

Evaluation of pronuclear morphology as the only selection criterion for further embryo culture and transfer: results of a prospective multicentre study

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BACKGROUND: The aim was to study zygote evaluation based on pronuclear morphology under the German embryo protection law, according to which only a maximum of three zygotes are allowed to be cultured for embryo transfer. **METHODS:** In this prospective multicentre study, a total of 512 treatment cycles was performed at 10 centres, between November 1999 and October 2000. Zygotes were classified into seven patterns (0A, 0B and 1–5). Pattern 0A and 0B zygotes were preferentially used for further culture and transfer. **RESULTS:** Cycles with transfer of at least one embryo derived from pattern 0B, but not pattern 0A, resulted in significantly higher pregnancy (37.9%) and implantation rates (20.5%) compared with non-pattern 0B cycles (26.4 and 15.7%; $P < 0.05$ and $P < 0.01$ respectively). In younger patients (aged ≤ 35 years), significantly more 0B zygotes were available for transfer than in older patients (34.2 versus 25.8%; $P < 0.005$). **CONCLUSIONS:** From these data, it is concluded that evaluation of pronuclear morphology is beneficial, especially for countries with legal restrictions regarding embryo selection.

Key words: ICSI/implantation/IVF/maternal age/pronuclear morphology

Introduction

The selection of embryos with the highest implantation potential for transfer is a crucial part of assisted reproduction. The introduction of sequential culture media enables the culture of a maximum number of embryos under optimal conditions to the blastocyst stage and consequently, high implantation rates have been reported following the transfer of selected blastocysts (Gardner *et al.*, 1998). Another approach was presented (Scott

and Smith, 1998) which proposed that the implantation potential of an embryo can be predicted at the pronuclear stage, based on morphological criteria. This idea was further supported by others (Tesarik and Greco, 1999), who showed that a single examination of pronuclear morphology, namely the number and distribution of nucleolar precursor bodies (NPB) in each pronucleus, can predict abnormal preimplantation development. The same group reported that following intracytoplasmic sperm injection (ICSI), embryos derived from a specific pronuclear pattern gave higher implantation rates compared with other patterns (Tesarik *et al.*, 2000); these observations were confirmed by another group (Wittemer *et al.*, 2000). More recently, it has been demonstrated that the morphology of human pronuclear stage embryos is related to blastocyst development (Scott *et al.*, 2000), which may explain the high implantation potential of embryos developing from distinct pronuclear morphology pattern.

The approach to identify human embryos with the highest implantation potential at the pronuclear stage is extremely interesting for countries with a strict embryo protection law, such as Germany and Switzerland. In these countries, the selection of cleavage stages is prohibited by law, and therefore no other embryos are available for later transfer than those selected at the pronuclear stage. At the pronuclear stage it has to be decided which cells are cultured and used for embryo transfer, and which are cryopreserved or discarded. A preliminary

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ary study on the use of a pronuclear score under these conditions has been published (Ludwig *et al.*, 2000). These authors assessed several morphological criteria in 74 non-selected ICSI cycles and found a higher pregnancy rate above a certain pronuclear score threshold. In accordance with German embryo protection law, the aim of the present study was to evaluate the potential use of pronuclear selection in IVF and ICSI cycles based on a single morphological observation in a prospective, non-randomized multicentre study.

Materials and methods

Study design and patients

This study was performed between November 1999 and October 2000 at 10 IVF centres in Germany. In total, 512 patients receiving either IVF ($n = 247$) or ICSI ($n = 265$) treatment were enrolled in this study. Criteria for inclusion were normal FSH concentrations during early follicular phase, fewer than four previous treatment cycles, and a negative test for hepatitis and HIV. Exclusion criteria were polycystic ovaries, endometriosis and poor ovarian response in previous stimulation cycles. Stimulation was performed by down-regulation in a long protocol in all centres, whereas the embryo culture system was not the same for all centres. On the day after follicular puncture, a maximum of three pronuclear stage oocytes were selected (see below) for further culture and transfer on days 2–5. All other pronuclear-stage oocytes were either cryopreserved or discarded, based on the policy of each centre. In order to fulfil the criteria of a prospective study, each centre had to register a treatment cycle for the study at the central study office on the day of pronuclear morphology assessment. On that day, detailed information on the pronuclear morphology pattern of those pronuclear-stage oocytes which were selected for further culture and transfer were reported to the study office. The study office requested for each cycle a second report form, containing data on human chorionic gonadotrophin (HCG) measurement, number of gestational sacs and fetal heart beat following the induction of a pregnancy.

Assessment of pronuclear and embryo morphology

Pronuclear morphology was classified 16–20 h after IVF or ICSI according to patterns previously described (0–5; Tesarik and Greco, 1999) with the following exception. In the original description, pattern 0 comprised two sub-patterns, one with >7 NPB showing an equal/symmetric distribution in each pronucleus and another with <7 NPB with polarization in each pronucleus at the area of contact. In this study, these two patterns were specified as pattern 0A (>7 NPB, equally distributed) and 0B (≤ 7 NPB, polarized). Each centre selected preferentially pattern 0A and/or 0B for further culture and transfer. When sufficient pattern 0 pronuclear stage oocytes were not available, other patterns were selected. Each pronuclear-stage oocyte was cultured individually. Embryo morphology was assessed on day 2, and each embryo was given an embryo score according to a published system (Steer *et al.*, 1992). Embryo transfers were performed on days 2–5 after follicular aspiration, depending on the transfer policy of each centre. According to the German embryo protection law, no embryos other than those derived from the pronuclear-stage oocytes selected for further culture and transfer were available.

Based on previously published data on the assessment of pronuclear morphology (Scott and Smith, 1998; Tesarik and Greco, 1999), it was considered unethical to perform a randomization with one group of patients receiving embryos from good prognosis pronuclear-stage oocytes and another group of patients receiving embryos from pronuclear-stage oocytes with a poor prognosis (Ludwig *et al.*, 2000).

Evaluations

For the calculation of the pregnancy rate only cycles with proven implantation, documented by ultrasound, were considered. The implantation rate was calculated from the number of gestational sacs divided by the total number of embryos transferred. For each pronuclear morphology pattern the mean embryo score was calculated based on all embryos derived from that specific pattern. Clinical pregnancy and implantation rates were calculated for all transfer cycles with at least one embryo derived from pattern 0B versus all remaining transfer cycles without embryos derived from pattern 0B. For all other patterns, pregnancy and implantation rates were calculated for all transfer cycles with at least one embryo derived from that specific pattern X ($X = 0A, 1, 2, 3, 4, 5$) versus all remaining transfer cycles without embryos derived from pattern X ($([0A+1+2+3+4+5] - X)$). For both of these calculations, all transfer cycles with at least one embryo derived from pattern 0B were excluded.

An evaluation of the data was performed for the number of presumable good pronuclear morphology pattern 0A/0B in relation to maternal age (≤ 35 years/ >35 years) and in relation to assisted reproduction technique (IVF/ICSI).

Statistical analysis

Statistical evaluation was performed with ANOVA and a χ^2 test where appropriate; a P -value < 0.05 was considered significant.

Results

Study characteristics

A total of 512 treatment cycles (variation between centres 1 to 180) were prospectively registered at the central study office. Among these, 495 treatment cycles fulfilled all criteria and were included in the final evaluation. In all, 495 transfers (IVF, $n = 239$; ICSI, $n = 256$) gave rise to 160 pregnancies (IVF, $n = 69$; ICSI, $n = 91$) with a mean clinical pregnancy rate per transfer of 32.3% and a variation of pregnancy rates between centres from 0–41% (0% pregnancy rate in the centre which reported one treatment cycle), with 203 out of 1114 transferred embryos implanting (implantation rate per embryo 18.2%). The mean maternal age was 33.2 ± 4.2 years.

Embryo development and implantation potential

Out of 1114 embryos which were transferred, 489 were derived from a pronuclear morphology pattern with a presumable good prognosis for implantation (patterns 0A/0B), and 625 from a pronuclear morphology pattern with a presumable poor prognosis for implantation (patterns 1–5; Table I). Embryos derived from a pronuclear morphology pattern with a presumably good prognosis showed a significant higher embryonic score (12.7 ± 5.0) compared with embryos derived from poor prognosis patterns (11.7 ± 5.3 ; $P < 0.001$).

Clinical pregnancy and implantation rates in transfer cycles involving at least one embryo derived from pattern 0B were compared with transfer cycles involving no embryos derived from pattern 0B (Table II). The pregnancy and implantation rates were significantly higher in transfer cycles with embryos derived from pattern 0B (37.9 and 20.5% respectively) compared with all remaining transfer cycles (26.4 and 15.7%; $P < 0.01$ and $P < 0.05$ respectively). In cycles where only two embryos were transferred, the pregnancy rate of two

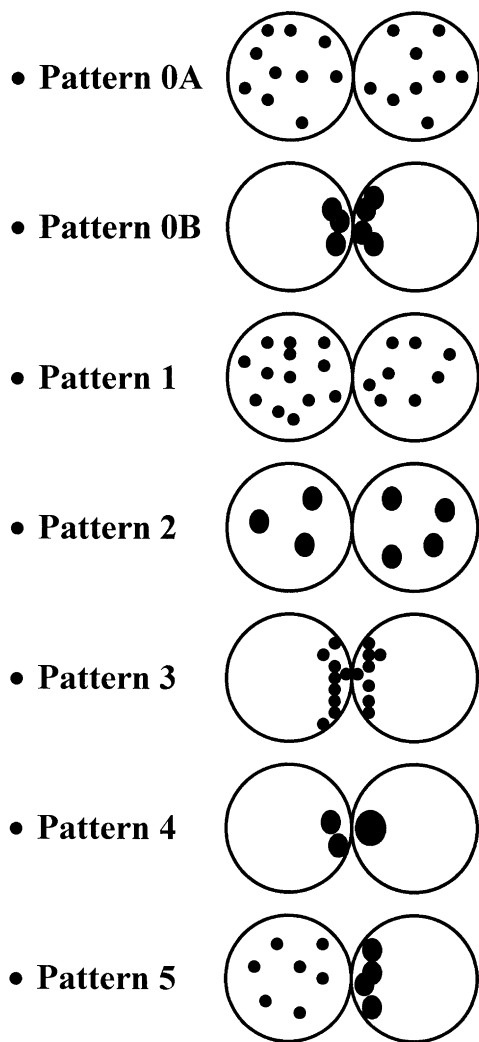


Figure 1. The different patterns of pronuclear morphology were adapted from Tesarik and Greco (Tesarik and Greco, 1999); however, the original pattern 0 was subclassified into pattern 0A and 0B as shown.

Table I. Numbers of pronuclear morphology patterns and corresponding embryo scores

Pattern	n (%)	Embryo score ^a
0A	139 (12.5)	12.7 ± 4.7
0B	350 (31.4)	12.7 ± 5.1
Total 0A + 0B	489 (43.9)	12.7 ± 5.0*
1	108 (9.7)	11.7 ± 5.8
2	118 (10.6)	12.1 ± 5.0
3	89 (8.0)	11.7 ± 5.3
4	44 (3.9)	10.1 ± 4.9
5	266 (23.9)	11.6 ± 5.3
Total 1–5	625 (56.1)	11.7 ± 5.3*

^aValues are mean ± SD.
*P < 0.001.

embryos derived from pattern 0B was 43.3% (26/60), compared with 26.8% (41/153; P < 0.025) for two embryos derived from other patterns (data not shown). On the basis of these results, it was decided to exclude all mixed cycles involving

pattern 0B for calculations of pregnancy and implantation rates of other patterns.

Transfer cycles involving pattern 3 gave significantly higher pregnancy and implantation rates. No significant differences were found for patterns 0A, 1, 2 and 4. Transfer cycles involving pattern 5 showed significantly lower implantation rates compared with cycles without pattern 5.

Maternal age and pronuclear morphology

The relationship between maternal age and pronuclear morphology was analysed (Table III). Significantly more pronuclear-stage oocytes of pattern 0B and 3 were available in younger patients (aged ≤35 years; 34.2 and 9.2%) than in older patients (aged >35 years; 25.8 and 5.4%; P < 0.005 and P < 0.05 respectively). For pattern 0A, 4 and 5 no significant differences were found, whereas significantly more pattern 1 and 2 oocytes were observed for transfer in older patients.

Compared with older patients, younger patients presented with significantly higher pregnancy rates (35.4 versus 25.5%; P < 0.05) and implantation rates (20.4 versus 13.9%; P < 0.001). Therefore, subgroups of younger and older patients were analysed and the number of transfer cycles involving at least one embryo derived from pattern 0B versus cycles without embryos from pattern 0B compared. No significant differences were found with regard to the number of cycles. The pregnancy rate in younger patients with transfer involving at least one embryo from a 0B pattern oocyte was significantly higher compared with all other subgroups. For the implantation rate, a significant difference was found only between younger patients with transfer of at least one embryo from 0B compared with older patients without pattern 0B (Table III). Older patients with pattern 0B attained the same pregnancy rate (28.8%) as younger patients without 0B (28.4%).

Pronuclear morphology and assisted reproductive technique

The distribution of pronuclear morphology patterns selected for transfer in IVF and ICSI treatment cycles is shown in Table IV. Significantly more 0B pattern were available in ICSI (36.3%) compared with IVF (27.9%; P < 0.025). For pattern 0A and pattern 1, significantly more oocytes were available in IVF, whereas for patterns 2–5 no significant differences were found. In ICSI, significantly more transfer cycles were noted involving pattern 0B (144/256) compared with IVF (109/239; P < 0.025); however, despite this trend the corresponding pregnancy (35.5 versus 28.9%) and implantation rates (19.3 versus 17.1%) were not significantly different. There was also no significant difference in maternal age (33.0 ± 4.3 versus 33.5 ± 4.1 years).

Discussion

In this prospective multicentre study, the possibility of selecting a maximum of three pronuclear-stage oocytes with a presumable high implantation potential for further culture and transfer was evaluated. The selection of only a limited number of pronuclear stages for subsequent embryo transfer (≤3) is a requirement of current German national legislation. This study differed from all previous investigations, as it is the first to be

Table II. Clinical pregnancy and implantation rates of pronuclear morphology patterns

Pattern	Cycles with at least one embryo derived from pattern X ^a			Remaining cycles without pattern X ^a			P (Pregnancy/ Implantation)
	Cycles (n)	Pregnancy rate (%)	Implantation rate (%)	Cycles (n)	Pregnancy rate (%)	Implantation rate (%)	
0B	253	37.9	20.5	242	26.4	15.7	< 0.01/< 0.05
0A	58	29.3	15.9	184	25.5	15.6	NS/NS
1	66	30.3	17.2	176	25.0	15.1	NS/NS
2	63	28.6	14.4	179	25.7	16.2	NS/NS
3	44	36.4	23.4	198	24.2	14.0	< 0.01/< 0.025
4	19	21.1	13.9	223	26.9	15.9	NS/NS
5	136	22.8	12.5	106	31.1	20.2	NS/< 0.025

^aCycles involving pattern 0B were excluded for all calculations in both groups (except for calculation of cycles with at least one embryo derived from pattern 0B).

NS = not significant.

Table III. Relationship between pronuclear morphology and maternal age

Pattern	≤35 years n (%)	>35 years n (%)	P
0A	96 (12.9)	43 (11.7)	NS
0B	255 (34.2)	95 (25.8)	< 0.005
1	60 (8.0)	48 (13.0)	< 0.01
2	69 (9.2)	49 (13.3)	< 0.05
3	69 (9.2)	20 (5.4)	< 0.05
4	27 (3.6)	17 (4.6)	NS
5	170 (22.8)	96 (26.1)	NS
Total 1–5	746	368	

Assisted reproduction technique results

	≤35 years		>35 years	
	≥1 0B	No 0B	≥1 0B	No 0B
Cycles	180	162	73	80
No. of embryos	403	343	183	185
Pregnancy rate (%)	41.7 ^{a,b}	28.4 ^a	28.8 ^a	22.5 ^b
Implantation rate (%)	22.6 ^{b,c}	17.8 ^c	15.8 ^c	11.9 ^b

^aP < 0.05; ^bP < 0.005; ^cNot significant for differences within rows.

Table IV. Relationship between pronuclear morphology and assisted reproduction technique

Pattern	IVF n (%)	ICSI n (%)	P
0A	80 (14.7)	59 (10.4)	< 0.05
0B	152 (27.9)	198 (36.3)	< 0.025
1	66 (12.1)	42 (7.7)	< 0.01
2	51 (9.4)	67 (11.8)	NS
3	42 (7.7)	47 (8.3)	NS
4	19 (3.5)	25 (4.4)	NS
5	135 (24.8)	131 (23.0)	NS
Total 1–5	545	569	

multicentre in nature, and because IVF and ICSI treatment cycles were included. The evaluation was based on a single observation of pronuclear morphology similar to that proposed by others (Tesarik and Greco, 1999) except that pattern 0 was subclassified into pattern 0A (>7 NPB showing an equal/symmetric distribution in each pronucleus) and 0B (≤7 NPB aligned at the contact of the two pronuclei). The results of

this multicentre study clearly showed that transfer cycles involving embryos derived from pattern 0B gave significantly higher pregnancy and implantation rates compared with those cycles in which selection of pattern 0B for transfer was not possible. In contrast to pattern 0B, transfer cycles with or without pattern 0A showed no difference with regard to pregnancy and implantation rates. Hence, these data imply that the subclassification of pattern 0 into 0A and 0B is a useful selection criterion.

In the current study, transfer cycles involving at least one embryo with pattern 3 versus transfer cycles without pattern 3 gave significantly higher pregnancy and implantation rates. As the primary aim of the study was the selection of pronuclear morphology pattern 0, the number of cycles involving pattern 3 are rather small and cannot be compared with transfer cycles with pattern 0B. It cannot be excluded that pattern 3 pronuclear-stage patterns can develop into pattern 0B with time however, and this point deserves further attention.

Another interesting point with regard to the subclassification of pattern 0 into 0A and 0B is embryo quality. Pattern 0A-derived embryos exhibited on day 2 an embryonic score comparable with that of pattern 0B, and significantly different from patterns 1–5. However, despite the higher embryonic score, transfers involving pattern 0A gave no higher implantation or pregnancy rates compared with transfers without pattern 0A. This suggests that a possible selection based on embryo quality on day 2 would not necessarily guarantee selection of embryos with the best implantation potential. In other words, at this stage of embryo development there are good quality embryos (derived from pattern 0B) with a high implantation potential, and also good quality embryos with a lower implantation potential (derived from pattern 0A). Consequently, embryo selection should not rely only on embryonic morphology, and other evaluation criteria such as pronuclear morphology or the developmental progression of the embryos must also be considered (Huisman *et al.*, 2000; Racowsky *et al.*, 2000; Shapiro *et al.*, 2000).

During the course of this study, another scoring system was published (Scott *et al.*, 2000) which is in fact a revision of the original zygote scoring system established previously by the same group (Scott and Smith, 1998). The revised system is mainly defined by alignment, number and distribution of

NPB. The different zygote morphology patterns were classified as Z1, Z2, Z3 and Z4. Scott and co-workers showed that Z1-scored zygotes possess a high potential to develop to blastocysts on days 5–6 and that these blastocysts exhibited a high implantation potential. Patterns different from Z1 showed significantly lower blastocyst formation rates and implantation rates. Interestingly, pattern 0B corresponds to zygote morphology Z1, patterns 0A and 1 correspond to Z2, and Z3–Z4 (Scott *et al.*, 2000) may be compared with patterns 2–5 classified by others (Tesarik and Greco, 1999) and by the current study. The results from the current prospective multicentre study correlate well with the data presented elsewhere (Scott *et al.*, 2000) in that preselection by a zygote score can give high implantation rates, although in the current study no double selection was possible by day 3 morphology. However, in contrast to results of the previously cited study (Scott *et al.*, 2000), a difference was found in the pattern distribution with regard to age and assisted reproduction technique. Therefore, the approach of pronuclear selection is a good option for countries with legal restriction of embryo selection at the blastocyst stage. However, from the patient's viewpoint, a better strategy would be pre-selection of pronuclear morphology patterns 0B/Z1, followed by selection of the best embryos derived from these pronuclear stages on the day of transfer, as proposed by others (Scott *et al.*, 2000). This treatment option offers undoubtedly the best basis towards single embryo transfer without compromising pregnancy rates.

The current study revealed that for patients with advanced maternal age (>35 years) significantly fewer pronuclear morphology pattern 0B were available for further culture and transfer. It should be noted that this analysis did not include all day 1 pronuclear oocytes, but only those selected for further culture and transfer. The reason for choosing the age of 35 years was due to an existing guideline in Germany, according to which only two embryos should be transferred in patients aged ≤35 years, and three embryos in those aged >35 years. A similar evaluation in choosing a cut-off at 38 years has already been made for 262 transfer cycles (Wittmer *et al.*, 2000). These authors found no significant difference for pattern 0 (0A + 0B) with regard to maternal age, although a trend was detectable towards reduced numbers of pattern 0 (39.3 versus 52.3%) in older patients (aged >38 years). The higher number of transfer cycles in this study (495 versus 262) may explain why statistical significance was reached. The current data also show that the implantation rate of cycles with pattern 0B is lower for older patients (>35 years; 15.8%) compared with younger patients (≤35 years; 22.6%). These differences were not significant, but such a trend shows that in older patients the success of implantation is also influenced by other factors.

The current study reports a significant difference between IVF and ICSI cycles with regard to the number of pattern 0B for further culture and transfer. In ICSI cycles, more pattern 0B were available compared with IVF cycles (36.3 versus 27.9%; $P < 0.025$), and this phenomenon may be explained by the difference in the course of development which was reported to be accelerated after ICSI (Nagy *et al.*, 1998; Sakkas *et al.*, 1998). Once the pronuclei are formed, further changes

occur in the nucleus which involve polarization of chromatin (Van Blerkom *et al.*, 1995) and NPB (Tesarik and Kopecky, 1989; Payne *et al.*, 1997). NPB are markers for chromosomes hosting rDNA-genes (Goessens, 1984; Tesarik and Kopecky, 1990), and consequently pattern 0B/Z1 characterizes the most advanced stage of nuclear polarization, which is also part of embryonic axis formation in the zygote (Edwards and Beard, 1997). Zygotes reach this stage earlier after ICSI than after IVF. All centres participating in this study usually screened the pronuclear stages at 16–20 h after insemination of oocytes, independently of the type of assisted reproductive technique used. It may be worthwhile exploring whether the difference observed would still exist if zygotes derived from IVF were to be screened later (18–20 h) than those from ICSI (16–18 h).

In conclusion, the current study provides further evidence that pronuclear morphology is an important addition to current assisted reproduction technique practice. In countries with legal restrictions of embryo selection, pronuclear morphology screening appears to be a useful and easy technique. In all other countries, pronuclear morphology offers an additional screening method which, in combination with extended embryo culture and a second selection based on blastocyst evaluation, represents a major step towards single embryo transfer.

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