Journal of in Vitro Fertilization and Embryo Transfer, Vol. 6, No. 1, 1989

Development of Spare Human Preimplantation Embryos in Vitro: An Analysis of the Correlations Among Gross Morphology, Cleavage Rates, and Development to the Blastocyst

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Submitted: June 29, 1988
Accepted: October 13, 1988 (European Editorial Office)

Following in vitro fertilization, the criteria commonly used to select human embryos for transfer are the cleavage rate and gross morphology, the contention being that those embryos which divide more rapidly and have regular, spherical blastomeres are more likely to lead to a pregnancy. In order to assess the validity of this assumption, the development in vitro of spare embryos was investigated. Eggs and embryos were cultured in Earle's balanced salt solution containing 10% heat-inactivated patient's serum, and insemination was performed at 40 hr post human chorionic gonadotropin (hCG). At 82–90 hr post hCG, up to four embryos were transferred. Any spare embryos were cultured in the same medium for up to 6 days and scored daily for cell number and morphology using a "quality" scale of 4–1 according to degree of fragmentation and shape of the blastomeres. Of 317 fertilized eggs, 55 (17%) developed to the fully expanded blastocyst stage. The remaining embryos ceased development at the one-cell (6; 2%), two-cell (49; 15%), four-cell (110; 35%), eight-cell (61; 19%), and cavitating morula (36; 11%) stages. The relationship between developmental arrest and gross morphology is discussed.

KEY WORDS: in vitro fertilization (IVF); cleavage rate; embryo morphology; blastocyst; developmental arrest; embryo quality; pregnancy prediction.

INTRODUCTION

The rate of successful pregnancy following in vitro fertilization (IVF) remains only 13% per embryo transferred (1). This low success rate can be attributed to uterine and/or embryonic factors. While poor uterine receptivity cannot be excluded in cycles that do not result in pregnancy, the fact that relatively few triplet or quadruplet pregnancies result from the transfer of three or four embryos suggests the existence of differential embryo "quality."

This variability between human embryos produced in vitro in both the quality and the rate of development (2–5) may reflect inherent differential embryo viability and/or suboptimal in vitro procedures and culture conditions. It is clear that differential embryo viability exists in natural conception (6–9), but the use of ovarian, hyperstimulation means that IVF cycles usually produce a cohort of embryos, from which the "best" must be selected for transfer. However, evaluation of embryo quality can be of clinical value only if it is noninvasive. Attempts to derive biochemical tests for embryo viability remain preliminary (10,11) and currently the only criteria of assessment available are the cleavage rate and gross morphology (12–14).

In an attempt to examine the validity of these criteria in predicting the potential of human embryos for implantation, they were correlated with the rate of development to the fully expanded blastocyst stage of supernumerary ("spare") embryos cultured in vitro.

MATERIALS AND METHODS

Patient Management

Ovarian stimulation was achieved with a combination of clomiphene citrate (Clomid, Merrell,

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0740-7769/89/0200-0030$06.00/0 © 1989 Plenum Publishing Corporation

Staines, UK), 100 mg daily for 5 days, and human menopausal gonadotropin (hMG; Pergonal, Serono, Welwyn Garden City, UK), 2–6 ampoules daily, as described previously (15). The response was monitored using daily ovarian ultrasound and assays of serum estradiol. Provided that estradiol levels were rising (16), human chorionic gonadotropin (hCG; Profasi, Serono, Welwyn Garden City, UK), 5000 IU, was administered when the leading follicle reached a mean diameter of 17 mm, and ultrasound-directed follicle aspiration performed 34–36 hr later.

Culture Conditions

Eggs were incubated at 37°C in an atmosphere of 5% CO_2 in air, in 1-ml drops of Earle's balanced salt solution (EBS; GIBCO, Paisley, UK) supplemented with 0.11 mg/ml sodium pyruvate (Sigma, St Louis, MO), 1.0% (w/v) sodium bicarbonate (AnalaR; BDH, Poole, UK) 0.02 mg/ml gentamicin (Flow Laboratories, Rickmansworth, UK), 0.06 mg/ml penicillin (Sigma), and 10% (v/v) heat-inactivated patient's serum (EBS + HIS). Drops were overlaid with a thin layer of sterile paraffin oil (BDH). Insemination was carried out at 40 hr post hCG, with 100,000 motile spermatozoa prepared in EBS + HIS using the swim-up technique (17).

After 16–18 hr (56–58 hr post hCG), eggs were examined for the presence of pronuclei. Only those embryos showing two pronuclei as evidence of normal fertilization were included in this study. Fertilized eggs were cleaned of cumulus and corona using a fine glass pipette and transferred to fresh 1-ml drops of EBS + HIS under oil for further incubation at 37°C in an atmosphere of 5% CO_2 in air.

The following morning (80–82 hr post hCG), embryos were scored for cell number and graded according to the shape of the blastomeres and degree of extracellular fragmentation (Fig. 1). A maximum of four embryos per patient was selected for transfer. Provided that patients had given their consent, the remainder (spare embryos) were left in the same culture drops, incubated as before, and scored daily for morphology. Any embryos which failed to cleave for 2 consecutive days or which showed evidence of pyknosis were destroyed.

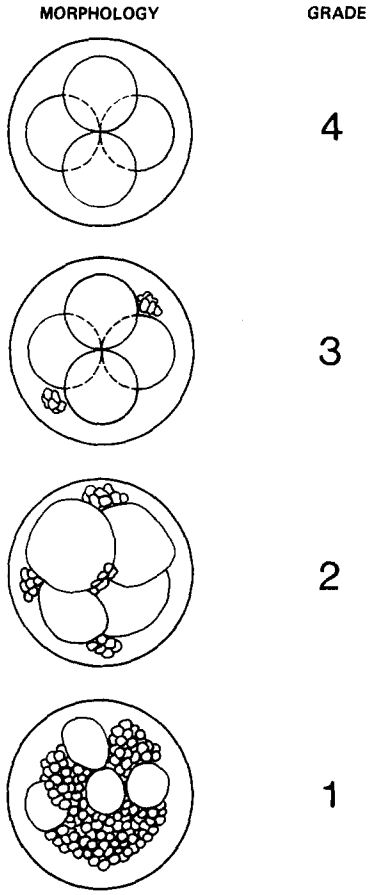

Fig. 1. Diagram illustrating the embryo grading system for four-cell embryos. Grade 4: regular, spherical blastomeres with no extracellular fragmentation. Grade 3: regular, spherical blastomeres with some extracellular fragmentation. Grade 2: blastomeres slightly irregular in size and shape with considerable extracellular fragmentation. Grade 1: barely defined blastomeres with considerable extracellular fragmentation.

RESULTS

Spare Embryo Quality and Quantity and Pregnancy

A total of 317 spare embryos was donated by 94 patients (mean, 3.4 per patient; range, 1–16). Of the donors, 72 had four embryos transferred, resulting in 18 pregnancies (25%). The remaining 22 patients had three embryos transferred, following a change in unit policy regarding the maximum number of embryos that should be replaced. Of these, 8 became pregnant (36%). Thus, overall, pregnancy occurred in 26 patients (28%).

The number of spare embryos produced by the patients who became pregnant was 101 (mean, 3.9 per patient; range, 1–16), while the nonpregnant group produced 216 spare embryos (mean 3.2 per patient; range, 1–16). Of the spare embryos from the pregnant group, 20 (20%) survived to the fully expanded blastocyst stage in vitro, while in the nonpregnant group, the corresponding figure was 35

(16%). This difference is not significant ($P = 0.43$; chi-square test).

The number of patients in the pregnant group who produced spare embryos which survived to the fully expanded blastocyst stage in vitro was 8 (31%). The corresponding figure for the nonpregnant group was 19 (28%). This difference is not significant ($P = 0.81$; chi-square test).

The Fate of Spare Embryos in Vitro

The percentage of embryos surviving through each cleavage division and morula and blastocyst formation in vitro is shown in Fig. 2. The majority of one-cell (98%) and two-cell (85%) embryos survived one further cleavage division. The greatest rate of developmental arrest occurred at the four-cell stage, with only 65% of embryos cleaving to the eight-cell stage. Thereafter, arrest occurred in 19 and 11% of eight-cell and morula-stage embryos, respectively. Ultimately, only 55 (17%) of the original 317 pronucleate-stage embryos developed into fully expanded blastocysts.

Correlation Between Cleavage Rate and Blastocyst Formation

At the time of embryo transfer (80 hr post hCG), of the embryos that had cleaved to the four-cell stage, 26% developed into fully expanded blastocysts (Fig. 3). Although marginally fewer two-cell, three-cell, and five- to eight-cell embryos survived (17, 21, and 16%, respectively), the difference is not significant (chi-square test). No embryos that were still uncleaved at 80 hr post hCG developed into fully expanded blastocysts.

At 110 hr post hCG, a higher percentage (38%) of embryos that had developed to the 9- to 16-cell stage survived in vitro, compared with embryos which were cleaving more slowly (e.g., 28% of 5- to 8-cell and 13% of 4-cell stages). No embryos that were still only one or two cells at 110 hr post hCG developed into blastocysts.

Although a small percentage of embryos that were only three cells at 130 hr post hCG survived to the blastocyst stage, the sample size in this group was small ($N = 8$). No embryos that were still two or four cells at 130 hr post hCG developed into blastocysts, compared with 63% of those that were already cavitating morulae.

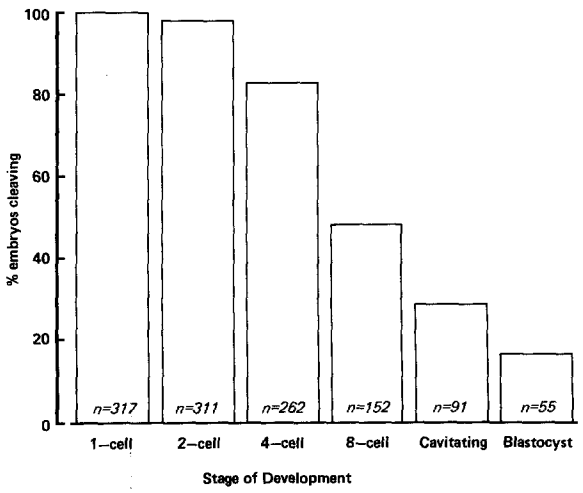

Fig. 2. Histogram illustrating the percentage of 317 spare human preimplantation embryos surviving each cleavage division through to blastocyst formation in vitro.

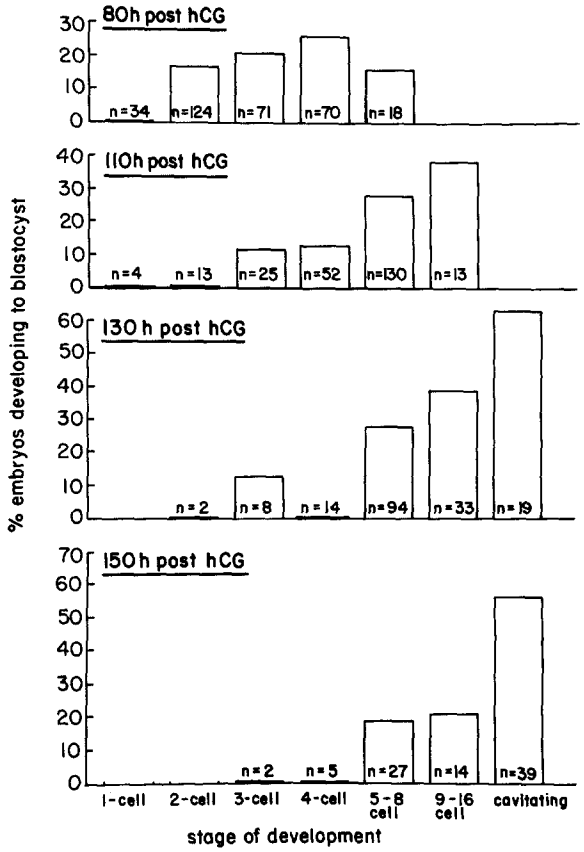

Fig. 3. Histogram illustrating the percentage of human preimplantation embryos at various times (hours post hCG) which developed to the fully expanded blastocyst stage in vitro, in relation to the cleavage rate.

Correlation Between Gross Morphology and Blastocyst Formation

Of the embryos that were graded 4 and 3 at the time of embryo transfer (80 hr post hCG), 18 and 23%, respectively, developed into fully expanded blastocysts, compared with 6 and 5% for grades 2 and 1, respectively. Overall, grade 4 and grade 3 embryos showed a better survival rate in vitro than grade 2 and grade 1 embryos, throughout the preimplantation period (Fig. 4). However, although the percentage of grade 1 embryos surviving to the blastocyst stage was consistently low (range, 0 to 16%), it is clear that even with such poor morphology, not all grade 1 embryos will undergo developmental arrest in culture.

Correlation Between Cleavage Rate *and* Gross Morphology and Blastocyst Formation

At the time of embryo transfer (80 hr post hCG), the best survival rate in vitro was achieved by grade 4 or grade 3 four-cell embryos (Fig. 5). At later times, more rapidly cleaving embryos with a good morphology (grades 4 and 3) survived more frequently than slower-cleaving embryos with a poor morphology (grades 2 and 1).

DISCUSSION

Although it has been suggested that there is a positive correlation between the survival of supernumerary embryos to the blastocyst stage in vitro and pregnancy in the donor (18), this is not supported by the above data. This may be explained by a discrepancy between units in scoring blastocysts; only those which were fully expanded, as opposed to those showing early signs of cavitation, were included in this study.

In the absence of objective, metabolic criteria for assessment of human embryo viability, the only

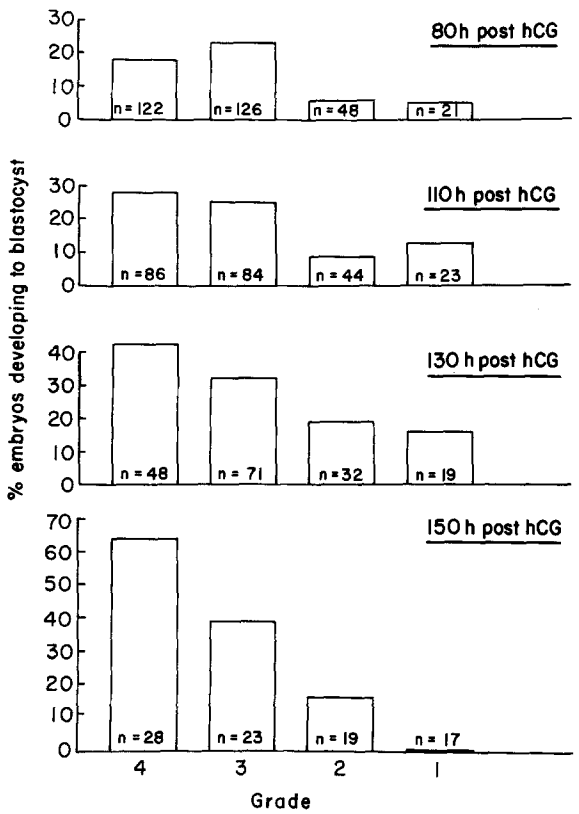

Fig. 4. Histogram illustrating the percentage of human preimplantation embryos at various times (hours post hCG) which developed to the fully expanded blastocyst stage in vitro, in relation to the gross morphology.

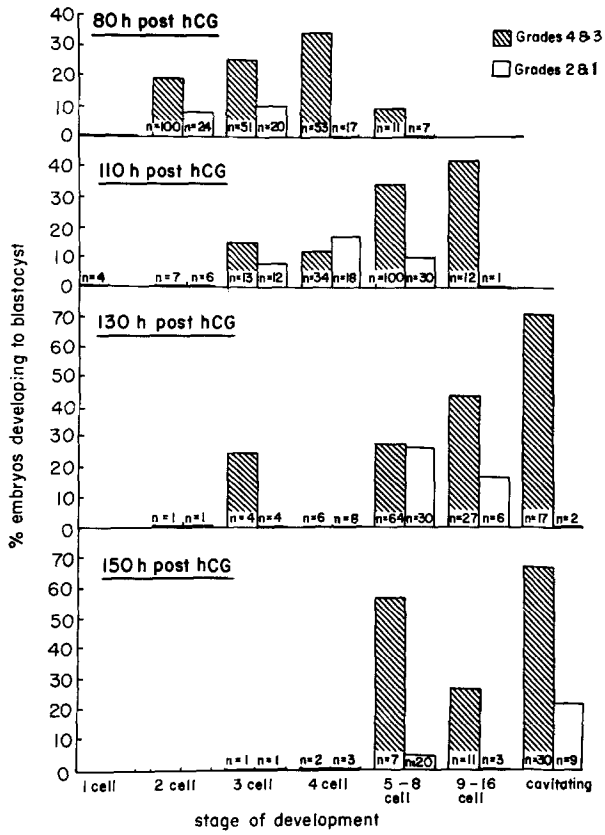

Fig. 5. Histogram illustrating the percentage of human preimplantation embryos at various times (hours post hCG) which developed to the fully expanded blastocyst stage in vitro, in relation to both the cleavage rate and the gross morphology.

noninvasive criteria currently available for selection of embryos for transfer following IVF are the rate of embryonic development and gross morphology. While a number of studies have demonstrated a correlation between the transfer of better-"qual-ity" embryos and subsequent pregnancy (12–14),the predictive value of these subjective estimates of embryo viability remains unsatisfactory. Even attempts to produce a formula (12) or scoring system (14) for embryos transferred in predicting pregnancy have provided no more than trends or correlations. It remains necessary to transfer three or even four embryos during each IVF cycle in order to achieve a pregnancy rate of 25 to 30%.

The facts that several embryos are usually transferred in IVF cycles that result in pregnancy, and that frequently not all the embryos transferred implant, mean that it is impossible to analyze accurately the properties of embryos that do have the potential to implant. By examining the development of individual spare embryos cultured in vitro, it is possible to evaluate the accuracy of the predictive criteria used in the assessment of embryo viability. Thus, it is assumed that those embryos which survive to the fully expanded blastocyst stage in vitro correspond to those which would have implanted, had they been transferred.

Cleavage rate alone is of little value in predicting whether or not an embryo will survive to the fully expanded blastocyst stage in vitro. Thus, at the time of embryo transfer, provided that an embryo has undergone at least one cleavage division, there is a possibility that it will survive in vitro. Although embryos that have reached the four-cell stage by the time of embryo transfer have the highest rate of survival (26%), a significant number of two- and three-cell embryos also survive (17 and 21%, respectively). The observation that relatively few embryos that have progressed beyond the four-cell stage at the time of embryo transfer survive to the blastocyst stage in vitro cannot be considered significant, in view of the small sample size. Nonetheless, it is a trend that is worth further investigation.

Gross morphology alone as a criterion of assessment indicates that embryos that are grade 4 or 3 at the time of embryo transfer have a better survival rate in vitro than those which are grade 2 or 1 (18 and 23 vs 6 and 5%, respectively). However, the best prediction of survival when examining embryo grade alone is only 23%, for grade 3 embryos.

A combination of the two criteria increases marginally the ability to predict embryo survival in vitro. Thus, at the time of embryo transfer, embryos which have undergone two cleavage divisions and which are grade 4 or 3 have a better survival rate than those which are lower grades. However, the predictive value of the combined criteria is, at best, only 34%.

The ability to predict embryo survival using the two criteria improves with the duration of culture. However, this can be explained largely by the fact that increasing numbers of embryos undergo developmental arrest with time. Therefore, it would seem that these criteria can be used with any accuracy only as exclusion, rather than inclusion criteria when selecting embryos for transfer. Thus, it appears that embryos which have not undergone at least one cleavage division and those which are of a poor morphology (grade 1) do not have the potential to implant. Embryos at any other stage, and of any other grade, do have some potential and should, therefore, be considered suitable for transfer.

It is important to consider the questions of why so many embryos fail to develop to the fully expanded blastocyst stage and why the rate of embryo failure is relatively insignificant before, but increases markedly after, the four-cell stage (Fig. 1). There are now considerable data suggesting that in the human, embryonic gene activation occurs between the four- and the eight-cell stages (19–21). It is likely that inherent viability of the human embryo, which may be compromised by suboptimal culture conditions, is manifested around the time of embryonic gene activation, i.e., after cleavage to the four-cell stage, and moreover, after the traditional time of embryo transfer.

The present study is based on the assumption that the observations of human preimplantation embryogenesis in vitro can be extrapolated to survival in vivo. Although the validity of this assumption is uncertain, it is supported by the observation that 17% of the spare embryos survived in vitro to develop into fully expanded blastocysts, compared with an in vivo implantation rate following IVF in our unit of 13% (22). However, firm conclusions cannot be drawn from a direct comparison, since the "best" embryos are selected for transfer, while embryos of varying quality were cultured in vitro in this study. Nonetheless, if the assumption is indeed correct, then perhaps the tradition of performing embryo transfer on the second day after egg collection requires reevaluation. If embryo transfer were to be delayed, at least until embryos have either succumbed to or overcome the hurdle that evi-

dently exists at the four- to eight-cell transition, then selection of the "best" embryos for transfer would be less of an approximation; only the best embryos would survive. A prospective clinical trial is under way in our unit, to evaluate the pregnancy rate achieved after delayed embryo transfer. If this demonstrates that transfer of blastocysts produces an equivalent or higher pregnancy rate than that achieved with earlier transfer, the implications for IVF will be considerable. (i) A number of patients would produce no embryos which survive to the blastocyst stage in vitro, thus removing false expectations of pregnancy. (ii) Those patients in whom blastocyst transfers are performed would have a realistic chance of pregnancy. (iii) The necessity for transfer of multiple embryos, with the potential of multiple pregnancy and ensuing arguments over embryo reduction, would be obviated. (iv) The extended duration of embryo culture may facilitate the development of techniques for preimplantation diagnosis of genetic disease. (v) The ultimate quality control of any IVF laboratory should be the ability to culture routinely human embryos to the fully expanded blastocyst stage in vitro.

ACKNOWLEDGMENTS

This work was approved by the King's College Hospital Ethical Committee and the MRC/RCOG Voluntary Licensing Authority.

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