The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: the German experience

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BACKGROUND: Due to improvements in embryo culture, it is now possible to transfer embryos 5 days after oocyte retrieval and IVF/ICSI at the blastocyst stage, giving a better synchronization with the female reproductive tract. In Germany it is illegal to culture more than three embryos. Therefore, there is need for a sufficient selection at the pronuclear (PN) stage to select the best zygotes and exclude those of poor quality. METHODS: A prospective trial was conducted in 168 IVF and ICSI cycles including the size, number and alignment of pronuclei and nucleoli, cytoplasmic halo effect, the presence of vacuoles and granularity of ooplasm. Based on the above criteria, the best zygotes were selected (score <15) for embryo transfer on day 5. Blastocysts were classified in eight grades based on the cleavage speed. RESULTS: A total of 1450 oocytes were collected, of which 1119 reached the pronuclear stage. Of the zygotes (n = 424) selected at the PN stage, 46% achieved the blastocyst stage after 5 days (grade 1–5), 26% the morula stage (grade 6–7) and 28% were arrested (grade 8). The mean zygote score showed a significant positive correlation with the mean blastocyst quality in ICSI, but not in IVF cycles. A cut-off of 15 was calculated for ICSI cycles giving the best discrimination with blastocyst grades (6 versus 7) and number of arrested embryos (23 versus 45%) below and above this cut-off. A total of 33 clinical pregnancies was achieved (20%). Women conceiving had a significantly better mean blastocyst development than those not conceiving. Strong cytoplasmic vacuolization and an extreme or no halo effect had a negative effect on blastocyst development. CONCLUSIONS: The data show that PN stage morphology is related to blastocyst development, but the rate of arrested embryos of almost 30% limits the chance of conception under the conditions of the German Embryo Protection Law.

Key words: embryo selection/human blastocyst/IVF/ICSI/pronuclear stage score

Introduction

In most IVF programmes, embryos are usually transferred to the uterus at the cleavage stage on the second or third day after oocyte retrieval. In the last few years it has been possible to culture embryos to the blastocyst stage due to the development of new sequential serum-free culture media capable of supporting the growth of viable blastocysts (Gardner and Lane, 1997). Blastocyst culture helps to improve the synchronization of the embryo with the female reproductive tract. The implantation rate per cleavage stage embryo transferred has not increased in the last 10 years beyond the range of 12.5–15% (Edwards and Craft, 1990). The implantation rates after blastocyst culture are reported to reach 50% in selected groups of patients (Gardner et al., 1998a, 1998b). High pregnancy rates, however, can only be achieved when a cohort of at least eight normal oocytes is available and selection can be done during the embryo culture and at the blastocyst stage (Bongso, 1999). The high implantation potential of blastocysts means that fewer embryos are required for transfer to achieve clinical pregnancy with a reduction of the incidence of multiple pregnancies.

One of the main problems in a German IVF unit is that selection of embryos after conventional IVF or ICSI has to be performed at the pronuclear (PN) stage, as the German Embryo Protection Law prohibits both the culture of more than three embryos and embryo selection. It is only allowed to culture as many zygotes as will be presumably transferred. Supernumerary zygotes can be cryopreserved or have to be withdrawn. Selection at the PN stage is not only important in Germany with its restrictive legislation, but also in other countries in patients with religious or ethical concerns regarding the destruction of non-selected but viable embryos.

Many studies have proposed a PN scoring system (Payne et al., 1997; Scott and Smith, 1998; Tesarik and Greco, 1999; Wittmer et al., 2000) with predictive value for the implantation rate, but relatively little is known about the development of selected zygotes after days 2 or 3.

The aim of this prospective study was to describe morphological criteria of zygotes that can predict the development of high quality blastocysts and therefore maintain a satisfactory rate of clinical pregnancies even under the conditions of the German Embryo Protection Law. In comparison with previous...
studies, we aimed at a more detailed description of the zygote morphology than has been done before, including the following criteria: number and size of pronuclei, juxtaposition of pronuclei, halo effect, alignment and number of nucleoli separately for both pronuclei, appearance of vacuoles and the appearance of the ooplasm. The use of this scoring system allows selection of those zygotes with favourable developmental potential for further embryo culture, therefore maximizing the chance of implantation.

Materials and methods

Patients

Between July 1999 and April 2000, 168 infertile couples were consecutively included in this prospective trial. Patients were unselected for age, sperm parameters or infertility criteria. In 101 patients, conventional IVF was performed and in 67 cases the ICSI procedure was applied. Couples were included only if at least one oocyte could be collected that showed signs of fertilization after 16 to 18 h (1PN or 2PN stage). All cases of unsuccessful oocyte retrieval or polyspermic zygotes were excluded. All patients underwent the IVF/ICSI programme at the University of Würzburg. The indications for assisted reproduction were one or several of the following: tubal factor (36%), endometriosis (5%), male factor (46%) or idiopathic infertility (17%). The indication for ICSI was male subfertility in all cases. The mean ± SD age of the women was 33.4 ± 4.1 years and that of their partners 36.1 ± 5.3 and did not differ significantly between the IVF and ICSI couples. The mean duration of infertility was 4.2 years.

Ovarian stimulation and sperm preparation

Ovarian stimulation, oocyte recovery, IVF and ICSI procedures were carried out using standard protocols as described previously (Zollner et al., 1996, 2001). Ovarian stimulation was induced by a mild step-down human menopausal gonadotrophin (HMG, Menogon®; Ferring, Kiel, Germany) or FSH (Gonal F®; Serono, Unterschleißheim, Germany; Puregon®, Organon, Oberschleißheim, Germany) protocol starting on day 3 of the cycle after pituitary down-regulation by 10 000 IU of HCG (Pregnesin®; Serono) was administered late in the evening of the day during which the mean diameter of the dominant follicle reached 18 mm after 6–7 days of a steady rise in serum oestradiol concentrations. Oocyte recovery was performed by the vaginal route 34–36 h after HCG administration.

All native semen samples and samples after preparation were evaluated manually according to published guidelines (World Health Organization, 1992) for volume, total sperm count, concentration, leukocytes, motility, vitality and morphology. The ejaculates were prepared by a PureSperm (Nidacon, Sweden) cushion centrifugation and a swim-up procedure. After several centrifugation and washing steps the sperm preparation was incubated for 30–60 min at 37°C for the swim-up.

Zygote and embryo scoring, embryo culture

After incubation for 16–18 h, the oocytes were checked for the presence of pronuclei as evidence of fertilization. Regular fertilization was defined as extrusion of the second polar body and presence of two pronuclei. In IVF cycles, the remaining cumulus and corona cells were stripped from the pre-embryos and then each zygote was placed individually into a Petri dish with microdroplets of 5–10 µl of IVF™ medium (Scandinavian IVF, Gothenburg, Sweden) overlaid with OvoiT™ (Scandinavian IVF). The zygotes were scored on an inverted microscope at magnification of ×400. Photodocumentation of the embryos were performed during this inspection and on days 3 and 5.

All cells were judged prospectively in detail for the following criteria: alignment and number of pronuclei and nucleoli, halo effect, size of pronuclei, appearance of vacuoles and the appearance of the ooplasm. Zygotes then were assigned a cumulative score as shown in Table I. For alignment and number of nucleoli, each pronucleus was evaluated separately. This assessment required the changing of focus during observation until the whole volume of both pronuclei could be inspected. With the use of an injection pipette to turn the oocyte around, an exact evaluation of the juxtaposition of pronuclei was achieved. The zygote score was calculated as a cumulative score of all items. The best score thereby was 10 and the worst score was 31 for 2PN stages and 37 for 1PN stages. Embryos with only one pronucleus were included in this scoring system but were not selected for culture except when no other pre-embryos were available. Figure 1 shows some examples of zygote evaluation. According to the patients’ age, individual history or desire, two or three zygotes with the lowest score were selected for further culture and transfer. A mean zygote score was calculated as the sum of all selected zygotes per number of selected embryos. For each PN stage embryo, the subsequent development at day 3 and the blastocyst development on day 5 was evaluated. Embryos were cultured separately to allow for individual comparisons at different times during the culture.

IVF™ (Scandinavian IVF) medium was used for all oocyte cultures. Oocytes were cultured at 37°C in 5% CO2 at pH 7.4. At 4 h after recovery the oocytes were inseminated with 10 000–20 000 motile sperm, or, in the case of severe andrological subfertility, the ICSI procedure was performed as described previously (Zollner et al., 2001). After injection, each oocyte was transferred separately in a microdroplet of fresh IVF medium. Fertilized oocytes in excess of two or three were cryopreserved or withdrawn. The selected embryos were transferred to G1.2™ medium (Scandinavian IVF), kept in culture for another 48 h and were then placed into G2.2 medium (Scandinavian IVF). Cleavage rate and embryo morphology were evaluated again at that time (day 3). If the embryos showed at least six blastomeres after 48 h they were placed into G2.2 medium. Embryos with fewer than six blastomeres were cultured for a further 4 h in G1.2 medium and then checked again. If the 6-cell stage was achieved, the embryos were transferred to fresh G2.2 medium.

The scoring system used on day 3 was based on the number of blastomeres and the proportion of anucleate fragments inside the embryo, allowing calculation of the embryo score (ES) and cumulative embryo score (CES) (Steer et al., 1992). The embryos were graded as follows: grade 4, equal-sized symmetrical blastomeres; grade 3, uneven blastomeres with <10% fragmentation; grade 2, 10–50% blastomeric fragmentation; grade 1, >50% blastomeric fragmentation or pronucleate single cell embryos. The morphological grade of the embryo was then multiplied by the number of blastomeres. The cumulative scores of all embryos selected at the PN stage per patient were calculated to obtain the cumulative embryo score (CES). The mean CES is calculated as CES divided by the number of transferred embryos.

Blastocyst culture, embryo transfer and pregnancy assessment

After 2 days of culture in G2.2 medium, blastocyst formation was evaluated. The assessment of embryo viability was based on embryo morphology and cleavage speed (Bongso, 1999): 1 = fully expanded blastocyst (distinct inner cell mass, trophoectoderm and blastocoel, thin zona pellucida, fully expanded diameter); 2 = expanding blastocyst (distinct inner cell mass, trophoectoderm and blastocoel, thin zona pellucida, substantial increase in embryo diameter, but not...
Blastocyst culture after selection at the pronuclear stage

Table I. Zygote scoring system

<table>
<thead>
<tr>
<th>No. of pronuclei</th>
<th>Alignment of nucleoli in PN 1</th>
<th>Alignment of nucleoli in PN 2</th>
<th>No. of nucleoli in PN 1</th>
<th>No. of nucleoli in PN 2</th>
<th>Appearance of vacuoles</th>
<th>Appearance of ooplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2PN</td>
<td>Strong together</td>
<td>Row</td>
<td>3–5</td>
<td>&gt;5</td>
<td>No vacuoles</td>
<td>Homogeneous</td>
</tr>
<tr>
<td></td>
<td>Near by</td>
<td>Beginning row</td>
<td>&gt;5</td>
<td>&gt;3</td>
<td>Light</td>
<td>granulated</td>
</tr>
<tr>
<td></td>
<td>Disjuncted</td>
<td>Confused</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>Strongly</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Only one pronucleus</td>
<td>Only one pronucleus</td>
<td>No nucleoli or no pronucleus</td>
<td>No nucleoli or no pronucleus</td>
<td>Strongly vacuolized</td>
<td>–</td>
</tr>
<tr>
<td>1PN</td>
<td>Only one pronucleus</td>
<td>Only one pronucleus</td>
<td>No nucleoli or no pronucleus</td>
<td>No nucleoli or no pronucleus</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

PN = pronucleus.

fully expanded); 3 = early blastocyst (distinct inner cell mass, trophoblast and blastocoele); 4 = late cavitating embryo with >50% blastocoele (distinct blastocoele, inner cell mass and trophoblast not laid down); 5 = early cavitating embryo with <50% blastocoele (first signs of blastocoele); 6 = compacted embryo; 7 = compacting embryo; 8 = arrested embryo.

A maximum of three embryos were transferred into the uterus 5 days after oocyte recovery. The luteal phase was routinely supported by vaginal progesterone (Crimone®; Serono) and by at least one injection of 5000 IU HCG.

A single serum β-HCG measurement was performed 14 days after embryo transfer. Clinical pregnancy was defined as presence of a gestational sac on ultrasound scan.

Statistical analysis

Statistical analysis was performed using SPSS 10.0. As not all parameters were normally distributed, results were expressed as median and range. The Mann–Whitney test was used to compare differences between groups while the χ² analysis was used to compare proportions. Multiple correlations were calculated using Spearman’s correlation. P < 0.05 was considered significant.

Results

Oocyte, zygote, embryo and blastocyst assessment

The oocyte characteristics and the fertilization data of the 168 patients included in this study are presented in Table II, showing the data from IVF and ICSI cycles separately. A total of 1450 oocytes were retrieved; 1119 were fertilized and reached the PN stage after 16–18 h. All oocytes with no fertilization and polyspermic zygotes were excluded. Only the data of the pre-embryos selected at the PN stage are presented here as the surplus zygotes had to be withdrawn or cryopreserved. A total of 424 zygotes was selected, five of which were at the 1PN stage. In 97 couples three pre-embryos were selected, in 62 couples two pre-embryos and in nine couples only one embryo was available.

In ICSI cycles significantly more oocytes were collected than in IVF cycles, but the fertilization rates were similar. Although significantly more embryos were transferred in ICSI than in IVF cycles, the pregnancy rates were not different. The mean zygote scores, mean and cumulative embryo scores and blastocyst data are given in Table II showing the data from IVF and ICSI cycles separately. There were no significant differences between conventional IVF and microinjection.

All morphological parameters of the PN embryos have been correlated with the rate of blastocysts and arrested embryos. If the zygotes showed strong vacuolization, 71% of them developed into arrested embryos versus only 27% of the zygotes with no or light vacuolization (P = 0.019). The median grade of blastocyst development was 8 (5–8) in strong vacuolized zygotes and 6 (1–8) in light or not vacuolized embryos (P = 0.019). The blastulation rate was 47% in embryos with normal or light halo effect versus only 29% in those with extreme or no halo effect (P = 0.041). The rate was 49% when pronuclei of equal size were apparent but only 36% when the pronuclei were of unequal size (P = 0.015). None of the zygotes with one pronucleus developed into a blastocyst. The blastulation rate of the 2PN stages was 47%. There were no further significant differences between the other morphological parameters of the pronucleate embryos and the rate of blastocysts and arrested embryos.

The mean zygote score was significantly lower in elective than in non-elective cycles after IVF as well as after ICSI (Table III). Patients were allocated to the elective transfer protocol if more than two or three zygotes were available, to the non-elective protocol if no surplus zygotes were available.

Of the zygotes (n = 424) selected at the PN stage, 46% achieved the blastocyst stage after 5 days. The distribution into blastocyst grades was as follows: (1) fully expanded blastocyst 2%, (2) expanding blastocyst 6%, (3) early blastocyst 12%, (4) late cavitating embryo 12%, (5) early cavitating embryo 14%. The morula stage was achieved by 26%, including (6) compacted embryo 17%, (7) compacting embryo 9%. Finally, 28% of the zygotes arrested during their embryonic development (8). These frequencies were not significantly different after IVF and ICSI.

A significant correlation between the mean zygote score
and the mean blastocyst development was found in ICSI ($P = 0.041$) but not in IVF cycles (not significant). For the zygote score, a cut-off value of 15 could be calculated for ICSI cycles with serial Mann–Whitney tests giving the best statistical power for the prediction of blastulation. If the PN score was >15, the blastocyst grade was significantly reduced (grade 7) compared with zygotes with a score ≤15 (grade 6, $P = 0.012$). With a score below this cut-off, significantly fewer embryos arrested during the cleavage stages than with a score >15 (Table IV). If the mean zygote score was >15, the clinical pregnancy rate was 14%, compared with 22% with a mean zygote score ≤15 (not significant). The zygote score also correlated with the female age ($P = 0.017$) and the number of oocytes collected ($P = 0.022$).
Pregnancy data

The overall pregnancy rate (PR) was 20% and was similar in IVF and ICSI cycles (Table II). All selected embryos were transferred, even if they were arrested at the cleavage stage. In 168 cycles studied, 33 clinical pregnancies occurred, of which nine ended as abortions, and six pregnancies were twin pregnancies, giving an overall implantation rate of 9.2%. The overall PR in non-elective (n = 61) embryo transfers was 15% (one embryo: PR 22%; two embryos: PR 13%; three embryos: PR 14%). In elective transfers (n = 107), it was 22% (two embryos: PR 21%; three embryos: PR 24%). Table III shows the fertilization and pregnancy data in elective and non-elective transfers.

There was no significant difference between women conceiving and not conceiving in terms of mean age, semen quality, number of transferred embryos and fertilization rate (Table III). Women achieving conception had a significantly better mean blastocyst grade (4 versus 6, \( P = 0.001 \)) than women who did not.

None of the patients, with transfer of grade 6–8 embryos
only, conceived. In 12 patients, only arrested embryos were transferred, resulting in no clinical pregnancy. In all successful cycles at least one blastocyst was transferred.

**Discussion**

In this prospective study, morphological parameters of PN stage pre-embryos that are predictive for blastocyst development were described. The zygote score correlated with subsequent blastocyst development in ICSI cycles. We did not observe a correlation in IVF cycles, perhaps due to different cleavage speeds of IVF and ICSI zygotes. With a mean PN score >15, a poor blastocyst development and a low pregnancy rate are to be expected. Those zygotes should not be selected unless they are the only ones available. Furthermore, it could be shown that the clinical pregnancy rate after selection and elective embryo transfer exceeds that after non-elective embryo transfers (not statistically significant), independent from the number of embryos transferred. As expected, the mean zygote score was significantly higher in those cycles where only two or three zygotes were present and therefore no selection was possible as in the cycles with supernumerary PN stages. We also correlated all morphological parameters at the PN stage with the blastulation rate and the rate of arrested embryos. It could be demonstrated that strong vacuolization or an extreme or no halo effect are negatively predictive for blastocyst development. The blastulation rates were also lower when the pronuclei were unequally sized.

Several authors have attempted to find selection criteria at the PN stage that are of similar predictive value for the implantation rate as those of a day 2 or day 3 embryo or a blastocyst. Scott and Smith published a new scoring system for zygotes, taking into account the alignment of pronuclei, the positioning of the nucleoli and the cytoplasm (Scott and Smith, 1998). Their score also included the time factor of embryo development and because the selection has to be done at the PN stage.

Scott and Smith developed a PN score that can be calculated at the PN stage. Initially, only the alignment of nucleoli at the junction of the two pronuclei was considered as a selection criterion for embryo transfer (Wright et al., 1990), but it could be shown that the appearance of the cytoplasm is also important. Tesarik and Greco developed a PN score that can be calculated by a single observation to predict the potential for implantation (Tesarik and Greco, 1999). They defined an optimal PN morphology as pattern 0. The rate of arrested embryos until the 4–8-cell stage was only 8.5% in pattern 0 embryos. Their score, however, contains just the number and arrangement of nucleoli. In contrast, our score is more detailed as we were aiming at the definition of morphological parameters correlating with the development of blastocysts. Our morphological description of the PN stage pre-embryos can be translated into a scoring system which may be evaluated by an experienced embryologist in ~30 s, so that it can be integrated in the routine work. Our score may also help to decrease the occurrence of multiple pregnancies without affecting the overall success rate, as we did not see a significant difference in pregnancy rates after the transfer of two versus three embryos. So in younger patients the choice of only two embryos may not affect the success rate.

As zygote morphology alone does not consistently identify embryos with high implantation potential, a graduated embryo score was developed (Fisch et al., 2001) combining PN morphology, first cleavage and day 3 morphology. Embryos with a high score had a significant better blastocyst formation than embryos with lower scores. In this scoring system, only the nucleolar alignment along the PN axis was taken into consideration. Another recent study found that not only the alignment of nucleoli but also the halo effect is important for further embryonic development (Salumets et al., 2001). In this study (Salumets et al., 2001), embryos derived from halo-positive zygotes had significantly better morphology compared to halo-negative-derived embryos.

It is generally accepted that the implantation and pregnancy rates can be increased when the selection of embryos can be done at the blastocyst stage. In Germany, however, selection of embryos and blastocysts is illegal. A significant proportion of fertilized oocytes is affected by chromosomal abnormalities, by deficient activation of the embryonic genome or other disturbances. Fluorescent in-situ hybridization diagnosis of human embryos in poor prognosis patients revealed that 57% were chromosomally abnormal (Magli et al., 1998). The natural selection occurring during development from gamete to blastocyst can take place in vitro (Menezo et al., 1997). Although development to the blastocyst stage cannot guarantee chromosomal normality, the majority of the embryos failing to continue to develop in extended culture show multiple aneuploidies for chromosomes X, Y, 16, 18 and 21 (Jones and Trounson, 1999).

Gardner et al. observed blastulation rates as high as 47%
(Gardner et al., 1998b) and 66% (Gardner et al., 1998a), but it was not possible to predict blastocyst development on the basis of embryo morphology on day 3. This observation is confirmed by the findings of our study. We found a positive correlation between PN and blastocyst morphology, but from a cohort of zygotes we could not identify those two or three that will develop into good quality blastocysts with certainty. In our study we found a blastulation rate of 46% after selection but only 2% of blastocysts were fully expanded. Only patients with the transfer of at least one blastulated embryo conceived, but none of the patients with transfer of only morulae or arrested embryos. If it is assumed that only blastocysts led to pregnancies, the implantation rate per transferred blastocyst would be 20%.

It has been postulated that implantation rates of day 2 embryos is comparable with that of blastocysts when the selection is done at the PN stage (Banerjee et al., 2000). It was proposed that prolonged selection of embryos in vitro until the blastocyst stage eliminates paternal chromosomal anomalies as only 45% of the fertilized oocytes reach the blastocyst stage, but they found that phenotypic manifestation of paternal genomic abnormalities does not occur prior to implantation. Nevertheless, blastocyst culture will facilitate the assessment of embryonic development as the genome is activated at the 8-cell stage, whereas the metabolism of the cleavage stage embryo reflects that of the oocyte (Gardner et al., 1998a).

For patients who produce only two or three embryos to begin with, the extension of culture beyond day 2 or 3 offers no additional advantage as selection will not be possible at any stage. Under the German Embryo Protection Law, each couple is allowed to produce only two or three embryos as only two or three zygotes are allowed to be cultured. We did not compare day 3 versus day 5 transfer after selection at the PN stage, but the pregnancy rates after blastocyst transfer were not higher than or equal to those during the early years when the embryo transfers were performed on day 2 or 3 in our programme (~20%). In summary, we think that under the legal conditions in Germany, blastocyst culture cannot improve pregnancy rates, in spite of a positive correlation between some parameters of PN and blastocyst morphology, when the selection has to be done as early as the PN stage. Nevertheless, in cases of repeated implantation failure, extended culture offers the advantage of in-vitro selection and elimination of chromosomally abnormal embryos and may be useful as a diagnostic procedure for embryonic development.

References


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