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CASE REPORT

Pregnancy achieved by transfer of a single blastocyst selected by time-lapse monitoring

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Csaba Pribenszky obtained a DVM degree in 1998 and a PhD degree in 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 sublethal hydrostatic pressure stress treatment to gametes, embryos, other cells and tissues in order to improve cell survival during subsequent ART procedures (e.g. cryopreservation). His current focuses are the application of the above method at the vitrification of stem cells and human oocytes, and the effects of different environmental factors on the in vitro embryo developmental dynamics and fragmentation.

Abstract Appropriate selection of a single blastocyst for transfer decreases the risk of multiple gestations. By using a compact time-lapse microscope system placed inside a regular incubator, combined with a microwell embryo culture dish, the development of all the embryos from a patient was continuously monitored by obtaining images at 10 min intervals. The embryos were not moved during the time-lapse observation. The system was switched off completely between image acquisitions in order to avoid exposure to electromagnetic radiation. The analysis of time-lapse records was used to choose a single blastocyst for transfer, which resulted in a singleton pregnancy and birth of a healthy boy on term.

KEYWORDS: early cleavage, fragmentation, single-embryo transfer, time-lapse monitoring

Introduction

Multiple gestations carry significant maternal and neonatal risks and are considered an adverse outcome of assisted reproductive technology (Pinborg, 2005). Appropriate selection of a single blastocyst for transfer may decrease the risk of multiple gestations while maintaining high pregnancy rates. Apart from the traditional morphological assessment before transfer, various methods were suggested for the selection procedure, including the measurement of oxygen consumption, protein production and other metabolic parameters (Nagy et al., 2009). Another approach is to evaluate morphological development over the whole period of in vitro culture. Time-lapse observation of in vitro development of mammalian embryos has quite a long history dating back to 1929 (Lewis and Gregory, 1929). Studies showed that the timing of early cleavages has strong correlation to in vitro embryo developmental competence in porcine,

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bovine, murine, hamster or human (Gonzales et al., 1995; Mateusen et al., 2005; McKiernan and Bavister 1994; Pribenszky et al., 2010; Racowsky et al., 2000). Fragmentation during the course of in vitro embryo development has been linked to compromised developmental competence; also reported is that the abundance and pattern of fragments define its severity (Mateusen et al., 2005; Van Blerkom et al., 2001). By using a compact time-lapse microscope and camera combined with the well of the well (WOW; Vajta et al., 2008) embryo culture system, Pribenszky et al. (2010) reported recently that the time-points of the first and second cleavages, as well as the occurrence of fragmentations, have a strong predictive value regarding the in vitro developmental competence in mouse. In the present study, the same time-lapse system was used to monitor the development of all embryos from a patient and aided in the selection of a blastocyst for single-embryo transfer.

Case report

The patient was a 37-year-old nulliparous single woman. She had regular 27—28 day cycles, a history of laparoscopic ablation for endometriosis and a subsequent depot gonadotrophin-releasing hormone agonist therapy in 2005. A recent hysterosalpingogram revealed bilateral patent Fallopian tubes and intact uterine cavity. Hormonal evaluation showed normal ovarian function. Following four failed donor insemination cycles, the clinic proceeded with IVF. Following 12 days of stimulation, recombinant human chorionic gonadotrophin (rHCG, Ovitrelle; Merck Serono) was administered to induce ovulation and transvaginal oocyte retrieval was performed 35 h later. All of the five retrieved eggs were subjected to intracytoplasmic sperm injection with donor spermatozoa and all of them presented two pronuclei 18 h later. Intracytoplasmic sperm injection was chosen because of the low number of oocytes retrieved and history of previous failed inseminations.

The main features of the digital time-lapse microscope/camera system have been described previously (Pribenszky et al., 2010). In brief, a custom-made, compact, sealed, digital inverted microscope/camera unit (Primo Vision System; Cryo-Innovation, Budapest, Hungary) was placed inside a water-jacketed CO_2 incubator (model 3111; Forma Scientific) (**Figure 1**).

All pronuclear-stage embryos of the patient were placed individually into the microwells of a specially designed WOW dish (nine microwells in 3×3 matrix; the diameters and the depth of a well are 550 μm × 450 μm and 170 μm; wells are 100 μm apart from each other; Cryo-Innovation), containing a single 50 μl micro droplet of G1.5 medium (Vitrolife, Sweden) over the microwells and covered with Ovoil (Vitrolife). The dish was placed on the top of the glass window of the microscope and the objective was mechanically focused (**Figure 2**). A 5 megapixel charge-coupled device captured approximately 4 mm^2 field of view, containing all the embryos. The camera was set to take a single picture every 10 min. The transfer of the images and the control of the digital inverted microscope were managed by a PC software program through an electric controlling unit that was located outside the incubator and connected to the microscope with a shielded firewire cable through the factory-made side port of the incubator. The computer screen displayed the actual developmental stage of the embryos, while all the recorded images were saved to be analysed later using a special software program developed for this purpose. The total magnification on the screen

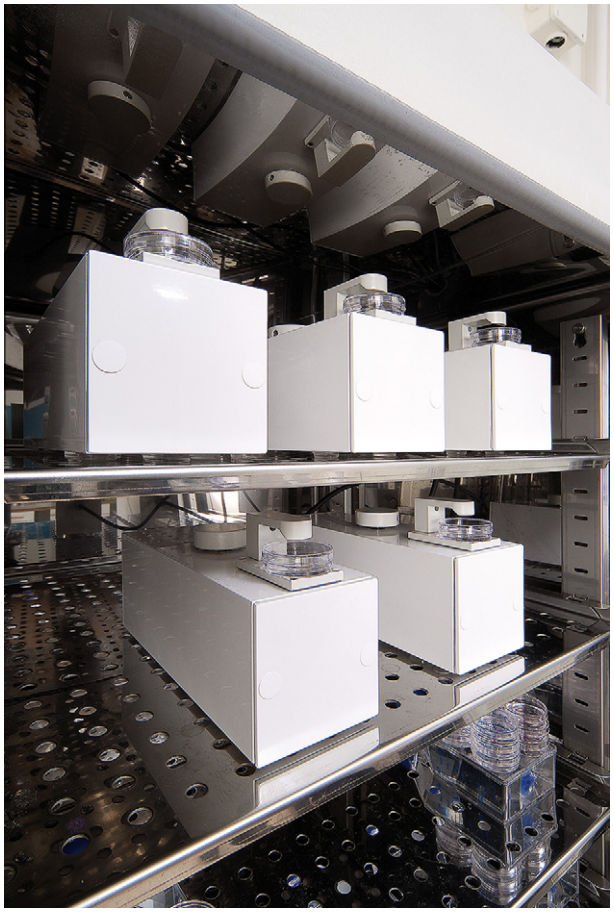

Figure 1 Compact digital inverted microscopes, placed inside the incubator. Microscopes are connected to a controlling hub outside the incubator that transfers the images to the PC for analysis and review.

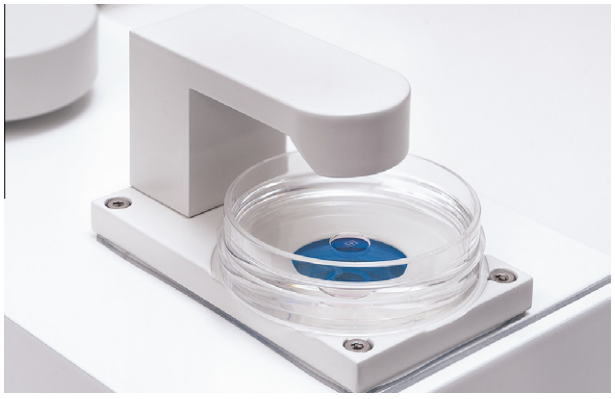

Figure 2 Position of the WOW dish on the console of the digital inverted microscope. The matrix of the nine wells keeping the embryos in the field of view is located in the middle of the dish.

was approximately ×150, the resolution of the image was 1 pixel/1 μm.

The culture medium was changed on day 3. The dish was removed from the incubator and all embryos were transferred to the same position into another WOW dish containing 50 μl microdroplet of G2.5 medium covered with Ovoil. The dish was then returned to the incubator, placed in the sample holder of the digital microscope and the time-lapse monitoring was continued.

By day 5, two embryos had reached the expanded blastocyst stage, one was at the blastocyst stage and two remained at early cleavage stages. According to the wish of the patient, a single blastocyst was selected and transferred on day 5 using a Wallace catheter. The selection was based on the retrospective analysis of the time-points of the first, second and third cleavages and the occurrence of fragmentations during embryo development. The times of these observations are summarized in **Table 1**. Embryo 5 was chosen for transfer on the basis of the lack of fragmentation, early cleavage to the 2- and 3-cell stages and synchronized cleavage to the 4-cell stage.

The luteal phase was supported by micronized progesterone (3 × 200 mg Utrogestan; Lab Besius). Twelve days after the transfer, the patient had a positive serum pregnancy test. The progesterone support was continued. At week 6 of pregnancy, a transvaginal sonogram showed an intact intrauterine gestational sac with a yolk sac. Two weeks later, an 18 mm intrauterine embryo with cardiac activity was detected. Progesterone support was maintained until week 9. Following an uncomplicated pregnancy a healthy boy was born on term.

Discussion

As far as is known, this case study is the first evidence that continuous monitoring of multiple human embryos with a compact time-lapse system between days 1–5 allows normal development to the blastocyst stage, followed by a successful pregnancy with the transfer of a single blastocyst that was selected based on the lack of fragmentation and times of cleavage.

Special features of the applied time-lapse system included the lack of movement of the dish between image acquisitions, because all the embryos of the patient were in the same field of view. Additionally, to achieve a stress-free environment, electricity was completely switched off between image acquisitions by the control box located outside the incubator. Accordingly, no continuous electric currents or electromagnetic radiation were present around the embryos. Electromagnetic radiation has been reported to affect in vitro embryo development by altering the speed and synchrony of cleavages, gene expression and enzymic activity of the embryos of different species and to reduce in vitro embryo survival (Beraldi et al., 2003; Cameron et al., 1993; Ravera et al., 2006). The approximate light intensity in the time-lapse imaging system was ~6 $\mu W/cm^2$ compared with the ~80 mW/cm^2 measured in a general inverted microscope, so even with imaging every 10 min, the total light energy to which the embryos were exposed for 5 days was approximately 10% of that which an embryo would be exposed to during routine daily checks. Other potentially harmful factors, including the light of the laboratory itself, the change in the environment during manual investigation (temperature, pH) and the sheer stress caused by moving of dishes were also eliminated with the time-lapse follow-up. The system has proved to be harmless in mouse (Pribenszky et al., 2010) and triploid human embryos (Matyas et al., unpublished observation).

It has been previously shown that the time-point of the first and second cleavage of mouse embryos (leading to 2- and 3-cell stages) was related to subsequent in-vitro development (Pribenszky et al., 2010). The earlier these cleavages occurred, the higher was the probability of reaching the blastocyst stage. It was also observed that all mouse embryos completing these two cleavages before a given

Table 1 Cleavage and blastulation times elapsed from the time of the ICSI and the occurrence of fragmentations during in vitro embryo development.

Embryo No.	*ICSI*	*2PN*		*2-Cell stage*		*3-Cell stage*		*4-Cell stage*		*Blastulation*	*Embryo transfer*
		Time	*Fragm*	*Time*	*Fragm*	*Time*	*Fragm*	*Time*	*Fragm*	*Time*	*Time*
1	00:00:00	00:18:00	PB	01:00:39	50	01:07:49	50	01:17:49	90		
2	00:00:00	00:18:00	1–2	01:06:09	0	01:15:19	0	01:17:59	70 after 8-cells		
3	00:00:00	00:18:00	2–3	01:02:39	2–3	01:16:27	2–3	01:21:09	Fragments not visible any more	04:05:16	
4	00:00:00	00:18:00	1–2	01:03:29	0	01:19:09	0	01:23:59	Only two blastomeres cleaved and then compacted	04:22:00	
5	00:00:00	00:18:00	0	01:02:19	0	01:13:00	0	01:14:49	0	04:02:34	05:00:00

Times are day:hour:minute.
PB: polar body; PN: pronuclei; Fragm: fragments in the embryo (%).

time-point will reach the blastocyst stage. Human data also supports this correlation, with additional reports that embryos cleaving too early may have potentially compromised developmental competence because of inappropriate imprinting and chromosomal abnormalities (Harper et al., 1994). However, the developmental potential is also influenced by the occurrence of fragmentation. Although most fragments disappear in a limited time frame, mouse embryos with temporary fragmentation have a lower chance to develop to blastocyst. If these correlations are confirmed in humans, time-lapse investigation will be useful in the selection of the embryo with the potentially highest developmental potential as early as the 3rd day for single-embryo transfer.

In the presented case, the analysis of time-lapse records was used to choose from two blastocysts of identical morphological quality on day 5. The blastocyst with faster cleavage and no fragmentation was selected for transfer, which resulted in a singleton pregnancy and birth of a healthy boy on term.

A single pregnancy is insufficient to prove the value of time-lapse investigation for these purposes. However, these results indicate that the monitoring process was not harmful for the development of preimplantation human embryos and that it could be used to provide data for the selection for single-embryo transfer resulting in an ongoing pregnancy. This case report should encourage further research to exploit the potential of time-lapse monitoring for prediction of preimplantation development and the selection of the most appropriate embryo for transfers in humans.

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Authors' contributions: C Pribenszky: development of the method, device and dish; P Kovács, MD: embryo transfer; S Mátyás, embryologist: development of the dish, care of the embryos and making the selection; E Losonczi, embryologist: mouse embryo assays during the development of the method; J Zádori, MD: licencing; G Vajta: idea and development of the dish.

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