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ARTICLE

Prediction of in-vitro developmental competence of early cleavage-stage mouse embryos with compact time-lapse equipment

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Csaba Pribenszky obtained a DVM degree (1998) and a PhD degree (2005) in the field of assisted reproductive technologies in Budapest, Hungary. Together with Mikos Molnar, he developed and patented the concept of 'stress for stress tolerance', utilizing sub-lethal hydrostatic pressure stress treatment to gametes, embryos, other cells and tissues in order to improve cell survival during subsequent procedures (e.g. cryopreservation, SCNT). His current focus is the application of the above method to human oocyte vitrification and the effect of different environmental factors on in-vitro embryo developmental dynamics and embryo fragmentation.

Abstract Single blastocyst transfer is regarded as an efficient way to achieve high pregnancy rates and to avoid multiple pregnancies. Risk of cancellation of transfer due to a lack of available embryos may be reduced by early prediction of blastocyst development. Time-lapse investigation of mouse embryos shows that the time of the first and second cleavage (to the 2- and 3-cell stages, respectively) has a strong predictive value for further development *in vitro*, while cleavage from the 3-cell to the 4-cell stage has no predictive value. In humans, embryo fragmentation during preimplantation development has been associated with lower pregnancy rates and a higher incidence of developmental abnormalities. Analysis of time-lapse records shows that most fragmentation is reversible in the mouse and is resorbed in an average of 9 h. Daily or bi-daily microscopic checks of embryo development, applied routinely in human IVF laboratories, would fail to detect 36 or 72% of these fragmentations, respectively. Fragmentation occurring in a defined time frame has a strong predictive value for in-vitro embryo development. The practical compact system used in the present trial, based on the 'one camera per patient' principle, has eliminated the usual disadvantages of time-lapse investigations and is applicable for the routine follow-up of in-vitro embryo development.

KEYWORDS: blastocyst, early cleavage, fragmentation, in-vitro culture, microwell culture dish, time-lapse monitoring

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Introduction

Multiple pregnancies following IVF treatment are a definite risk and should be kept at the lowest level possible while maintaining overall efficiency, measured usually by the healthy baby per treatment cycle ratio (Quea et al., 2007). A feasible way to achieve this goal is to transfer a single embryo with the highest developmental competence. Transfer of advanced-stage preimplantation embryos (blastocysts) offers better selection possibilities and results in higher implantation rates (Bhattacharya and Templeton, 2004; Gardner and Lane, 2003; Pandian et al., 2005). However, there are some concerns regarding this approach. Freezing of blastocysts is usually less efficient than that of cleavage-stage embryos, therefore future frozen embryo transfers utilizing blastocysts may be compromised (Loutradi et al., 2008). Additionally, in spite of improved culture systems, embryos may not reach the blastocyst stage *in vitro*, resulting in cancellation of transfer.

New vitrification techniques provide an efficient solution for cryopreserving blastocysts and maintaining excellent pregnancy rates (Youssry et al., 2008). Accurate prediction of the *in vitro* and *in vivo* developmental competence of early cleavage embryos creates a tool for making appropriate choice between a day-3 or day-5 transfer and to avoid cancellations. Unfortunately, traditional and widely used methods of embryo evaluation, e.g. blastomere symmetry, cytoplasmic appearance, extent of fragmentation and blastomere nuclear status in cleavage-stage embryos, seem to be insufficient to obtain the required accuracy (Cummins et al., 1986; Emiliani et al., 2006). Recently, alternative techniques have been suggested, including the assessment of the zona pellucida under polarized light, analysis of the amino acid turnover, oxygen consumption, protein production or cumulative metabolic activity of the embryo (Booth et al., 2007; Brison et al., 2004; Katz-Jaffe et al., 2009; Montag and van der Ven, 2008; Nagy et al., 2009; Scott et al., 2008; Singh and Sinclair, 2007; Sturmey et al., 2008).

Another approach is to consider the time points of early cleavage as quality indicators. Several studies have demonstrated strong correlation between short initial cleavage cycles and the subsequent development potential of individual embryos. (Bos-Mikich et al., 2001; Edwards et al., 1984; Fenwick et al., 2002; Giorgettic et al., 2007; Hesters et al., 2008; Luna et al., 2008; Lundin et al., 2001; Montfoort et al., 2004; Petersen et al., 2001; Rienzi et al., 2005; Windt et al., 2004). Other studies regard rapidly cleaving embryos as potentially compromised because of inappropriate imprinting and chromosomal abnormalities (Alikani et al., 2000; Harper et al., 1994; Magli et al., 1998; Ziebe et al., 1997).

According to recent reports, fragmentation, even if reabsorbed during the course of embryo development, may dramatically reduce pregnancy rate (Alikani, 2007; Ragione et al., 2007). Transfer of fragmented embryos may also cause significantly higher rates of malformations (Ebner et al., 2001). However, most embryologists regard fragmentation as an irreversible process although a fragment may disappear by reabsorption within 10 min (Hardarson et al., 2002).

The need for more frequent observation has been pointed out by several studies (Arav et al., 2008; Lemmen et al., 2008; Lundin et al., 2001). However, frequent visual observations and the associated moves from the incubator to a microscope can induce a physical stress that may impede or even stall embryo development. It is also time consuming and difficult to incorporate into the daily routine of IVF clinics.

Time-lapse investigations have illustrated that continuous monitoring of early embryo morphology may provide data with a predictive value for the subsequent embryo developmental competence (Arav et al., 2008; Lemmen et al., 2008). However, large-scale routine application of most available time-lapse techniques is hampered by the high costs, complicated structure and the limited capacity of available equipment. Potentially dangerous technical solutions to these problems may cause heat accumulation, continuous low voltage electromagnetic field and shear stress by embryo movements, which may affect embryo viability negatively.

The purpose of this study was to evaluate the correlation between the time of initial cleavage stages, occurrence of fragmentation and the in-vitro development to the blastocyst stage by using a simplified compact time-lapse system, suitable for routine continuous monitoring of the development of all embryos in human IVF laboratories.

Materials and methods

Embryo production

Eight 12-week-old NMRI (Naval Medical Research Institute; Charles River, Hungary) female and ICR (Institute for Cancer Research; Charles River, Hungary) male mice were housed under standard conditions (22 ± 2°C; 12 h dark/12 h light; water and food *ad libitum*). Females were superovulated by intraperitoneal injection of 10 IU of pregnant mare serum gonadotrophin (PMSG; Werfaser, Alvetra & Werfft, Wien, Austria) followed by injection of 5 IU of human chorionic gonadotrophin (HCG; Choragon 5000 IU, Ferring, Kiel, Deutschland) 48 h later. After HCG administration, females were mated with fertile males in monogamous pairs.

Two pro-nuclei (2PN)-stage embryos were harvested from the oviducts of copulated females on the day when the vaginal plug was observed (17—18 h after HCG injection; approximately 6—8 h after copulation) by flushing the oviduct with flushing medium (Medicult, Jyllinge, Denmark). Procedures were approved by the animal care and use committee of Szent István University, Faculty of Veterinary Science. Zygotes with spherical and symmetrical appearance, without extruded material in the perivitelline space, with an intact zona pellucida and polar body and with detectable pronuclei were included in the experiment.

Embryo culture

Six to nine embryos were cultured in 30 μl microdrops of potassium simplex optimized medium, supplemented with amino acids (EmbryoMax; Millipore, Billerica, USA) under mineral oil (Ovoil; Vitrolife, Kungsbacka, Sweden) at 37°C with 6% CO_2 and 90% humidity in air. For all of the time-lapse analyses, up to nine 2 PN-stage embryos were placed one by one into the microwells of a well-of-the-well (WOW) dish (see detailed description below), the wells were covered

with a single 30 μl microdrop. Embryos in 30 μl microdrops were cultured in normal commercially available 35 mm Petri dishes (BD Falcon, BD Biosciences, San Jose, CA, USA) as controls for in-vitro culture. In this study, no embryo transfers were made.

Well-of-the-well dish

The WOW dish (Cryo-Innovation, Budapest, Hungary) consists of a 35-mm diameter polystyrene Petri dish containing nine microwells aligned in three rows and three columns in the centre of the bottom of the dish (**Figure 1**). The approximate dimensions and function of the microwells follows the principle described in earlier publications (Vajta et al., 2000, 2008); however, the dishes used in this study were industrially produced. The wells are 200 μm in diameter, 100 μm deep, flat U-shaped to achieve the appropriate optical clarity. The distance between the wells (the embryos are approximately 150—200 μm apart from each other) meets optimal embryo density requirements according to earlier publications (Gopichandran and Leese, 2006; Stokes et al., 2005).

This design of the culture system may provide the benefits of communal culture while permitting individual continuous morphological monitoring of embryos during development.

Time-lapse system

A purpose-made, compact, digital inverted microscope (Primo Vision; Cryo-Innovation, Budapest, Hungary) was placed inside a common water-jacketed CO_2 incubator (Nuaire, US Autoflow, Plymouth, USA). The outer dimensions of the microscope were $220 \times 80 \times 110$ mm for length, width and height, respectively. The microscope (consisting of a ×10 objective, a prism, projective lens, a 1.3 megapixel charge-coupled device and a light emitting diode light source) was covered completely by corrugated aluminum except for a glass window for the inverted objective and a shielded firewire cable for intermittent power source and communication (**Figure 2**). The firewire cable of the microscope was led through the factory-made side port of the incubator and was connected to an electric controlling unit that was able to control up to six of these microscopes. The images were transferred through this controlling unit to a laptop computer via a USB cable. The custom-made software running on the PC made it possible to adjust the interval of the image acquisitions and the duration of the recording.

WOW dishes were placed on the top of the glass window, into the sample holder of the microscope and the objective was mechanically focused. The WOW culture dish enabled all the embryos to be positioned in the field of view. Illumination was provided by a reflected warm white light. The system was set to take a single picture every 10 min. Apart from these expositions (3—5 s length for each), no electricity passed through the firewire cable to the microscope, because both the power source of the light and the camera were shut down in the electric controlling unit. (**Figure 3**). The computer screen displayed the actual developmental stages of the embryos, while all the images recorded earlier were saved to be analysed later with software.

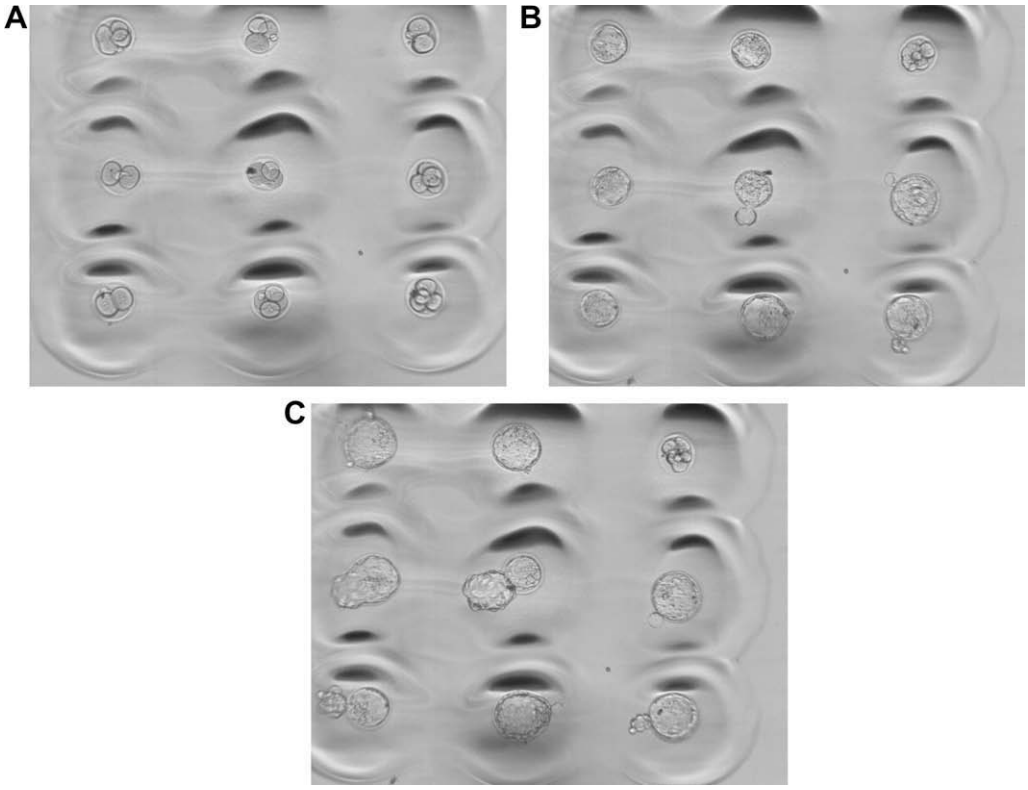

Figure 1 Embryos in the microwells in the bottom of a 35-mm Petri dish as presented online by the controlling PC; (**A**) 2- to 4-cell-stage embryos on day 2; (**B**) embryo development on day 5; and (**C**) embryo development on day 6.

Figure 2 Compact, digital inverted microscopes inside the incubator. The well-of-the-well dishes are positioned under the lamp console.

Figure 3 Observing embryo development online on the screen of the PC.

The embryos were not moved or disturbed in any way for the whole period (5 days) of development, completely eliminating sheer stress. Lubricants were not used; moreover, neither additional heat nor continuous low voltage electric currents were affecting embryo development.

Analysis

A total of 38 time-lapse series were recorded, observing 345 embryos altogether, for 5 days and compared with the control group (215 embryos) in terms of development to the blastocyst stage.

For the analysis of the effect of fragmentation and cleavage times on embryo development, 12 series with a total of 102 embryos distributed in groups of six to nine, were chosen randomly. The time between the midpoint of the dark period (mating) and the cleavage to the 2-cell stage represents the first cleavage time. The second cleavage time is the time elapsed from the 2-cell stage until the 3-cell stage and the third cleavage time is the time elapsed between 3- and 4-cell stage. Embryos were regarded as expanded blastocysts if their cleavage course went through the 1-cell—2-cell—3-cell—4-cell—8-cell—16-cell—32-cell—compaction—blastulation expansion. For statistical analysis, the generalized linear-mixed model was used (Agresti, 2002). Results with $P \leq 0.05$ values were considered as significant.

On the basis of the exact fragmentation times recorded with time-lapse monitoring, this study also calculated the detection rate if embryo evaluation would have taken place only at 9 a.m. in the morning each day or every second day (D1, D3 and D5). Assuming that each fragmented embryo is detected independently by equal probability, the binomial distribution was applied to construct 95% confidence intervals for the detection rates.

Results

Blastocyst development rates of embryos cultured in the WOW dishes and monitored by the time-lapse system did not differ from those of the controls cultured in regular microdrops in BD Falcon dishes (288/345, 83.5% versus 182/215, 84.7%, respectively).

The time of the first and second cleavage of the embryos significantly influenced the probability of reaching the blastocyst stage ($P = 0.007$ and 0.0008, respectively). The shorter the time of the cleavage to the 2-cell stage and from the 2-cell stage to the 3-cell stage, the higher the chance of the embryo developing to blastocyst stage. **Figure 4** shows the cleavage times to the 2- and 3-cell stages of each embryo that developed without the occurrence of any fragmentation. The time elapsed between fertilization until the 3-cell stage alone has an even stronger influence on the chance of an embryo reaching blastocyst stage ($P = 0.0002$). Cleavage from the 3-cell to the 4-cell stage had no effect on the blastocyst formation rate (data not shown).

The probability of reaching the blastocyst stage was reduced significantly ($P < 0.01$) if fragmentation occurred during embryo development. **Figure 5** shows the cleavage times to the 2- and 3-cell stages of the embryos where fragmentation occurred.

Fragmentation occurred in 12 embryos (12%), sometimes repeatedly in the same embryo. Altogether, 22 events of fragmentation were detected during the development from 1-cell to the blastocyst stage. In one embryo, three fragmentations were observed at the blastocyst stage, so this event was excluded from the statistical analysis. Seventeen fragmentations reabsorbed within 24 h, while two fragmentations remained visible at the end of the investigated period. The average duration for fragmentation—reabsorption was 9.1 h (±7.3 h).

The duration of individual fragmentation is shown on **Figure 6**. The occurrence of fragmentations was inversely related to subsequent blastocyst development (**Figures 4** and **5**). **Figure 7** illustrates the reduced probability of an embryo reaching the blastocyst stage if fragmentation occurs during in-vitro embryo development. Fragmentation of embryos with rapid or slow cleavage to the 2- and 3-cell stage does not influence probability: early cleavage times predict 100%, while late cleavage times predict 0%, theoretical probability of an embryo reaching the blastocyst stage. At intermediate cleavage times, the chance for a fragmented embryo to reach the blastocyst stage might be reduced by up to 60% compared with non-fragmented embryos.

In the present study, 36.4% or 72.7% of fragmentation would not have been noticed in daily or bi-daily (D1, D3

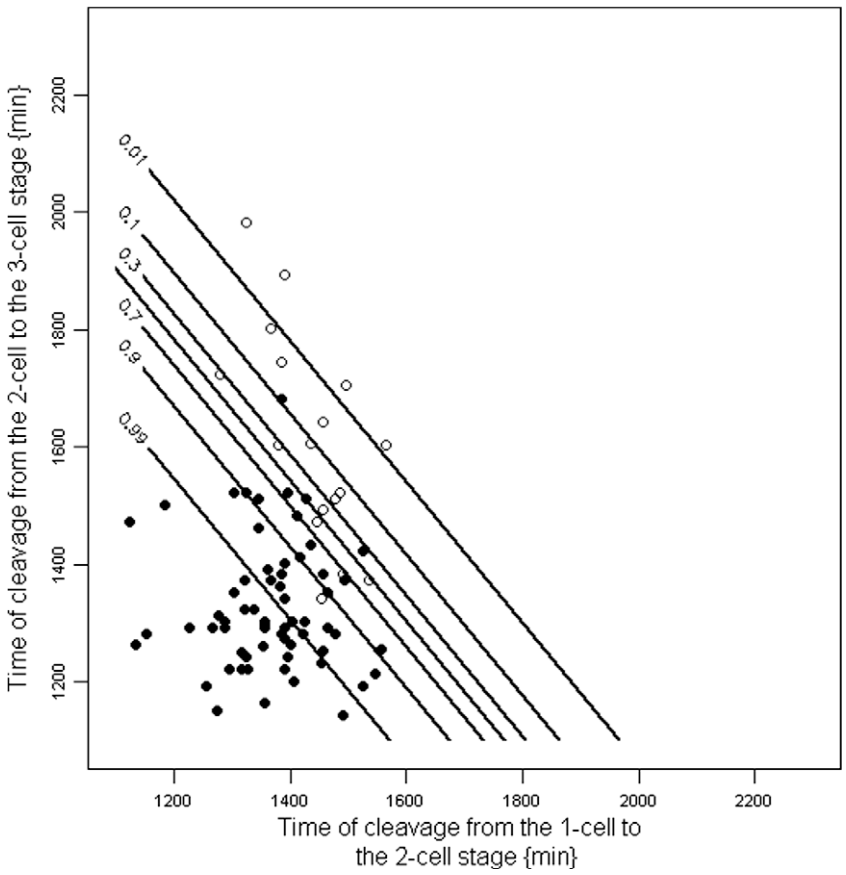

Figure 4 Probability of a non-fragmented embryo developing to the blastocyst stage in relation to the time of the first and second cleavages. The cleavage data of 90 embryos are presented on the image. Each data point (circles) represents an embryo with the corresponding cleavage times. Filled circles = embryo reached the blastocyst stage; open circles = embryo did not reach the blastocyst stage.

and D5) monitoring at 9 a.m. It can be concluded that, in a general case with 95% confidence interval, a minimum 15% or 39% and a maximum 61% or 94% of fragmentation will not be noticed at daily or bi-daily embryo monitoring, respectively.

Discussion

In contrast to the daily or bi-daily check of embryo development routinely used in human assisted reproduction, semi-continuous time-lapse microscopic investigation generally offers the following benefits. The length of phases and occurrence of morphological events including cleavage, compaction, blastulation, fragmentation and reabsorption can be exactly determined. Misinterpretation of certain phenomena can be avoided (for example a fixed time-point investigation may evaluate a large fragment as cleavage). The significance of some processes (fragmentation, blastocyst collapse) can be properly evaluated. A more authentic, dynamic view regarding embryo development can be obtained, including the discovery of new correlations. Embryos are spared from the potentially harmful effects of environmental changes and shear stress during removal from the incubator, observation under a microscope and returning to the incubator. The time-lapse approach provides embryologists with a greater decision-making process based on the real-time embryo status as well as the process of embryo development.

The possibility for establishing such time-lapse observations has existed for decades (Cole, 1967; Massip and Mulnard, 1980; Wright et al., 1976); however, technical, financial and conceptual drawbacks have hampered its application. Two solutions are commonly applied to solve these problems: to use a commercially available inverted microscope and to build an incubator chamber on/around the stage (Bavister, 1988; Hardarson et al., 2002; Holm et al., 2002; Van Blerkom et al., 2001; Stage-top Incubator, Tokai-hit, Japan) or to use a common laboratory incubator with an in-built modified inverted microscope and robotics or carousel to move the embryo holding dishes (Arav et al., 2008; InCu-Cell Live, Sanyo, Japan; Bio-Station, Nikon, Japan).

The time-lapse system applied in the present study consisted of a purpose-designed compact digital microscope, offering the possibility for routine embryo monitoring in everyday laboratory practice. Due to its small size it could be easily placed onto a single shelf of a common laboratory incubator. The simple design allows rapid loading and focusing of embryos and the compact arrangement minimizes the chance and also the consequences of accidental infection. Combined with the WOW systems, a parallel observation

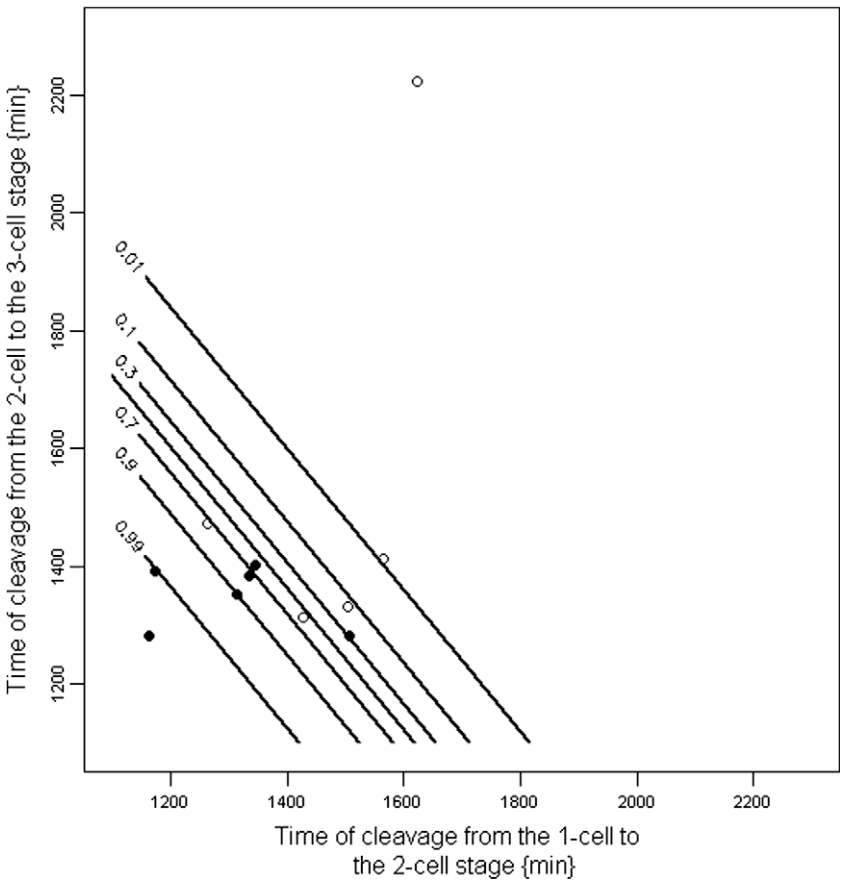

Figure 5 Probability of a fragmented embryo developing to the blastocyst stage in relation to the time of the first and second cleavages. The cleavage data of 11 embryos are presented on the image. Each data point (circles) represents an embryo with the corresponding cleavage times. Filled circles = embryo reached the blastocyst stage; open circles = embryo did not reach the blastocyst stage.

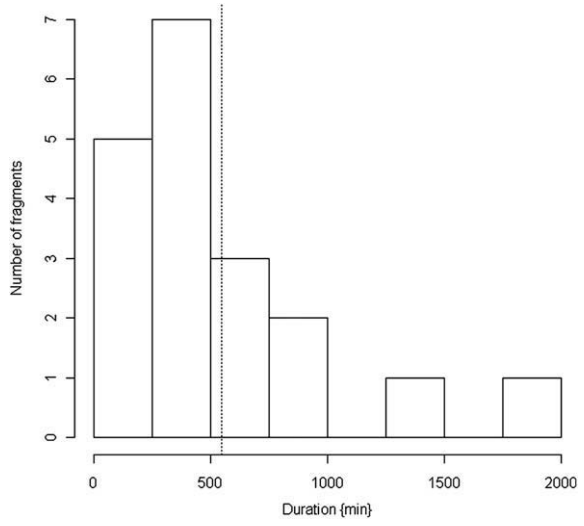

Figure 6 Duration of the appearing/disappearing fragments. The average time for fragments to appear/disappear was 546 ± 442 min (dotted line).

of nine embryos with a fixed position is possible. Due to the latest tendency to decrease hormonal stimulation, one camera may follow the development of all the embryos of one patient. One personal computer could control six such compact digital microscopes, allowing the time-lapse control of all patients in a medium sized ART unit. As a single microscope was assigned to one group of embryos, no movement of the embryos (via robotics or carousel) was needed. In this way, sheer stress, which may reduce embryo viability (Xie et al., 2006), was completely avoided. Furthermore, the safety of the developing embryos was increased by the protection from low-voltage electromagnetic field and extra heat accumulation caused by the presence of constant electric currents in digital microscopic units (Pribenszky et al., unpublished data).

The experiments with in-vitro-produced 2PN-stage mouse embryos cultured until the blastocyst stage (a standard test system in laboratory embryology) showed that embryo development was not compromised as a result of the time-lapse investigation.

The time-point of the first, and especially the second, cleavage shows a strong association with further development to the blastocyst stage. The predictive value of these data appears to be higher than in previous investigations made with fixed-point detection of developmental stages (referred to earlier) and is in accordance with the time-lapse investigations of Arav et al. (2008) and Lemmen

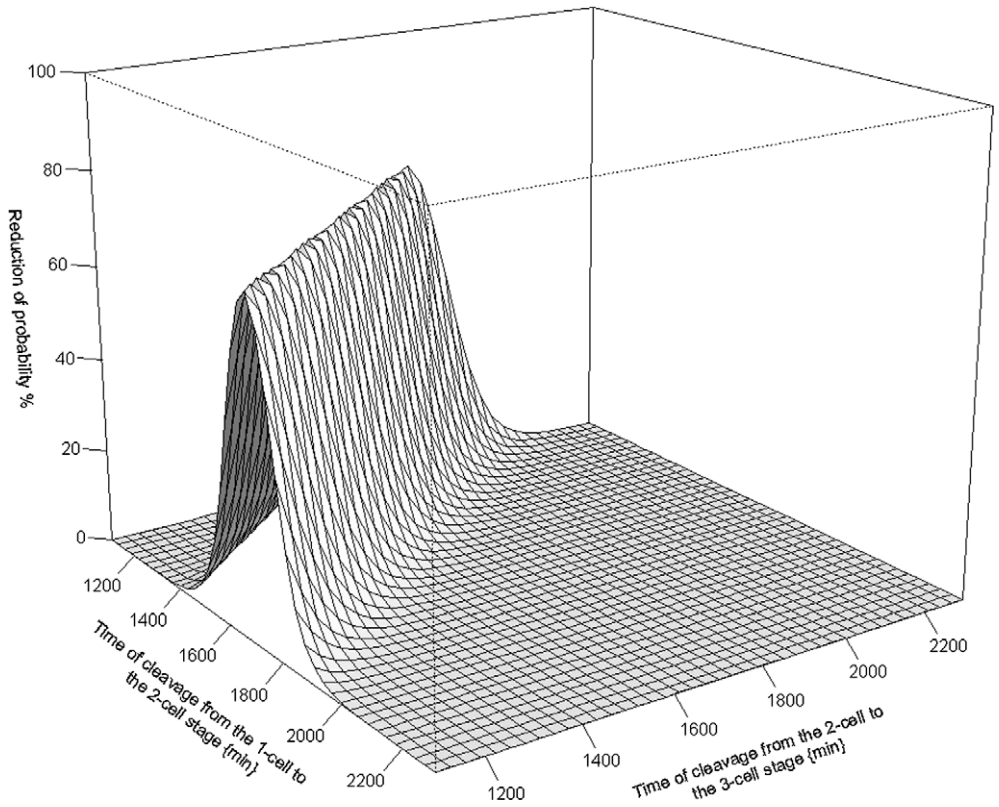

Figure 7 Reduction of the probability of reaching the blastocyst stage if a fragmentation occurs during embryo development. Data for 11 fragmented embryos were analysed. If the first and second cleavage of an embryo occurred before 1300 and 1200 min, respectively, the chance of blastocyst development (~100%) is independent of the occurrence of fragmentation. If the first and second cleavage of an embryo occurred after 1500 and 1700 min, respectively, the chance of blastocyst development (~0%) is also independent from the occurrence of fragmentation. However, if the first and second cleavage of an embryo occurred between these time-intervals, respectively, a fragmented embryo may have a 60% less chance to develop to blastocyst than a non-fragmented embryo.

et al. (2008). As far as is known, there has been no previous data published showing the extremely strong relationship between the time-point of the second cleavage (to 3-cell stage) and blastocyst development. If such a relationship also exists in humans, it will provide considerable help in determining the optimal day for transfer and other related procedures. The third cleavage, from the 3-cell to the 4-cell stage, occurred within 10—90 min after the second cleavage. Since the duration of the third cleavage had no effect on the continued embryo development, this study can conclude that synchronized cleavage did not show any relationship with embryo quality. Late cleavers can also develop to the blastocyst stage but most of these embryos showed reduced quality: blastomere necrosis at 4—8-cell stage or early compaction.

Another important predictive factor is fragmentation during embryo development. Although internalization of these fragments has been suggested and/or documented earlier (Hardarson et al., 2002; Van Blerkom et al., 2001), the dynamics, significance and predictive value of fragmentation is not entirely clarified yet. According to this study's observations in the mouse, fragmentation was usually a reversible process and the formation and internalization of fragments was a relatively rapid event with an average length of 9 h that may occur anytime during pre-hatching development. If the dynamic is similar in human embryos, the current routine morphological investigation of embryos may detect fragmentation rather accidentally, with a probability of 63.6% and 27.3% for daily and bi-daily control, respectively, although more experiments need to be conducted to confirm these percentages. In mouse embryos, a strong negative relationship was observed between fragmentation and blastocyst development. Previous investigations in human embryos described similar fragmentation events (Hardarson et al., 2002; Lemmen et al., 2008). Accordingly, the routine application of time-lapse investigation may have a strong predictive value in humans.

In conclusion, time-lapse investigation of developing mouse preimplantation embryos has uncovered two important events with strong predictive value regarding further developmental competence, i.e. the time of the first and especially second cleavage, and the occurrence of fragmentation. The simple, compact time-lapse instrument combined with the WOW system was suitable for monitoring these events without compromising embryo development. As previous studies indicate that similar events occur during human embryo development, time-lapse investigation may become a routine tool in human assisted reproduction laboratories to predict the developmental competence of

embryos and to support the right decision for further procedures.

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