Articles

Does pronuclear morphology and/or early cleavage rate predict embryo implantation potential?

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After completing her basic education, Dr Lundqvist started clinical work at the department of Pathology, University Hospital, Uppsala. At the same time, she pursued research on neuro-endocrine gastro-intestinal tumours, and in 1987 defended her dissertation entitled "Gastrointestinal endocrine cells and carcinoids" which reported a histopathogenetic study with a comparison of silver-staining and immunohistochemical characteristics. She continued her research at the Ludwig Institute for Cancer Research in Uppsala, culturing neuro-endocrine tumours and conducting molecular investigations. Her career in assisted reproductive technology began in 1990. She currently works as a laboratory director at the Centre for Reproduction, University Hospital, Uppsala. Her research interests involve the oocyte and embryo, its morphology and development, primarily identifying characteristics by which the "right" embryo can be recognized.

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Abstract

A total of 340 patients referred for in-vitro fertilization was included in a retrospective, comparative study in which zygotes were studied regarding alignment and polarization of nucleolar precursor bodies (NPB) and also early cleavage in relation to implantation and pregnancy rates for the 680 transferred embryos. At assessment of the pronucleus 18–19 h after sperm injection, NPB were checked for alignment/polarization. Twenty-six hours after sperm insemination the zygotes were assessed for early cleavage. At embryo transfer the two embryos with the best morphological score, irrespective of polarization and early cleavage, were selected for transfer. The overall rate of positive HCG tests 17 days after embryo transfer was 42% and the implantation rate 23%. Fourteen percent of the patients received two embryos with polarized NPB, with a positive HCG test of 51%. Embryo transfer with early-cleaved embryos was carried out in 21% of the cycles, with a pregnancy rate of 45%. Embryos with polarized NPB and/or early cleavage were transferred in 34% of the cycles, with a pregnancy rate of 51%, compared with a pregnancy rate of 38% when none of the embryos fulfilled these criteria (P-value 0.02). In this study the pregnancy rate was significantly higher when one or two embryos were polarized and/or early cleaved. It is concluded that in a cohort of morphologically good embryos, assessment for alignment/polarization of NPB and/or early cleavage can, together with conventional morphological criteria, serve as a simple non-invasive method for selection of embryos with high implantation potential.

Keywords: *: early cleavage, human IVF, implantation rate, nucleolar precursor bodies, pronuclear morphology*

Introduction

In connection with in-vitro fertilization (IVF), every possible effort should be made to find the "right" embryos for uterine embryo transfer with the aim of achieving a maximal chance of pregnancy and at the same time avoiding the risk of multiple childbirth. Multiple pregnancy is known to entail a risk of premature birth, involving an increased risk for the child, and also necessitates expensive hospital care (Berg *et al*.,1999; Templeton and Morris, 1998). In clinical practice the embryos are selected for embryo transfer according to the morphological score. Only non-invasive methods of selection are of value in the clinical work.

Several embryo-scoring systems have been presented. One of them is an evaluation of the embryo quality and embryo developmental rate, by a calculation of quality and expected growth rate for development (Cummins *et al*., 1986). An overall embryo quality score based on the amount of fragmentation and the developmental rate was proposed by Puissant and co-workers and an average morphology score by Roseboom and co-workers (Puissant *et al*., 1987; Roseboom *et al*., 1995). It is now well known that embryos with symmetrical, well-defined mononucleated blastomeres, without anuclear fragments, which have reached the 4-cell-stage on day 2 after ovum retrieval, have the best implantation potential (Ziebe *et al*., 1997).

Other methods of embryo selection, based on pronuclear formation, orientation and size and also polar-body extrusion and placement, in relation to the development of the zygote and subsequent development into an embryo have been presented. These techniques are not easy to incorporate in the laboratory routine work, since they require special microscope equipment

and are time-consuming (Garello *et al*., 1999; Payne *et al*., 1997; Van Blerkom *et al*., 1995).

However, better selection criteria are still needed in order to improve the implantation and pregnancy rates. The possibility of selecting embryos with high implantation potential permits transfer of a reduced number of embryos.

Nuclear precursor bodies (NPB) are intracellular structures, which appear after fertilization and move around and coalesce within the pronuclei and later transform into nucleoli. Among other indicators, characterization of the pronuclear stage morphology has been reported to be of value as an additional and important tool for prediction of embryo development (Tesarik and Kopecny, 1990; Payne *et al*. 1997; Scott and Smith, 1998; Tesarik and Greco, 1999; Tesarik *et al*., 2000).

The early cleavage rate of the embryo 25 h post-fertilization has also been proposed as a simple method for selection of the embryos with the highest potential implantation and pregnancy rates (Shoukir *et al*., 1997; Scott and Smith, 1998). The aim of this study was to compare the implantation and pregnancy potentials of embryos with aligned and polarized NPB and/or an early cleavage rate, with those of embryos not fulfilling these criteria.

Materials and methods

The study comprised 340 patients referred to our clinic for IVF during May 1999 to March 2000. The patients were not selected for age (mean 32.8 years, range 23–41) or cause of infertility. Most commonly the infertility was caused by tubal disease, endometriosis, anovulation or male factors, or was unexplained. Ovarian stimulation was achieved with the long protocol. After down-regulation with a gonadotrophin-releasing hormone (GnRH) agonist (buserelin or nafarelin nasal spray), the ovaries were stimulated with recombinant follicle stimulating hormone (Puregon; Organon, Oss, The Netherlands or Gonal-F; Serono Laboratories, Aubonne, Switzerland). Human chorionic gonadotropin (HCG) was administered when at least three follicles measured >18 mm. Transvaginal oocyte retrieval was performed 36–38 h after the HCG injection. Included in the study were treatments with both conventional IVF and intracytoplasmic sperm injection (ICSI) with both ejaculated and testicular spermatozoa.

For oocyte culture a standard protocol was followed: the oocytes were first cultured all together in 750 µl IVF culture medium overlaid with paraffin oil (both from MediCult a/s, DK-40 Jyllinge, Denmark) in a centre well dish. For conventional IVF, sperm insemination (2.5 x 10 5 / 750 µl) was performed 3–5 h after ovum retrieval.

In cases of ICSI the oocyte–cumulus complex was gently removed enzymatically with hyaluronidase and mechanically by gentle pipetting. The ICSI procedure was the same as described earlier (Fishel and Symonds, 1993).

Assessment regarding pronuclei was made 18–19 h after sperm insemination and the zygotes were placed in individual 20 µl IVF-medium droplets under oil, in order to follow the development of each zygote/embryo separately. In connection with the check for fertilization, NPB were counted and observed for alignment and polarization according to a procedure described by Tesarik and co-workers and Scott and co-workers, using an inverted microscope (Nikon LaboPhot 300, Hoffman equipment) with a final magnification of 200–400 times (Tesarik and Greco, 1999; Scott *et al*., 2000). For practical reasons, the time for assessment of pronuclear morphology followed our routine procedure. From Tesarik's original classification as pattern 0, only zygotes with both pronuclei containing aligned/polarized NPB were included as aligned/polarized, but not the pronucleus containing NBP dispersed in the pronucleus due to difficulty in separating them from pattern 1 (Tesarik and Greco, 1999). Twenty-six hours after sperm insemination the oocytes were checked for early cleavage (Shoukir *et al*., 1997).

On day 2 or 3, depending on the day of ovum retrieval, the embryos were given a morphological score on the basis of a standard scoring system, i.e. assessment of the developmental rate, percentage of anuclear fragments, equality and clarity of the blastomeres, and the filling-out under the zona pellucida. The blastomeres were also checked for mono or polynucleation. The two embryos with the best morphological score, irrespective of polarization and early cleavage, were selected for transfer. However, if several embryos had the same morphological/developmental score and some of these showed aligned/polarized NPB and/or early cleavage, those embryos were selected for transfer. Pregnancy was defined as a positive urinary HCG test 17 days after embryo transfer, and clinical pregnancy as implanted embryos, diagnosed by a fetal heart beat as observed by transvaginal sonography.

As this was a retrospective study in which an evaluation of the pronuclear morphology and a check for early cleavage were performed in addition to use of standard selection criteria for embryo transfer, no specific approval by an institutional ethical review board was needed.

Statistical analysis was performed by a χ^2 test using a Statview computer program.

Comparisons were made between different groups regarding alignment/polarization (both embryos with polarization, versus one polarized and one not, versus neither with polarization) and cleavage (both embryos with early cleavage, versus one cleaved and one not, versus neither with cleavage) and also between sub-groups regarding similar criteria.

Results

This study comprised 196 conventional IVF treatment cycles and 144 ICSI cycles with a mean oocyte retrieval of 11.5 oocytes per cycle (range 1–40). The overall pregnancy rate obtained from the tests performed 17 days after embryo transfer was 42%. For conventional IVF and ICSI it was 43.4% and 40.3% respectively. The overall implantation rate was 23.1% (157/680). For conventional IVF and ICSI it was 25.8% and 20.1% respectively. Altogether, 63.7% of the patients received two embryos which, irrespective of early cleavage or alignment/polarization of NPB, had a good morphological score and were cleaved to four or eight symmetrical mono-nucleated blastomeres without fragmentation on days 2 and 3 respectively, after ovum retrieval.

Table 1. Pregnancy rates for pre-embryos differing in pronuclear morphology and early cell division

	P/P[a]	*P/NP*[b]	*NP / NP*[c]	*C/C*[d]	*C/NC*[e]	*NC / NC*[f]
Positive serum HCG	24/47	43/99	70/186	32/71	43/90	52/129
% positive serum HCG	51.1	43.4	37.6	45.0	47.8	40.3
% implantation rate	25.5	21.7	18.8	22.5	23.9	20.1

[a–f]Two embryos transferred: [a] both with polarized NPB, [b] one polarized and one not, [c] neither with polarization, [d] both with early cleavage, [e] one cleaved and one not [f] and neither with cleavage.

Table 2. Pregnancy rates in cycles in which embryos with polarized NPB and/or early cleavage were transferred compared with those in cycles in which the transferred embryos did not fulfil these criteria

	Polarized NPB and/or EC[a]	*No polarized NPB or EC*[b]	*P-value*
Positive serum HCG	58/114	85/226	
% positive serum HCG	50.9	37.6	0.024
% of cycles	33.5	66.5	
% implantation rate	24.6	19.1	0.019

[a,b]Embryos transferred: [a]one or two with polarised NPB and/or early cleavage, [b]no embryo with polarised NPB and/or early cleavage

Out of the total of 3946 oocytes studied, 2803 (71%) had two visible pronuclei and were included in this study of the pronuclear morphology/early cleavage rate. A further 495 zygotes (18%) showed aligned/polarized NPB in both pronuclei and of these, 275 (56%) developed to embryos with a good morphological score. Regarding early cleavage, 604 (22%) zygotes were cleaved 26 h after sperm insemination and 438 (72.5%) of these showed good morphology on day 2 or 3 after ovum retrieval.

Fourteen per cent of the patients received two embryos with aligned/polarized NPB. In this group 51% became pregnant (**Table 1**). Transfer of two embryos, both with early cleavage, was achieved in 21% of the cycles, with a pregnancy rate of 45% (**Table 1**). A difference was observed between conventional IVF and ICSI groups regarding early cleavage, 14% more embryos showing early cleavage in the ICSI group. With regard to NPB and early cleavage nine different combinations for embryo transfer could be identified. For example only 14 patients received two embryos that had both polarized NPB and early cleavage. Out of these eight became pregnant (57%) with an implantation rate of 35.7%. These groups, however, were too small to be used for statistical analysis and therefore the groups were combined for further analysis and presented in **Table 2**. In 34% of the cycles the patients received one or two embryos that displayed alignment/polarization of the precursor bodies and/or early cleavage (**Table 2**). In this group, the pregnancy rate was 51% (58/114). In 66% of the cycles there were no embryos with aligned/polarized NPB or with early cleavage available for transfer. In this group the pregnancy rate was 38% (85/226), which is significantly lower when compared with the group which received one or two embryos with aligned/polarized NPB and/or early cleavage (*P*-value 0.02).

Discussion

Today, the choice of which embryo to transfer is mainly based on the morphological and developmental scores. This is a subjective, microscopical classification system, but it is relatively blunt. In order to decrease the number of multiple pregnancies in the future, the number of embryos transferred has to be decreased and there is therefore a great need for better non-invasive selection criteria that are easy to include in the routine laboratory work. So far, the embryos with the highest morphological score, as judged with our conventional protocol, have been chosen for transfer, with consideration paid mainly to the cleavage rate, percentage number of anuclear fragments and the presence of mononucleated blastomeres. By virtue of the selection criteria applied in this study, with the addition of assessment regarding alignment/polarization of NPB and early cleavage, sub-groups of embryos with polarized NPB and/or early cleavage were obtained. The results of embryo transfer from this group could therefore be compared with those of transfer from the group of embryos selected and transferred according to our conventional scoring method.

The use of pronuclear stage morphology has been claimed by Tesarik and co-workers to be a powerful method for selection of embryos with high implantation potential (Tesarik and Greco, 1999; Tesarik *et al*., 2000). In the present study a tendency towards higher implantation and pregnancy rates was observed when embryos with polarized NPB were transferred. However, not all fertilized oocytes with aligned/polarized NPB

developed into 4-cell embryos with a good morphological score. Nearly half of the embryos that had aligned/polarized NPB were not transferred or frozen, because of a poor morphological score. At the pronuclear inspection at 18–19 h after sperm insemination, two distinct pronuclei were recognized in most of the oocytes, but in occasional zygotes the pronucleus had already started to fuse, and hence assessment regarding NPB alignment/polarization was not possible. In a study using thymidine incorporation for studying DNA synthesis in human zygotes, Balakier and co-workers found that 20 h after insemination 89% of the oocytes had two pronuclei. After 24 h 41% no longer had visible pronuclei and a further 5% had already cleaved to the 2-cell stage (Balakier *et al*., 1993).

Tesarik and co-workers had a different observation time in their studies, ranging from 14–20 h and recommended the best examination time to be 14–18 h after sperm insemination (Tesarik and Greco, 1999; Tesarik *et al*., 2000). In a cohort of zygotes, the optimal time for recognition of NPB alignment/polarization differ, due to the speed of pronuclear development. For practical reasons it was necessary in this study to choose 18–19 h after sperm insemination for assessment of NPB alignment/polarization. Hence, it is likely that the chosen time gave a better possibility of recognizing all the aligned/polarized NPB. Tesarik and Greco (1999) found among 67 treatment cycles that if at least one transferred embryo had polarized NPB 12–20 h after sperm insemination, the pregnancy rate reached 50%, compared to 9% if none of the embryos had polarized NPB. Further, in a retrospective study comprising 380 embryo transfers, Tesarik and co-workers obtained significantly higher pregnancy and implantation rates (44.8% and 30.2% respectively) in a group of patients receiving embryos with NPB, derived from the pattern 0 group in their study, than in a group which received embryos, with NPB, from groups with patterns 1 to 5 (22% and 11.2% respectively) (Tesarik *et al*., 2000). Embryos derived from pattern 0 are, according to Tesarik's classification, associated with a significantly higher rate of development into morphologically good embryos compared to embryos derived from pattern 1–5. Scott and co-workers, using a slight modification of Tesarik`s pronuclear morphology pattern, found a relationship between the aligned/polarized NPB (Z1) pattern and the subsequent ability of the embryo to form blastocysts (Scott *et al*., 2000).

Fourteen per cent of the patients in this study had two embryos with aligned/polarized NPB suitable for transfer and 29% had one embryo with aligned/polarized NPB that was used for transfer. In cycles where embryos with aligned/polarized NPB were transferred, somewhat better pregnancy and implantation rates were obtained compared with transfers with embryos without such NPB, but the marked difference as suggested by Tesarik *et al*. (2000) was not observed. The study reported here suggests that evaluation concerning the pronuclear morphology and alignment/polarization of NPB should nevertheless be considered as an additional non-invasive method for selection of zygotes/embryos with high implantation potential.

Also the timing of the first cell cleavage to the 2-cell stage 25 h after sperm insemination has been considered to be a valuable tool for selection of embryos for transfer in connection with IVF (Shoukir *et al*., 1997). In a study using thymidine incorporation on spare human zygotes, 38% of the zygotes had cleaved to the 2-cell stage 27 h after insemination (Balakier *et al*., 1993). In another study of 143 IVF cycles, early cleavage was observed 25 h post-insemination in 18.9% of the cycles and a significant increase in the pregnancy rate was obtained using these embryos for transfer (Scott and Smith, 1998). In the study reported here, early cleavage was observed 25–26 h after insemination. Forty-seven per cent of the patients received one or two embryos with early cleavage, with a pregnancy rate of 46.4%, compared with 40.3% when none of the embryos showed early cleavage. Different observation times for micro-injected and conventionally inseminated oocytes are recommended. Nagy and co-workers compared ICSI and conventional IVF regarding pronuclear formation and polar-body extrusion (Nagy *et al*., 1998). They concluded that pronuclear formation and the first cell cleavage generally occur 4 h earlier after sperm micro-injection than after conventional IVF, and suggested that different observation times be used depending on the method of fertilization.

In a cohort of oocytes from a patient, the time intervals before fertilization, formation of pronuclei and early cleavage obviously differ between individual oocytes. In a comparison between the ICSI group and the conventional IVF group in the present study, the ICSI group showed a 14% higher cleavage rate on day 1 at the time of assessment, probably as a result of earlier sperm de-condensation and pronuclear formation. The optimal time interval for assessment of pronuclear and early embryo morphology in a cohort of embryos can thus be discussed. It is still not known what are the optimal lengths of time from fertilization of the oocyte to possible NPB alignment/polarization and early cleavage in the case of embryos with high developmental and implantation potential.

In conclusion, we have combined two different proposed methods for selection of human embryos with high implantation potential. Significant differences in implantation rates were found when comparing the groups of patients receiving one or two embryos with aligned/polarized NPB and/or early cleavage with the group that received embryos that did not fulfil these criteria. Thirty-four per cent of this unselected group of women received one or two embryos with aligned/polarized NPB and/or early cleaved embryos. In this group the pregnancy rate was 51%, compared to 38% in the group in which none of the transferred embryos contained aligned/polarized NPB or showed early cleavage. A close examination of the early zygote/embryo morphology can perhaps serve as an alternative to extended, more complicated methods for embryo selection such as blastocyst culture methods. These latter methods are only suitable for patients who have a large number of oocytes to start with, since many embryos may arrest during the culture period. Extended culture methods are also time consuming and more expensive. A better understanding of the cell cycle events taking place during early embryo development can help us to predict the embryo implantation potential more accurately. The results obtained in this investigation suggest that in a cohort of morphologically good embryos, evaluation of early pronuclear morphology and early cleavage rate can serve as an additional simple, powerful and non-invasive method for selection of embryos with high implantation potential.

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