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Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization

S.Ziebe[1,4], K.Petersen[2], S.Lindenberg[3], A.-G.Andersen[1] A.Gabrielsen[2] and A.Nyboe Andersen[1]

[1]The Fertility Clinic, The Juliane Marie Center, Section 4071, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, [2]Ciconia Fertility Clinic, Århus, Ildervej 9, DK-8270 Højbjerg, and [3]The Fertility Clinic, Herlev University Hospital, Herlev Ringvej, DK-2730 Herlev, Denmark

[4]To whom correspondence should be addressed

This retrospective study of 1001 in-vitro fertilization (IVF) cycles included a consecutive series of single transfers (*n* = 341), dual transfers (*n* = 410) and triple transfers (*n* = 250) where all the transferred embryos in each cycle were of identical quality score and identical cleavage stage. In our 2 day culture system, transfer of 4-cell embryos resulted in a significantly higher implantation rate and pregnancy rate (23 and 49%) compared with 2-cell embryos (12 and 22%) and 3-cell embryos (7 and 15%). Furthermore, the transfer of 4-cell embryos resulted in a significantly higher pregnancy rate compared with embryos that had cleaved beyond the 4-cell stage (28%). The implantation rate (21%) and pregnancy rate (43%) after transfer of embryos of score 1.0 were significantly higher than after transfer of embryos of score 2.0 (14 and 32% respectively). Transferring embryos of score 2.1 resulted in significantly higher implantation rates (26%) and similar pregnancy rates compared with score 1.0. Transferring embryos of score 2.2–3.0 resulted in a significantly lower implantation rate (5%) and pregnancy rate (15%). A striking finding was that embryos of quality score 2.0 had a significantly lower implantation rate compared with embryos of quality score 1.0 and 2.1 and a significantly lower pregnancy rate compared to embryos of quality score 1.0. We also found a lower implantation rate and pregnancy rate when transferring 3-cell embryos. These findings may indicate periods of increased sensitivity to damage during the cell cycle. In conclusion, these results substantiate the idea of the superiority of 4-cell embryos and demonstrate that minor amounts of fragments in the embryo may not be of any importance. These findings may call for a shift when weighing the two main morphological components (quality score and cleavage stage) in the sense that reaching a 4-cell cleavage stage even with the presence of a minor amount of fragments should be preferred to a 2-cell embryo with no fragments.

Key words: cleavage/embryo selection/fragmentation/quality score

Introduction

After in-vitro fertilization (IVF), the pregnancy rate increases with the number of embryos transferred (Edwards and Steptoe, 1983; Kerin *et al.*, 1983; Tan *et al.*, 1990) and the implantation rate is positively correlated to a good morphology and to the cleavage stage of the embryos (Staessen *et al.*, 1992; Shulman *et al.*, 1993; Cummins *et al.*, 1986; Puissant *et al.*, 1987).

Embryo morphology and cleavage stage can be combined in a scoring system that is predictive of pregnancy rate (Puissant *et al.*, 1987; Steer *et al.*, 1992). Claman *et al.* (1987) found high pregnancy rates when the transfer included at least one embryo at the 4-cell stage at 40 h after insemination. Regardless of the number of embryos transferred, the pregnancy rate remained low when the embryos transferred consisted of those with fewer than four blastomeres. In most previous studies, pregnancy rates have been analysed after transfers of two or more embryos of different quality and/or cleavage stage, making it impossible to define which of the embryos implanted. In spite of these earlier studies there only seems to be one conclusive clinical study (Giorgetti *et al.*, 1995) regarding the association between the cleavage stage, degree of fragmentation and the implantation rate. The study included 858 single embryo transfers and it was found that embryos transferred at the 4-cell stage implanted twice as often as did 2-cell embryos.

However, a study of single embryo transfers may not be considered a representative model for IVF, where the majority of the patients have more than one embryo available for transfer. The present study aims to extend and substantiate the work by Giorgetti *et al.* (1995) on the relationship between embryo morphology, cleavage stage and implantation rate. This study includes single embryo transfers and dual and triple transfers of embryos of identical quality score and identical cleavage stage.

Materials and methods

Patient selection

This retrospective analysis includes couples undergoing IVF at one of the three participating clinics between January 1993 and May 1996. The patients were referred to IVF mainly due to tubal infertility or unexplained infertility. The study represents a consecutive series of transfers of single embryos (n = 341) and those dual (n = 410) or triple (n = 250) embryo transfers where only embryos of identical morphology score and identical cleavage stage were included. Patients who had intracytoplasmic sperm injection (ICSI) or replacement after cryopreservation were excluded. A total of 1001 transfers of 1918 fresh embryos in 880 patients were included in the study. The mean age of the women was 33.2 ± 3.8 years (range 20–45 years). Of the

880 women, 18 were aged >40 years and 88% of the patients were aged 28–38 years at the time of treatment.

The clinics

The heads of the three clinics have all been trained at the same IVF unit (Rigshospitalet) and most clinical and laboratory procedures were basically the same. All three clinics transferred between one and three embryos, although the policy concerning transfer of two versus three embryos may have changed within the clinics during the study period.

Hormonal stimulation

Ovarian stimulation was achieved by human menopausal gonadotrophins (HMG, Humegon; Organon or Pergonal or Fertinorm; Serono, Geneva, Switzerland) in various protocols of which the vast majority involved the long protocol. In a minor number of cycles the ultra-short protocol or clomiphene was used. The long and ultra-short protocols involved down-regulation with gonadotrophin-releasing hormone analogues. Details of the stimulation protocols were as follows:

Long protocol

Down-regulation began on day 21 of the cycle, using buserelin (Hoechst, Denmark) or nafarelin (Syntex, Denmark). Stimulation with follicle stimulating horomone (FSH)/HMG was initiated at least 2 weeks after initiation of down-regulation.

Ultra-short protocol

Down-regulation was initiated on day 2 of the cycle using buserelin for 3 days. Stimulation with FSH/HMG was initiated at day 3.

Clomiphene stimulation

Clomiphene and HMG were used; clomiphene (Serono, Denmark), 100 mg/day, was given on days 2–6, combined with HMG from day 4.

Human chorionic gonadotrophin (HCG; Serono, Denmark) was injected 36 h before oocyte retrieval. As luteal support, progesterone vagitoris (3–6 per day) was given in all clinics.

IVF procedure

IVF was performed according to the routine protocols of each clinic. In each clinic oocytes were aspirated 36 h after HCG injection and fertilized 4–6 h later. On the following day the oocytes were checked for fertilization and cultured for another 24 h. Transfer was carried out 48–50 h after aspiration. In all three clinics the embryos were evaluated for cleavage stage and scored prior to transfer in accordance with the following morphological criteria: (i) morphology score 1.0: equally-sized symmetrical blastomeres; (ii) morphology score 2.0: uneven sized blastomeres; (iii) morphology score 2.1: embryos with <10% fragmentation; (iv) morphology score 2.2: embryos with 10–20% blastomeric fragmentation; (v) morphology score 3.0: 20–50% blastomeric fragmentation; (vi) morphology score 4.0: >50% blastomeric fragmentation. The present study only includes transfers of single embryos and transfers of two or three embryos of identical morphology and identical cleavage stage. Thus, in each transfer the morphology and cleavage stage of all implanted embryos were known.

If a pregnancy occurred, ultrasound evaluation was performed to ensure the presence of an intrauterine gestational sac ('clinical pregnancy'); otherwise the pregnancy was registered as 'biochemical'.

Implantation rate was defined as the fraction of transferred embryos resulting in an implanted embryo or gestational sac.

Statistical analysis

Statistical analysis done by the Fisher test and χ^2 test. Values were considered significant when $P < 0.05$.

Table I. Percentage implantation rate in relation to embryo morphology and cleavage stages

Cleavage stage	Embryo morphology				
	1.0	2.0	2.1	2.2–3.0	Total
2-cell	13 (32/238)	12 (4/33)	11 (13/117)	4 (2/47)	12 (51/435)[e]
3-cell	0 (0/4)	6 (1/16)	11 (2/19)	6 (1/16)	7 (4/55)[f]
4-cell	23 (194/856)	15 (18/118)	32 (106/336)	4 (2/57)	23 (320/1367)[g]
>4-cell	21 (3/14)	20 (2/10)	26 (6/23)	14 (2/14)	21 (13/61)[h]
Total	21 (229/1112)[a]	14 (25/177)[b]	26 (127/495)[c]	5 (7/134)[d]	20 (388/1918)

[a,b]$P < 0.05$.
[b,c]$P < 0.01$.
[c,d]$P < 0.0001$.
[a,c]$P < 0.05$.
[e,g]$P < 0.0001$.
[f,g]$P < 0.01$.
[g,h]Not significant.

Results

A total of 7912 oocytes were aspirated in 1001 aspirations (7.9 oocytes per aspiration). Of these, 4785 cleaved (4.8 per aspiration), resulting in a cleavage rate of 61%. The average number of blastomeres at embryo transfer was 3.5 (range 2–8). Following transfer of 1918 fresh embryos (1.9 embryos per transfer, range 1–3) 390 pregnancies were recorded. In 341 cycles only one embryo was transferred. In these cases the average age of the women was 34 years. In 660 cycles more than one embryo was transferred and the average age of these women was 33 years.

Implantation rate

Cleavage stage

Transfer of 4-cell embryos resulted in a significantly higher implantation rate (23%) than transfer of 2-cell embryos (12%) ($P < 0.0001$) or 3-cell embryos (7%) ($P < 0.01$) (Table I). Transfer of embryos with more than 4 cells did not result in a further increase in implantation rate (Table I), rather there was a non-significant trend towards a lower implantation rate.

Embryo morphology

The implantation rate was 21% after transfer of embryos of score 1.0 (Table I). This was significantly higher than after transfer of embryos of score 2.0 (14%) ($P < 0.05$), but slightly although significantly lower than after transfer of embryos of score 2.1 (26%) ($P < 0.05$). Additionally, transferring embryos of score 2.1 resulted in a significantly higher implantation rate than embryos of score 2.2–3.0 (5%) ($P < 0.0001$) (Table I).

If only one embryo was transferred the overall implantation rate was significantly lower ($P = 0.0015$) compared with multiple embryo transfers. However, no difference in implantation rate was found between single and multiple transfers of 2-cell embryos and 4-cell embryos of good quality (score 1.0, 2.0, 2.1) (Figure 1). No difference in implantation rate was found between transferring two or three embryos. Further, no difference was found between implantation rate in women

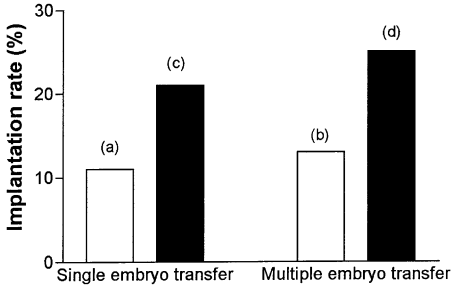

Figure 1. Implantation rates of good quality embryos in single embryo and multiple embryo transfer. Single embryo transfers of 2-cell (white bars; $n = 115$) and 4-cell embryos (dark bars; $n = 106$) and multiple embryo transfer of 2-cell (white bars; $n = 273$) and 4-cell embryos (dark bars; $n = 1192$). All transferred embryos had <20% fragmentation. At each transfer all embryos had an identical morphology score and identical cell number. [a,b]Not significantly different, [c,d]Not significantly different

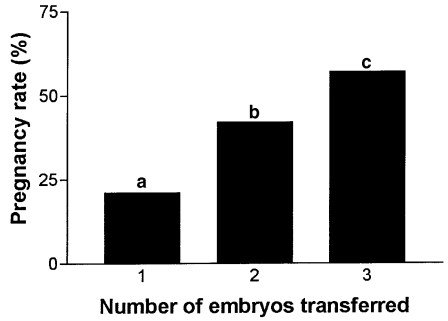

Figure 2. Pregnancy rates in relation to number of transferred embryos. At each transfer all embryos had identical morphology score and identical cell number. Data represent 341 single embryo transfers, 410 dual embryo transfers and 250 triple embryo transfers.
[a,b]$P < 0.0001$.
[b,c]$P < 0.001$.

Table II. Pregnancy rate in relation to embryo morphology and cleavage stages

Cleavage stage	Embryo morphology				
	1.0	2.0	2.1	2.2–3.0	Total
2-cell	22 (30/134)	23 (6/26)	25 (18/73)	9 (3/32)	22 (57/265)[e]
3-cell	0 (0/4)	17 (2/12)	24 (4/17)	7 (1/15)	15 (7/48)[f]
4-cell	51 (192/378)	39 (24/61)	54 (90/166)	19 (7/36)	49 (313/641)[g]
>4-cell	40 (4/10)	33 (2/6)	22 (4/18)	23 (3/13)	28 (13/47)[h]
Total	43 (226/526)[a]	32 (34/105)[b]	42 (116/274)[c]	15 (14/96)[d]	39 (390/1001)

[a,b]$P <0.05$.
[b,c]Not significant.
[c,d]$P <0.0001$.
[a,c]Not significant.
[e,g]$P <0.0001$.
[f,g]$P <0.0001$.
[g,h]$P <0.01$.

Table III. Pregnancy outcome in relation to embryo quality score

	Embryo score 1.0, 2.0 or 2.1	Embryo score 2.2 or 3.0
Ongoing/delivery	66 (250/376)[a]	36 (5/14)[b]
Biochemical	18 (67/376)[c]	57 (8/14)[d]
Abortion before week 12	10 (38/376)	7 (1/14)
Abortion after week 12	2 (9/376)	0
Ectopic pregnancies	3 (12/376)	0

[a,b]$P <0.02$.
[c,d]$P <0.001$.

aged <40 and >40 years. In the subgroup of women aged >40 years, four out of 18 became pregnant (22%). A total of 28 embryos were transferred and five implanted (18%).

Pregnancy rate

Cleavage stage

In accordance with the implantation rates, the transfer of 4-cell embryos resulted in a significantly higher pregnancy rate (49%) than transfer of 2-cell embryos (22%) ($P <0.0001$) or 3-cell embryos (15%) ($P <0.0001$) (Table II). Furthermore, transfer of embryos with more than four cells resulted in a significantly lower pregnancy rate (28%) compared with 4-cell embryos (49%) ($P =0.01$) (Table II).

Embryo morphology

Transferring embryos of quality score 1.0 resulted in a significantly higher pregnancy rate (43%) than transferring embryos of quality score 2.0 (32%) ($P <0.05$). No other significant differences concerning pregnancy rates were found, when transferring embryos of quality 1.0, 2.0 or 2.1 respectively. However, when transferring embryos of quality 2.2–3.0, the pregnancy rate decreased significantly ($P <0.0001$) compared with quality score 2.1 (Table II).

Pregnancy outcome

Overall, 390 pregnancies were achieved after 1001 embryo transfers in 880 patients. A total of 768 patients had one treatment, 103 patients had two treatments and nine patients had three treatments. Of these 255 (65%) were ongoing/delivered and 75 (19%) were biochemical pregnancies. 39 pregnancies (10%) ended as abortion before week 12 and 9 pregnancies (2%) ended as abortion after week 12. A total of 12 pregnancies (3%) were ectopic.

The pregnancies achieved after transfer of embryos of quality score 1.0, 2.0 or 2.1 resulted in significantly more ongoing pregnancies ($P <0.02$) and significantly fewer biochemical pregnancies ($P <0.001$) compared with pregnancies achieved after transfer of embryos of quality score 2.2 or 3.0 (Table III).

Figure 2 illustrates the pregnancy rates in relation to the number of embryos transferred. The pregnancy rate after transferring one embryo was 21.4% which was significantly lower ($P <0.0001$) than after transfer of two embryos, which resulted in a pregnancy rate of 42.5%. Additionally, transfer of three embryos increased the pregnancy rate significantly to 57.2% ($P <0.001$) (Figure 2).

Discussion

In contrast to most studies relating embryo quality to the implantation rate, Giorgetti *et al.* (1995) based their study on single embryo transfers only, in order to define the embryos that implanted. They found that 4-cell embryos implanted twice as often as embryos with more or fewer cells. Extending this study to include multiple transfers we also found the 4-cell embryo to be the optimal cleavage stage. There now seems to be substantial clinical evidence for an optimal cleavage speed for the embryos, as reported 10 years ago by Cummins *et al.* (1986), who suggested that slowly or rapidly cleaving embryos implanted less frequently than embryos at the 4-cell stage after 2 days of culture. Similarly, in a series of multiple embryo transfers, Claman *et al.* (1987) noted a significantly higher pregnancy rate in women who received at least one embryo at the 4-cell stage. In 312 single embryo transfers Staessen *et al.* (1992) found a higher implantation rate for good quality embryos with at least three blastomeres compared with 2-cell embryos of similar quality. Further, when transferring two or three good quality embryos of different cleavage stages, Staessen *et al.* (1992) found that the pregnancy rate increased proportionally to the number of 3- or 4-cell embryos. The results of the present study substantiate the case for the superiority of 4-cell embryos when transferring more than one embryo.

When transferring embryos of score 2.1, the pregnancy rate was not significantly different from transferring embryos of score 1.0 (Table II). Thus, it seems that these subgroups could be considered together as one group of good quality embryos and the presence or absence of minor fragmentation has no clinical importance. With regard to embryos of score 2.2–3.0 we found the expected significant decrease in implantation rate and pregnancy rate. These findings are in accordance with Staessen *et al.* (1992), who classified embryos as type A (no fragments), B (<20% fragmentation) or C (>20% fragmentation) and found no difference between the implantation rate of the types A and B embryos, while type C embryos had a significantly reduced implantation rate compared to the two other categories. Thus, in relation to daily clinical practice it seems that the detailed scoring systems which are often used today are of very limited use; a less stringent classification of good and bad is sufficient when selecting embryos for transfer. As presented in Table III, significant differences were found in the number of ongoing pregnancies and biochemical pregnancies, when looking at the quality of the embryos transferred. Transfer of embryos of quality score 2.2–3.0 gave predominantly biochemical pregnancies, while transferring embryos of quality score 1.0, 2.0 or 2.1 resulted in predominantly ongoing pregnancies. This indicates that even though an embryo quality score of 2.2–3.0 is capable of initiating the early events of pregnancy, the chance of this pregnancy leading to the birth of a child is low. However, the abortion rate per clinical pregnancy and per implanted embryo was unrelated to the quality of the embryos, which is similar to the findings of Staessen *et al.* (1992) and Giorgetti *et al.* (1995).

A striking finding in this study was that embryos of score 2.0 (containing blastomeres of irregular size), had a significantly reduced implantation rate both compared with embryos with uniformly sized blastomeres (score 1.0) and to embryos with a small amount of fragmentation (score 2.1). Furthermore, transfer of embryos of score 2.0 resulted in a significantly reduced pregnancy rate compared to embryos of score 1.0 (Tables I and II). This finding is also in line with the findings by Giorgetti *et al.* (1995) who found a significantly decreased pregnancy rate when transferring embryos having irregular sized blastomeres.

One may speculate that blastomeres are more sensitive to damage just prior to cleavage than at other stages of the cell cycle and therefore more vulnerable when transferred. As the 3-cell stage is a transient stage, often with irregular sized blastomeres some of which are in the process of cleavage, these embryos may be damaged during transfer and this could explain the low implantation rate of 3-cell embryos as found in the present study as well as by Giorgetti *et al.* (1995).

Further, this is supported by the speculations that embryos with uneven numbers of blastomeres may be more sensitive to damage when cryopreserved (Lassalle *et al.*, 1985). We do not believe that the findings reported in this study are biased by differences in the age of the women between the various groups. This is based on the fact that 88% of the women were between the ages of 28 and 38 and that the average age of the women, which was 33.2 years, had an SD of 3.8 years. Furthermore, only 2% of the women were aged >40 years. Most importantly no differences were found in the age between those women who had single or multiple embryo transfers and the implantation rate was not lowered in the small group of women age >40 years.

In regard to the implantation rate in single and multiple embryo transfers, we find a decreased implantation rate when transferring only one embryo. However, when only good quality embryos were analysed no difference was found in the implantation rates between single and multiple transfers (Figure 1). This finding was also reflected in the marked increase in pregnancy rates by adding a third embryo (Figure 2). Previous studies have shown no increase or only a marginal increase in pregnancy rate after triple versus dual embryo transfers (Waterstone *et al.*, 1991; Staessen *et al.*, 1993). In the present study, the marked increase in pregnancy rate after transfer of three versus two embryos should be considered in the context that we transferred embryos of the same quality and not of different quality. Transferring embryos of the same quality improves the chances that the implantation rate would be the same for each of the three embryos. In contrast, transfer of embryos of different quality often includes embryos with lower quality which may have a lower implantation rate. In this latter situation only a marginal increase in pregnancy rate can be expected by adding a third embryo.

In conclusion, these results may call for a shift when weighing the two main morphological components (quality score and cleavage stage) in the sense that reaching a 4-cell cleavage stage, even with the presence of a minor amount of fragments, should be preferred rather than a 2-cell embryo with no fragments. As a consequence, when considering the group of 'good' embryos (i.e. embryos scoring 1.0–2.1), 4-cell

embryos should be valued higher than embryos with no fragmentation.

We think that defining the principles for good embryo selection may help us in selecting the 'right' embryos for transfer. However, as we increase the quality of the embryos transferred we should always consider the number of embryos that we transfer together with the risk of establishing multiple pregnancies (Walters, 1996). The data presented here as well as those presented by others (Kodama *et al.*, 1995, Tasdemir *et al.*, 1995) suggest that by selecting the right embryos we will increase implantation and pregnancy rates and thus obtain the additional benefit of allowing us to reduce further the number of embryos transferred.

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