentioned in the study. The randomization procedure was based on the day of the week oocyte retrieval took place. Two further retrospective studies arrived at opposite conclusions but may have suffered from selectively allowing better prognosis patients to proceed to day 3, whilst worse prognosis patients had their transfer on day 2. In a retrospective comparison of 661 embryo transfers on day 3 with 567 embryo transfers on day 2 (Dawson *et al*., 1995), the patients were compared for age, response to ovarian stimulation, number of oocytes retrieved and embryos transferred. Although the mean number of embryos transferred was significantly higher in patients having embryo transfer on day 2, the pregnancy rate was similar in both groups. The implantation rate was significantly higher following transfer on day 3 than after transfer on day 2. In a retrospective analysis (Carillo *et al*., 1998), patients with a relatively large number of retrieved oocytes were selected for embryo transfer on day 3 ($n = 176$). These patients were then matched on the basis of age, diagnosis and number of oocytes collected with day 2 patients treated in the same year. Pregnancy and embryo implantation rates were significantly higher in patients with day 3 transfers compared with day 2 (51 and 24% versus 29 and 13% respectively). It should be mentioned that glucose- and phosphate-free media were used, which is different from the culture conditions in all other studies. In an earlier study (Edwards *et al.*, 1984), the incidence of miscarriages after day 3 embryo transfer was reported to be higher but this finding was not confirmed either by our own results or by other authors (Van Os *et al*., 1989).

It can be argued that a delay of 1 day is too short to better differentiate the quality of embryos. In recent years, therefore, a more extended delay of embryo transfer, up to the blastocyst stage, has been tried by several investigators. The transfer of cavitating morula stage embryos was found to give an implantation rate of 41% per embryo (Huisman *et al*., 1994). The disadvantage of culture until day 4 is that only a small fraction of the embryos shows a cavitating morula (18.4% in

the study of Huisman *et al*., 1994). In an early study (Scholtes and Zeilmaker, 1996), the implantation rate of embryos after 3 and 5 days of IVF culture were compared. They observed that the overall embryo transfer results were comparable. In a more recent study (Scholtes and Zeilmaker, 1998) these authors analysed the effects of patient age and treatment cycle number on the occurrence of blastocyst transfer and subsequent implantation. They reported a decreasing implantation rate after cycle 2 and observed that biological ovarian age is a determining factor on the frequency of blastocyst transfer or pregnancy rate.

In the above-mentioned studies, a single culture medium formulation was used to support embryo development from the 1-cell stage to the blastocyst stage. More recently, sequential media have been introduced for culture of later stages of embryo development. Gardner *et al.* developed sequential serum-free media and showed that >50% of embryos became blastocysts. Transfer of these blastocysts resulted in an implantation rate of ~50% (Gardner and Lane, 1998). An improved IVF culture system where the use of sequential 'stage-specific' culture media allegedly increased the implantation rate from 11.1% without this system to 30.6% has been described (Mortimer *et al*., 1998). The clinical pregnancy rate increased from 19.6 to 45.9%.

The pregnancy and implantation rates for transfer of blastocysts versus transfer of day 2 or day 3 embryos is in the similar range. A pregnancy rate of 38% and an implantation rate of 23% has been reported (Jones *et al*., 1998). In the present study, the pregnancy rates were 47.9% in the day 2 group and 46.8% in the day 3 group and the implantation rate was 23.8% in both groups. The patient selection in both studies was similar. In the study by Jones *et al.* only patients aged <40 years with more than five fertilized oocytes were included while in the current study only patients with at least seven fertilized oocytes were included and randomized for age.

In conclusion, this prospective, randomized study demonstrates that (at least in cases where a sufficient number of fertilized oocytes are available), it does not make a difference whether transfer is performed on day 2 or day 3, since similar pregnancy and implantation rates can be achieved. Moreover, these findings indicate that embryo transfers can be safely scheduled at the convenience of the patient and the centre.

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