Prediction of the developmental potential of hamster embryos *in vitro* by precise timing of the third cell cycle

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Time-lapse videomicrography was used to determine the timing of early developmental events in hamster embryos *in vitro*. The time intervals from pronuclear envelope breakdown to the completion of the first cleavage (Dt_2), second cleavage ($Dt_4 = 2-4$ cells), third cleavage ($Dt_8 = 4-8$ cells), blastocyst formation, and zona escape were precisely measured to determine whether the variable 'time' (t) can be used to predict the developmental potential of preimplantation embryos. The range of the developmental time interval (Dt) from the second to the third cleavage divisions (Dt_8) provided the best indicator for predicting the probabilities of blastocyst formation and zona escape ($P = 0.015$ and 0.041 , respectively). Dt_8 was subdivided into consecutive time cutoff points of ≤ 750 , ≤ 800 , ≤ 850 and ≤ 900 min. Of the embryos that took ≤ 750 min to complete the third cleavage division, 92% developed into blastocysts and 69% escaped from their zonae pellucidae. When the completion of Dt_8 extended to ≤ 900 min, the percentages decreased to 75% and 49% for blastocyst formation and zona escape, respectively. This study identifies a specific developmental time interval and a model whereby time can be used as a noninvasive parameter to predict embryo developmental potential *in vitro*.

Introduction

Culture of fertilized eggs or embryos at the early cleavage stage retards their further development, and can reduce or even destroy embryo viability (Bowman and McLaren, 1970; Harlow and Quinn, 1982; Sakkas *et al.*, 1989; Van Soom *et al.*, 1992; Barnett and Bavister, 1992; McKiernan and Bavister, 1994; Gonzales and Bavister, 1995). Frequently, embryos cultured *in vitro* arrest before the blastocyst stage or fail to escape from their zonae pellucidae (Maurer *et al.*, 1970; Fishel *et al.*, 1985; Bavister, 1988a; Lindenberg and Hyttel, 1989; Lopata and Hay, 1989; McKiernan and Bavister, 1990; Kishi *et al.*, 1991; Gonzales and Bavister, 1995). These problems not only provide incentives for devising improved culture media (Bavister, 1995) but also necessitate methods for selecting the most competent and viable embryos for transfer to recipients in both animal and human programmes. The high frequency of embryonic loss after transfer in human IVF clinics (Amso and Shaw, 1993) has also stimulated the search for a noninvasive tool to assess embryo viability and potential for subsequent transfer.

Several parameters have been used to assess the quality of preimplantation embryos. Most methods involve use of morphological criteria (Lindner and Wright, 1983; McKinnon and Squires, 1988; Wolf, 1988; Bernardini *et al.*, 1993; Tasdemir *et al.*, 1993), or biochemical procedures (Leese *et al.*, 1986; Gardner and Leese, 1993) that may or may not be compatible

with continued development or successful embryo transfer (Conaghan *et al.*, 1993; Bavister, 1995; Barnett and Bavister, *in press*). The variability of biochemical measurements (for example uptake of pyruvate or glucose) among embryos and at different stages of development diminishes the value of this approach for selecting the most competent embryos (Leese *et al.*, 1986; Conaghan *et al.*, 1993). Fluorescein diacetate has been used for evaluating embryos (Mohr and Trounson, 1980; Hutz *et al.*, 1985; Noto *et al.*, 1991) but requires expensive equipment, is invasive and may not correlate with embryo viability (Hoppe and Bavister, 1984). Use of subjective morphological criteria has not improved the frequency (which is approximately 10% for individual human embryos) of implantation and subsequent development through gestation (Edwards, 1986; Rogers *et al.*, 1986; Bolton *et al.*, 1989; Van Blerkom, 1989; Amso and Shaw, 1993). Although the success rate of embryo transfer improved in cattle up to the 1980s, there has been little improvement during the last 10–15 years in the commercial success rate (which is 50–70% of transferred embryos, depending on stage and morphological criteria; Hasler *et al.*, 1987; Hasler, 1992). Therefore, morphological assessment is not very effective for separating embryos with high versus low potential for further development and there remains a need for improvement in domestic species.

Developmental endpoints used in conventional experimental protocols, such as the proportion of embryos reaching morulae or blastocysts, give information that does not directly indicate embryonic potential or viability (Angell *et al.*, 1987) and can even be misleading about the efficacy of culture protocols (Bavister, 1995). Determination of the mean number of cells,

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especially with a differential cell count (inner cell mass versus trophoctoderm; Handyside and Hunter, 1984), can be very informative but present methods for this destroy the embryo.

An alternative to using proportions of embryos developing into blastocysts after a predetermined culture period is to measure the time taken for development to different stages. The rationale for using time as an indicator of developmental competence stems from observations that embryos that develop faster in culture have a greater potential to reach the morula and blastocyst stages than do those that develop more slowly (Bavister *et al.*, 1983a; Plante and King, 1992; Van Soom *et al.*, 1992; McKiernan and Bavister, 1994; Grisart *et al.*, 1994). In addition, cultured bovine embryos reaching the blastocyst stage on days 6–8 implant at higher rates than do day 9 blastocysts after transfer (Hasler *et al.*, 1987, 1995). However, timing data from most studies are derived from infrequent examination of embryos in culture (usually not more than once every 12 or 24 h). Substantial differences among embryos can occur in the timing of cleavage events, and these may be missed if examinations are infrequent, possibly misdirecting the selection of embryos for transfer (Bavister, 1995). The timing of early cleavage events may be critical for assessing cultured embryos, since development is often delayed *in vitro* (Grisart *et al.*, 1994; Gonzales and Bavister, 1995). Moreover, measurement of developmental timing has the advantage of being noninvasive.

Using time-lapse videomicrography of cultured hamster embryos, we set out to test the hypothesis that the precise timing of early cleavages can predict the developmental potential of embryos. The aim was to validate a model for selecting embryos with a high probability of success (success being defined as either reaching the blastocyst stage or escaping the zona pellucida), thereby providing a noninvasive means to select embryos with a greater likelihood of further development and pregnancy after embryo transfer.

Materials and Methods

Animals

Female golden hamsters used in this study were sexually mature (3–4 months old, mass 110–130 g) and naturally cyclic (i.e., not gonadotrophin-stimulated). Animals were maintained under a photoperiod of 14 h light:10 h dark (lights on at 06:00 h and off at 20:00 h central standard time; CST). Females were mated on day 4, the evening of the oestrous vaginal discharge (Orsini, 1961; Bavister, 1989) at 18:30 h CST to proven fertile males. Consistent timing in the experimental protocol was controlled by excluding from the study females that did not mate within 15 min after placement with males. Five mated female hamsters were used in this timing study and three in the control group.

Collection and culture of embryos

One-cell embryos were flushed from the oviducts the day after mating (day 1) at 16:30 h CST, approximately 12.5 h after egg activation (Orsini, 1961; Bavister *et al.*, 1983b) with hamster embryo culture medium-4m (HECM-4m; Gonzales and

Bavister, 1995). Egg activation is defined as the time when a spermatozoon penetrates the egg; in this case at approximately 04:00 h on day 1 (Bavister *et al.*, 1983b). Each culture consisted of the embryos from a single female and embryos were placed in 60 mm × 50 mm Falcon Petri dishes (Becton Dickinson and Co., Lincoln Park, NJ) containing 80 µl drops of HECM-4m overlaid with silicone oil. Culture drops were kept at 37°C, 10% CO₂ and 5% O₂ in an incubator for a minimum of 2 h before embryo collection. There were five replicate time-lapse cultures, because only one culture could be monitored at a time with the equipment available. There were 10, 10, 19, 13 and 10 embryos, respectively, in cultures one to five.

Each embryo provided complete timing data for the duration of the culture, providing a total of $n = 62$ discrete data sets. Embryos were cultured either in a standard incubator or on the stage of a Nikon Diaphot inverted microscope (Nikon Corp., Tokyo). The microscope was equipped with an environment control chamber surrounding the optics and the stage. Within the chamber, a controlled, humidified atmosphere of 10% CO₂, 5% O₂ and 85% N₂ at 37°C was maintained. Embryos were cultured until they either escaped their zonae or began to degenerate (5–7 days in culture). The microscope light source was covered with an orange-red filter to protect the embryos from light toxicity (Umaoka *et al.*, 1993).

Time-lapse data collection

Details of the system used in this study for culturing embryos on the microscope stage and for data collection by time-lapse videomicrography were described by Bavister (1988b) and Gonzales and Bavister (1995). Briefly, embryo cultures were monitored under Nomarski DIC (Nikon) optics with a ×10 objective, and recorded using a video camera fitted with a Newvicon detector tube (Series 70; Dage-MTI Inc., Michigan City, IN) and a Panasonic Super-VHS recorder (S-VHS, AG-6720A; Panasonic, Secaucus, NJ). A zoom lens fitted between the microscope port and the detector tube was set at ×1.0 or ×1.5 to obtain magnifications of ×10 or ×15. At these magnifications, the male and female pronuclei of the zygotes were easily observed at the initiation of culture (12–13 h after egg activation; Fig. 1). Timing was initiated ($t = 0$) at pronuclear envelope breakdown for each embryo (Figs 1 and 2). The timing of developmental events such as cleavage, blastocoel formation and zona escape was determined separately for each embryo.

Time-lapse videos were recorded at 1/120 normal rate, so that 1 h of development was compressed into 30 s of real time. Precise temporal information on development was obtained from the time/date generator of the S-VHS recorder. The first developmental interval was the time from pronuclear envelope breakdown to the completion of the first cleavage division (Dt_2 ; i.e., AB to A + B; Fig. 2). The second interval (Dt_4 ; Fig. 2) began at the completion of the first cleavage division and extended to the completion of the second cleavage division (from two to four cells; i.e., B to B₁ + B₂). The time interval from four to eight cells (Dt_8) began with the completion of the second cleavage division and ended with the completion of cytokinesis of blastomere B₂ (i.e., B₂ to B_{2a} + B_{2b}; Fig. 2). It was not possible to determine the timing of cleavages past the third

Fig. 1. Computer-generated digital image of pronucleate hamster embryos (a), with some undergoing pronuclear envelope breakdown (b) and spindle pole formation (c).

cleavage division because of the decreasing size of individual blastomeres, the progressively obscured view by the increasing number of blastomeres, and the increasing cell–cell adhesion as a result of morula formation. The time of blastocyst formation was established as the time when a blastocoel was first apparent in the plane of focus. Zona escape was discerned when a focal area of dissolution penetrated the complete thickness of the zona wall (Gonzales and Bavister, 1995).

Digital images were generated for analysis and for publication. A detailed description of computer generated digital imaging of cultured embryos is given by Gonzales and Bavister (1995). Briefly, at fixed intervals (5, 10 or 20 min), eight digital video images were collected by a personal computer (486/33 MHz; Gateway 2000, Sioux City, SD). The eight digital images were averaged and the averaged image was stored in the computer for analysis and photography. This process used the computer as a time-lapse device that gathered static data over time. Images for publication (Fig. 1) were made by photographing the screen of the video monitor with a Nikon N-2000 camera, using a 55 mm macro lens and TMAX 400 film (Eastman Kodak Co., Rochester, NY).

Experimental design

Experiment 1: control experiment. Three replicate experiments using three female hamsters (replication over days) were conducted ($n = 34$) to determine whether there was a difference in the development of videotaped embryos (cultured under continuous red light) compared with embryos cultured in the incubator (absence of light; Umaoka *et al.*, 1993). These three replicates were run at the same time as the first three replicates in Expt 2 and consisted of one-cell embryos cultured in an incubator (model 3187, Forma Scientific, Marietta, OH) under the same temperature and gas conditions as on the microscope stage but in the absence of light. Embryos were collected and placed in culture, as described above, for the timing of embryo development.

Experiment 2: timing of embryo development. Five replicates were conducted (five female hamsters over days), with each culture initiated at the same time after egg activation. The timing data of early developmental events were collectively and individually analysed using logistic regression analysis, to determine whether time could be used as a tool for predicting embryo development potential *in vitro*. To detect differences in the five cultures, comparisons were made by ANOVA (Snedecor and Cochran, 1989) of the mean times of early cleavage events, blastocyst formation and zona escape.

Embryos in Expt 1 (control group) and Expt 2 (timing group) were assessed after 48 h and 72 h of culture for the percentages of eight cells and blastocysts, respectively. These results were compared using Student's *t* test (Snedecor and Cochran, 1989).

Statistical model for predicting developmental potential

Logistic regression (Agresti, 1990) was used to estimate the probability of blastocyst formation and of zona escape as a function of time, during early cleavages in preimplantation embryo development. Three different time intervals (Dt_2 , Dt_4 and Dt_8) were examined separately and in all combinations in the analysis. The logistic model for the probability (P) of blastocyst formation as a function of the variable time (t) is written as:

$$P(\text{blastocyst}|t) = \frac{e^{(\alpha + \beta t)}}{1 + e^{(\alpha + \beta t)}}$$

where the parameter β determines the association between t and P (Agresti, 1990). In this model, if $\beta < 0$, the probability decreases with t ; if $\beta > 0$ the probability increases with t ; and if $\beta = 0$, the probability does not depend on t . Therefore, the parameter β establishes the dependence between the probability and time. A property of the logistic model is that it can be easily linearized. The log odds ratio (Agresti, 1990) of $P_t = P(\text{blastocyst}|t)$ in the logistic model is a linear function of t and is written as $\log(P_t/1 - P_t) = (\alpha + \beta t)$. The parameter α is the y intercept and the parameter β is the slope of the log odds ratio. The time interval with a significant P value ($P = 0.05$) for the null hypothesis that $\beta = 0$ can be used to predict developmental potential. A similar model was used for the probability of zona escape.

Practical application of the model

In the developmental interval that was best able to predict blastocyst formation and zona escape, time cutoff points were made to compare success rates as a function of time. The percentages of embryos that became blastocysts and escaped their zonae after completing the third cleavage division within the designated time cutoff points were calculated. The time cutoff points were determined following logistic regression analysis.

Results

Experiment 1 (control group)

There were no significant differences in the numbers of eight-cell embryos and blastocysts between the control group

Fig. 2. Schematic of hamster embryo generation time *in vitro*. Circles enclosing cleavage notations represent individual blastomeres. The time intervals designated between arrows (Dt_2 , Dt_4 and Dt_8) were tested by logistic regression analysis.

(Expt 1) and the ‘timing’ group (Expt 2) at 48 h and 72 h, respectively ($P > 0.1$ for both observation times). After 72 h of culture, 70.6% of the embryos in the control group had reached the blastocyst stage.

Experiment 2 (timing group)

No significant differences were found among the five replicate cultures in the mean times of early cleavage events, blastocyst formation and zona escape; therefore, the data were pooled (Table 1). The percentages of blastocyst formation and zona escape were 68% and 45%, respectively. By logistic regression analysis, the developmental intervals Dt_2 and Dt_4 (Fig. 2) had P values that were not significant for predicting blastocyst formation and zona escape (Table 2). The predictive value of the combined intervals ($Dt_2 + Dt_4$) was also not significant. The developmental time interval that best predicted blastocyst formation and zona escape was Dt_8 (Table 2). As the developmental interval Dt_8 increased, the estimated probabilities of blastocyst formation and of zona escape decreased (Fig. 3); the estimated 95% confidence intervals for the probabilities are also represented. Table 3 provides the estimates of the parameters α and β and their standard errors for the developmental interval Dt_8 . Both estimates of β are negative

Table 1. Time intervals of development events in preimplantation hamster embryos *in vitro*^a

Event ^b	<i>n</i> ^c	Mean elapsed time $\pm$ SEM (h)	Range (h)
PNEB ^d to first cleavage (Dt_2)	62	1.4 $\pm$ 0.1	0.5–2.4
First to second cleavage (Dt_4)	62	28.1 $\pm$ 0.2	25.1–36.8
Second to third cleavage (Dt_8)	62	11.9 $\pm$ 0.1	9.8–26.5
PNEB to blastocyst	42	61.7 $\pm$ 1.1	53.4–83.9
PNEB to zona escape	28	88.82 $\pm$ 2.0	78.2–105.3

^aEmbryos were flushed from the oviducts of five female hamsters at 16:30 h on day 1 of pregnancy.

^bDescribed in Fig. 2.

^cNumber of observations (= number of embryos).

^dPronuclear envelope breakdown, from which timing was initiated for each embryo.

for Dt_8 , indicating that the probability of blastocyst formation and zona escape decreases with t . The concordance, which provides a measure of fit in logistic regression analysis (Agresti, 1990), was 74.2% for blastocyst formation and 61.2% for zona escape.

Table 2. *P* values for the developmental intervals tested by logistic regression analysis

Developmental time interval	<i>P</i> value for blastocyst formation	<i>P</i> value for zona escape
PNEB ^a to first cleavage (Dt_2)	0.214	0.075
First to second cleavage (Dt_4)	0.288	0.585
Second to third cleavage (Dt_8) ^b	0.015	0.041

^aPronuclear envelope breakdown.^bOnly Dt_8 provided significant *P* values for estimating the probability of reaching the endpoint shown. **Fig. 3.** Logistic regression analysis of (a) probability of blastocyst formation and (b) probability of zona escape in hamster embryos cultured *in vitro*. Time on the *x* axis is elapsed time during the developmental interval Dt_8 . The *P* values of the estimated parameter β of the fitted lines (—) for (a) blastocyst formation and (b) zona escape are shown in Table 2; (····) represents the plot of the 95% pointwise confidence limits for the estimated probabilities in logistic regression analysis.

Empirical evidence for using time as a variable in the model

The time interval of 725–900 min within Dt_8 represents the time when most of the embryos (71% of total observations) completed the third cleavage division (Fig. 4). Another 11% of

Table 3. Estimated parameters for Dt_8 in the logistic regression model

Response ^a	α (SEM)	β (SEM)
Blastocyst formation ^b	7.13 (2.63)	− 0.008 (0.003)
Zona escape ^b	4.68 (2.37)	− 0.006 (0.003)

^aTotal number of embryos (observations) = 62.^bConcordance (success of predictions) = 74.2% for blastocyst formation and 61.2% for zona escape. **Fig. 4.** Observed incidence of (a) blastocyst formation and (b) zona escape in 50 min subsets during the developmental interval Dt_8 in hamster embryos cultured *in vitro*. The *x* axis is elapsed time during Dt_8 . The time points were chosen based on the logistic regression model. Within (a) and (b), each response is significantly different from all other time cutoff points ($P \leq 0.002$ and $P \leq 0.03$, respectively). Within the time cutoffs for completion of Dt_8 , ≤ 750 , ≤ 800 , ≤ 850 and ≤ 900 min, the proportions of embryos (percentage of total, inclusive values) were 21, 48, 71 and 82%, respectively, and the numbers (inclusive) of embryos within each cutoff were 13, 30, 44 and 51, respectively.

the embryos completed the third cleavage division before 725 min, and the remaining 18% after 900 min. Within the interval 750–900 min, time cutoff points were established at 50 min intervals to compare success (% blastocyst and % zona escape) as a function of time (Fig. 4). Each time cutoff point is inclusive, i.e., includes all embryos that had completed the third

cleavage division up to that time, and excludes slower-cleaving embryos. In all cases, cleavage was completed within one time cutoff, i.e., there was no overlap of embryos between consecutive time cutoffs. Within the time cutoff of ≤ 750 min for completion of Dt_8 , 92% of embryos subsequently reached the blastocyst stage and 69% escaped their zonae. As the time taken to complete the third cleavage division increased, the cumulative percentage of embryos reaching the blastocyst stage or escaping their zonae decreased (Figs 3 and 4). Although cleavage divisions within Dt_4 and Dt_8 were asynchronous, there was no correlation between the degree of asynchrony and blastocyst development.

Discussion

In clinical IVF laboratories, embryos are selected for transfer based primarily on morphology (Wolf, 1988). However, morphological criteria do not take into consideration potential aberrations that cannot be seen under a light microscope (Winston *et al.*, 1991) but may affect embryonic potential for development *in vitro* or viability after embryo transfer. Using Nomarski optics and transmission electron microscopy, Sathananthan *et al.* (1990) found that some cultured embryos that appeared morphologically normal had multiple nuclei. Such embryos are incapable of normal development (Angell *et al.*, 1987; Winston *et al.*, 1991), but under a conventional light microscope, are indistinguishable from euploid embryos (Van Blerkom *et al.*, 1984; Sathananthan *et al.*, 1990). In addition, there may be subtle yet potentially lethal defects, such as mitochondrial damage (Dorland *et al.*, 1994), that are not detectable at this stage (D. K. Barnett and B. D. Bavister, unpublished).

Culturing preimplantation embryos, from any stage, retards their development and alters their ability to escape from the zona pellucida (Bowman and McLaren, 1970; Harlow and Quinn, 1982; Sakkas *et al.*, 1989; Van Soom *et al.*, 1992; Gonzales and Bavister, 1995). Embryos that develop faster are more likely to reach the blastocyst stage or escape their zonae than are embryos that develop slowly (Bavister *et al.*, 1983a; Plante and King, 1992; Van Soom *et al.*, 1992; McKiernan and Bavister, 1994) and have higher pregnancy rates after embryo transfer (Hasler *et al.*, 1987, 1995). These observations support the hypothesis that time can be used as a noninvasive indicator of embryo potential.

The notion that time can be used as an indicator of viability was demonstrated by McKiernan and Bavister (1994), who reported that one-cell hamster embryos that develop faster *in vitro* are more viable than are slower-developing embryos. In their study, although morulae and blastocysts derived from faster versus slower embryos were morphologically indistinguishable under the light microscope, twice as many fetuses were produced by the faster cleaving group after embryo transfer.

In the present study, the overall incidence of blastocyst formation and zona escape was 68% and 45%, respectively. These data provide developmental endpoints but cannot predict which individual embryos will become blastocysts or escape their zonae before these endpoints are attained, and there is no assurance that the embryos reaching these stages

are 'healthy'. By means of the logistic regression model, the embryos cultured from the one-cell stage that have a higher probability of reaching the blastocyst stage or undergoing zona escape can be determined, giving an early indication of the developmental potential of the embryo. The time interval best able to predict developmental potential during preimplantation development of hamster embryos *in vitro* is Dt_8 . By subdividing Dt_8 , embryos with substantially greater probability of reaching the blastocyst stage or escaping their zonae pellucidae can be selected. The ability to select noninvasively embryos with the highest probability of completing preimplantation development has considerable implications for improving the success of embryo transfers when embryo selection is made during the first few cleavage divisions, as in human IVF.

Several studies have attempted to correlate cleavage timing of human IVF embryos with development and viability, to incorporate the data into a scoring protocol for selection of embryos for transfer (Trounson *et al.*, 1982; Cummins *et al.*, 1986; Steer *et al.*, 1992). However, all of these studies used periodic inspection of embryo development, an approach that is inherently flawed (Bavister, 1995). By inspecting embryos only at fixed intervals (usually 12 or 24 h), the time schedule of observations is out of synchrony with embryo cleavages, and, as a result, the range of timing established for each cleavage becomes so broad as to be of little use (Bavister, 1995), and the apparent timing of successive cleavages may even overlap. For example, in one study of human IVF embryos using periodic observations, the time range for the first cleavage division was determined as 27–43 h, for the second cleavage division 36–64 h, and for the third cleavage division 44–73 h (Trounson *et al.*, 1982). The only way to avoid this problem is to increase the frequency of observations. However, repeated disturbance of the cultured embryos for visual inspection is harmful to their development and impractical from the viewpoint of laboratory personnel. These problems are avoided by using a computer to capture digital images at frequent intervals (Gonzales and Bavister, 1995), time-lapse video (present study), or ciné recording (Grisart *et al.*, 1994). The problems here are the high cost of the equipment and the labour intensive initial analysis of the data, but once the critical timing is established for any species and culture system (as in this study), it should be possible to use this information to select the most competent embryos in a practical or clinical setting.

In the present study, we confirmed that faster-cleaving embryos reached the blastocyst stage or escaped their zonae pellucidae at a higher frequency than did slower-cleaving embryos. In addition, we provide evidence that precise timing of embryo development can be used to predict developmental potential *in vitro* with an accuracy as high as 92%. Finally, we identified Dt_8 (the developmental interval from the second to the third cleavage division) as the time interval most able to provide predictive data. We do not know why the third cell cycle is so critical for subsequent development and can serve as a predictor of the developmental capacity of embryos. Study of biochemical and molecular events taking place during this cell cycle could be informative. To test the general applicability of these observations we and other workers (Grisart *et al.*, 1994) are collecting precise timing data on embryos from more species, including humans.

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