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Blastocyst collapse as an embryo marker of low implantation potential: a time-lapse multicentre study

Research Article

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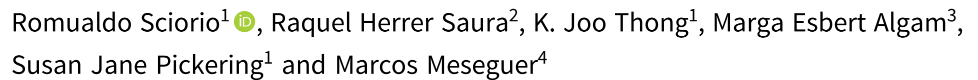

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Summary

Spontaneous blastocyst collapse during *in vitro* embryo development has been suggested as a novel marker of embryo quality. Therefore, the aim of this multicentre study was to carry out a retrospective multicentre analysis to investigate the correlation between blastocyst collapse and pregnancy outcome. Here, 1297 intracytoplasmic sperm injection (ICSI)/*in vitro* fertilization (IVF) fresh cycles, with an elective single blastocyst transfer (eSET) were included in this study. Embryos were cultured individually in 6.0% CO_2, 5.0% O_2, 89.0% N_2, using single step medium (GTL^{TM} VitroLife, Sweden) or sequential medium (Cook^{TM}, Cook Medical, Australia) and selected for transfer using standard morphological criteria. With the use of time-lapse monitoring (TLM), blastocysts were analyzed by measuring the maximum volume reduction and defined as having collapsed, if there was ≥ 50% volume reduction from the expanded blastocyst and the collapse event. Following embryo replacement, each blastocyst was retrospectively allocated to one of two groups (collapsed or not collapsed). Here, 259 blastocysts collapsed once or more during development (19.9%) and the remaining 1038 either contracted minimally or not collapsed (80.1%). A significantly higher ongoing pregnancy rate (OPR) of 51.9% (95% CI 48.9–59.9%) was observed when blastocysts that had not collapsed were replaced compared with cycles in which collapsed blastocysts were transferred 37.5% (95% CI 31.6–43.4%). This study suggests that human blastocysts that collapse spontaneously during development are less likely to implant and generate a pregnancy compared with embryos that do not. Although this is a retrospective study, the results demonstrated the utility of collapse episodes as new marker of embryo selection following eSET at blastocyst stage.

Introduction

Assisted reproductive technologies (ART) have evolved considerably over the past 4 decades, with several key advances and historical achievements (Steptoe and Edwards, 1978; Wang 2011; Zhao *et al.*, 2011). Despite these improvements, live birth after IVF treatment is not guaranteed, with almost half of the patients treated having an unsuccessful outcome, even after several treatments. Currently, embryo selection is primarily based on morphology assessments that can have high interobserver variability (Wang, 2011; Braude, 2013). The introduction of more complex culture medium has facilitated the generation of better quality embryos thereby enabling extended culture to blastocyst stage (day 5/6) prior to transfer in many more patients. Extending embryo culture allows selection of embryos at more advanced stage, and improves both uterine and embryonic synchronicity, therefore resulting in better live birth rates (De Vos *et al.*, 2016). Reviews published by Blake *et al.* (2007) and Glujovsky *et al.* (2016) reported a significant difference in live birth rate in favour of blastocyst transfers compared with transfers at cleavage stage. Embryo development is a dynamic process in which the morphology changes significantly in a short time (Lemmen *et al.*, 2008). Recently, continuous embryo culture using TLM has allowed embryologists to analyse the dynamic process of embryo development and follow the whole sequence of embryonic evolution from fertilization to the blastocyst formation (Meseguer *et al.*, 2011b; Basile *et al.*, 2014; Campbell 2014; Aparicio-Ruiz *et al.*, 2016). Cruz *et al.* (2012) and Wong *et al.* (2010) found that development of human embryos to the blastocyst stage was linked to key timing events in early embryo development such as the duration of the first cleavage (cytokinesis), and the length of the interval between divisions in the first stages of embryonic development. In addition, TLM provides a stable environment that avoids the need to move embryos or expose these to non-optimal conditions outside the incubator such as fluctuating temperatures, humidity, pH and gas concentrations (Zhang *et al.*, 2010). The most widely used blastocyst grading system is that originally proposed by Gardner and Schoolcraft (1999). Although the system does not cover all aspects of blastocyst morphology, it has been very

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effective in classifying the appearance and compactness of the inner cell mass (ICM), the cohesiveness and number of trophectoderm (TE) cells and degree of expansion of the blastocoels cavity.

Formation of the blastocoel cavity and collapse episode

Preimplantation development is a time of dynamic changes and reprogramming. The fertilization process is normally followed by several cleavages that takes place at an interval of roughly 12 h. During the 8-cell stage, around day 3, the first signs of morphological differentiation appear: following the activation of intracellular adhesion, blastomeres begin to flatten on each other in a process known as compaction (Fleming *et al.*, 2001). Eventually, cells become indistinct from each other and the embryo forms a compact morula. Cells become polarized with apical and basolateral domains on all blastomeres. As cell division continues, some cells are confined exclusively to the centre of the embryo and some to the outside layer. These two distinct groups of cells receive different signals, and this eventually leads to the formation of two different cell types with the respect to their positions in the embryo. The cells on the inside of the morula generally give rise to the ICM, which is the only pluripotent cell lineage in the blastocyst. The ICM forms the epiblast and hypoblast layers, which develop into the embryonic tissues and extra-embryonic tissue, respectively. The cells confined to the outer portion of the embryo differentiate exclusively into the trophectoderm (TE), which gives rise to outer chorionic sac and the fetal component of the placenta and has a major role in embryonic implantation following embryo transfer (Yamanaka *et al.*, 2006). Tight junction formation in the TE layer is crucial for the formation of the blastocoel cavity. Junctional complexes (adherens, tight, desmosomes and gap junctions) are established between trophectoderm cells and allow the formation of the first tissue-like structures within the embryo. The TE acts as permeability barrier for ions and water molecules (Ducibella *et al.*, 1975; Hastings and Enders, 1975; Eckert and Fleming, 2008) and facilitates fluid transportation across the epithelium leading to an accumulation inside the blastocyst, eventually leading to the formation of a blastocoel cavity (Biggers *et al.*, 1988). The blastocoel gradually expands during development as more fluid accumulates. An osmotic gradient is created between the cavity and the external environment, mainly generated by actions of the sodium pump Na+/K+-ATPase (Watson *et al.*, 2004). The osmotic pressure alone is not enough to move water molecules across the plasma membrane (Biggers *et al.*, 1988). Therefore, designated water channels, aquaporins, are mandatory in increasing the water influx through the cells (Marikawa and Alarcon, 2012). The increased water pressure leads to expansion of the cavity, which continues throughout blastocyst formation causing progressive enlargement of the blastocyst with progressive thinning of the zona pellucida (ZP) and finally the hatching of the blastocyst. When blastocysts expand, fluid gradually accumulates in the blastocoel resulting in increased pressure on both the TE and ZP (Baltz *et al.*, 1997). At the same time, TE cells secrete lysine that is involved in ZP thinning and hatching. Expansion and ZP thinning occur in mammalian blastocysts prior to hatching (Biggers, 1998). As well as blastocyst expansion and ZP thinning, blastocyst collapse episodes have also been documented by time-lapse monitoring (TLM) in mouse (Glass *et al.*, 1973) and hamster blastocysts (Gonzales and Bavister, 1995). To start the implantation process, it is essential for the embryo to escape the ZP and start to contact endometrial cells. Several studies have described the process of ZP escape *in vitro* in which the blastocyst expands, causing the ZP to become stretched, thin and then to rupture, allowing the blastocyst to extrude in a characteristic 'dumb-bell' shape, the so-called 'hatching' process. The sequence of events during hatching *in vitro* has been described in different species including rabbit, mouse, rat, pig, cow and monkey (Sireesha *et al.*, 2008; Seshagiri *et al.*, 2009; Erbach *et al.*, 2013). The process seems to be regulated by a variety of autocrine and paracrine molecules and by the presence of trophectodermal projections (TEPs). TEPs are cytoplasmic extensions of trophectodermal cells that protrude through the ZP into the extra-embryonic environment (Gonzales *et al.*, 1996). In addition, it is known that implantation serine proteinase 1 (ISP1) and serine proteinase 2 (ISP2) play a critical role in the processes of embryo hatching and implantation, at least in mouse model (O'Sullivan *et al.*, 2001; Sharma *et al.*, 2013). ISPs are necessary for blastocyst hatching *in vitro* and the initiation of invasion. Inhibitors of ISPs are able to prevent murine embryo implantation pointing to a key role in this critical process (Huang *et al.*, 2004; Sharma *et al.*, 2006; Sun *et al.*, 2007). Collapse episode was first described in mammalian embryos in 1929 (Lewis and Gregory, 1929). They monitored *in vitro* cultured rabbit blastocyst for 8 days by time-lapse cinematography, and found that blastocysts repeatedly contracted and re-expanded during development. In mice, early blastocysts have been reported to contract from four to 15 times during an 18 h culture period, and to exhibit a strong robust collapse and several modest and small contractions (Cole, 1967; Massip and Mulnard, 1980). Cattle blastocysts required 13–17 min to collapse and 6–10 h for re-expansion, and this was repeated up to three times before completion of the hatching process (Gonzales *et al.*, 1996). Despite demonstration of the collapse episode in several mammalian species, the role and the mechanism of blastocyst collapse *in vitro* remains unclear. Huang *et al.* (2016) in a recent study analyzed 38 embryos from egg donation cycles with known implantation outcome (KID; Alikani *et al.*, 2000). The study described abnormal kinetics of blastocyst expansion and slower blastocyst formation in embryos that present collapse events and further a negative effect on implantation and pregnancy rates. Another retrospective study with egg donation and autologous cycles showed that some kinetic parameters such as time to reach morula stage and blastulation were significantly shorter in blastocysts that display a collapse event during their development. The results showed a reduction in implantation rate from 48.5% to 35.1% in those blastocysts that displayed a collapse episode (Marcos *et al.*, 2015). However, that was a small study, therefore the goal of this study was to provide additional evidence and establish in a multicentre study, the correlation between collapse event and pregnancy outcome for unselected IVF/ICSI patients. To our knowledge, this is the first multicentre attempt to monitor blastocyst collapse *in vitro* using TLM and correlate with implantation and pregnancy outcome.

Materials and methods

This is a retrospective multicentre study, carried out between January 2016 and February 2017 in four different units: Edinburgh Assisted Conception Programme, Edinburgh Fertility and Reproductive Endocrine Centre (EFREC), Royal Infirmary of Edinburgh, UK, IVIRMA Valencia, IVIRMA Barcelona and IVIRMA Zaragoza in Spain. *In vitro* embryo culture was performed using an EmbryoScope time-lapse incubator (EmbryoScope^{TM} Time-lapse system, VitroLife, Göteborg, Sweden) which is CE certified and conformed to the health and safety requirements for equipment in the European Union. In this

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study, it was used for the purposes for which it was approved. The procedure and protocol for analyzing embryos were approved by an Institutional Review Board (IRB reference 1507-BCN-052-ME), which controls and approves database analyses and clinical IVF procedures for research at IVIRMA. In total, 1297 eSET at blastocyst stage were analyzed and particularly we looked for the presence of blastocyst collapse event(s). For this study, the blastocyst volume reduction was measured using EmbryoViewer^{TM} workstation drawing tools. We have defined blastocyst collapse if there was a volume reduction ≥50%, from the expanded blastocyst and the collapse episode. If the volume reduction was <50% the event was considered as a contraction. Blastocyst transfer was scheduled on the morning of day 5, approximately 110–120 h after insemination. We included only fresh eSET, as this avoids the presence of confounding information for cycles with double embryo transfer and only one implanted embryo. Embryo implantation was confirmed by an ultrasound scan for gestational sac with fetal heartbeat after 7 weeks of pregnancy. These embryos were defined as known implantation data (KID) (Alikani *et al.*, 2000).

Ovarian stimulation

All patients received controlled ovarian stimulation with gonadotropin-releasing hormone (GnRH) antagonist (subcutaneous Cetrorelix 0.5 mg daily, Merck Serono) treatment. Ovarian stimulation was carried out using either Gonal F (Merck Serono) or Menopur (Ferring) based on individual patient characteristics. Follicular development was monitored by transvaginal ultrasound and ovulation was triggered when three follicles were 18 mm or above. Each patient received hCG (Ovitrelle 0.25 mg, Merck Serono) to trigger ovulation. Oocyte recovery was carried out under conscious sedation with transvaginal ultrasound guidance at 35 h after Ovitrelle injection.

Oocyte collection, fertilization and embryo culture: Assisted Conception Programme, EFREC, RIE, Edinburgh, UK

Cumulus–oocyte complexes (COC) were isolated from follicular fluid and then rinsed and cultured in 0.5 ml equilibrated G-IVF^{TM} medium (VitroLife, Sweden) at 37ºC and 6.0% CO_2 in air atmospheric in a Hera cell 240 incubator (Thermo Scientific). Sperm used for either routine insemination or intracytoplasmic sperm injection (ICSI) procedure was collected by masturbation and processed using a standard method as described by Bourne *et al.* (2004). All oocytes were cultured in G-IVF^{TM} on the day of insemination (day 0) as described by Sciorio *et al.* (2018). These were inseminated by IVF or ICSI according to the patient's aetiology and history. Fertilization was identified by the presence of two pronuclei approximately 16–19 h after insemination or microinjection. At this stage, normally fertilized pronuclear stage embryos were allocated to the EmbryoScope time-lapse incubator for culture in a 12-well EmbryoSlide^{TM}, which is a specifically designed dish for the EmbryoScope^{TM} imaging system (VitroLife, Sweden). This slide has 12 individual wells for embryo culture, each well contains 25 µl of culture medium with 1.4 ml overlay of mineral oil (VitroLife, Sweden) to prevent evaporation. *In vitro* culture was performed in an atmosphere of 6.0% CO_2, 5.0% O_2, 89.0% N_2 at 37ºC, using VitroLife single step time-lapse medium, G-TL^{TM} (VitroLife, Sweden) which is designed for the time-lapse incubator, allowing undisturbed culture.

Oocyte collection, fertilization and embryo culture: IVI Valencia, IVI Barcelona and IVI Zaragoza, Spain

Follicles were aspirated and the oocytes were washed in HEPES medium (LifeGlobal, Canada) and cultured using a fertilization medium (Cook^{TM}, Sydney, Australia) at 6.0% CO_2, 5.0% O_2, 89.0% N_2 at 37°C for 3 h before oocyte denudation. Removal of granulosa cells from the oocyte was carried out by mechanical pipetting in 1:1 hyaluronidase and fertilization medium (final concentration of hyaluronidase of 40–80 IU/ml) prior to ICSI. Sperm were analyzed for count, motility and concentration followed by washing performed in Global fertilization medium (LifeGlobal, Canada) and ICSI procedures were performed in Cook fertilization medium containing HEPES (Cook^{TM}, Sydney, Australia), as described by Motato *et al.* (2016). Finally, the zygotes were placed inside a pre-equilibrated culture slide (EmbryoSlide^{TM} VitroLife, Sweden) containing 12 wells each of 25 µl medium with 1.4 ml overlay of mineral oil (Cook^{TM}, Sydney, Australia). Slides containing zygotes were placed in the EmbryoScope immediately after ICSI and incubated for 5 days. Embryo culture was performed using cleavage medium up to day 3, changing to new pre-equilibrated blastocyst medium from day 3 to day 5 (Cook^{TM} Sequential medium, Sydney, Australia). Slides were incubated at 6.0% CO_2, 5.0% O_2, 89.0% N_2 at 37ºC. To determine the exact timing of cell divisions, images of each embryo were acquired every 15 min at resolution 1000 × 1000 pixels at seven different focal planes, starting from the placement of the embryos in the incubator a few minutes after ICSI until about 120 h after ICSI.

Embryo scoring and selection: Assisted Conception Programme, EFREC, RIE, Edinburgh, UK

In the EmbryoScope embryo culture, images were acquired every 15 min at seven different focal planes and morphological assessment was made by examining a video of development using the associated EmbryoViewer^{TM} software (EmbryoViewer^{TM} VitroLife, Sweden) without moving embryos from the incubation. Embryos were assessed morphologically at cleavage stage according to British Fertility Society and Association of Clinical Embryologists guidelines, published by Cutting *et al.* (2008). Blastocysts were classified according to degree of expansion of the blastocoel cavity (1–6), quality and cohesiveness of the ICM and trophectoderm cells (A–C), using Gardner's score (Gardner and Schoolcraft, 1999). Embryos were selected for transfer by combining blastocyst morphology and KID score (VitroLife, Sweden).

Embryo scoring and selection: IVI Valencia, IVI Barcelona and IVI Zaragoza, Spain

Embryo morphology was evaluated on day 2 at 48 and day 3 at 72 h post insemination, as previously described by Meseguer *et al.* (2011a) and Bellver *et al.* (2010). Embryo scoring and selection was performed by analysis of time-lapse images of each embryo on an external computer with software developed for time-lapse image analysis (EmbryoViewer^{TM} software, VitroLife, Sweden). Embryo morphology and developmental events were annotated, including the precise timing of the observed cell divisions in hours post ICSI. We used the hierarchical classification of embryos described by Meseguer *et al.* (2011b). Human blastocysts were scored on day 5 (110–120 h post insemination) according to the Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) criteria, which are based on assessing the expansion

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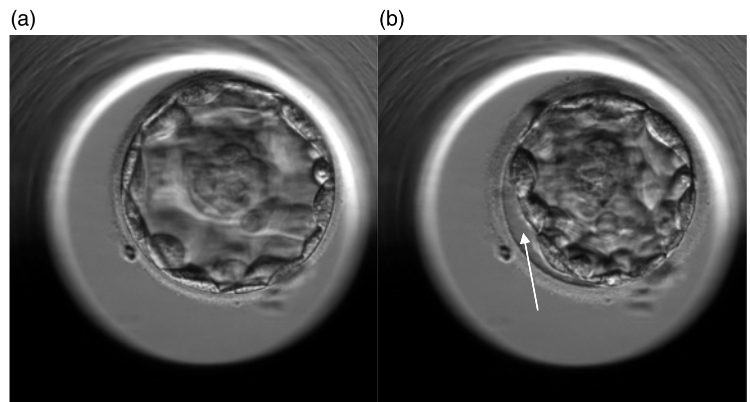

Figure 1. Time-lapse monitoring of cultured human blastocyst. (a) Embryo at the maximum expansion. (b) Embryo after the contraction event. Arrow shows a weak contraction (volume reductions less than 50%).

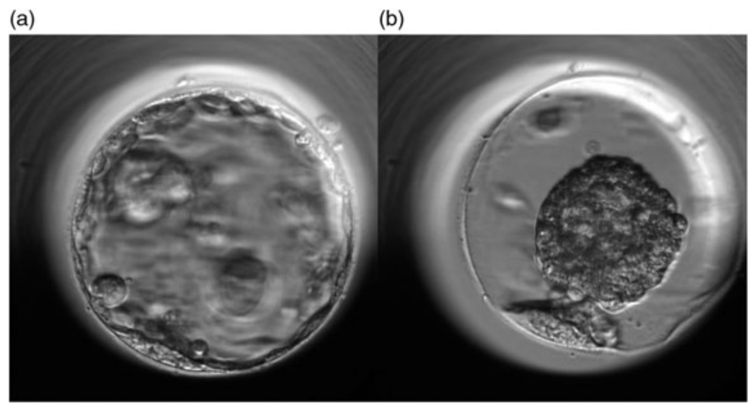

Figure 2. Time-lapse monitoring of cultured human blastocyst, collapse event with volume reductions of more than 50%. (a) Embryo at the maximum expansion. (b) Embryo after the collapse episode.

degree, ICM and trophectoderm appearance as described by Motato *et al.* (2016). Blastocysts were selected for transfer combining morphology and the morphokinetic algorithm described by Motato *et al.* (2016).

Definition, measurement of the collapse event

To analyze blastocyst collapse event, we used the EmbryoViewer™ workstation drawing tools to verify a blastocyst volume reduction ≥50%, from the expanded blastocyst and the collapse episode. We categorized the degree of collapse into the following two categories according to the percentage of volume reduction: weak when the volume reduction was <50% and we called 'contraction' (Fig. 1*a, b*), strong when it was ≥50% (Fig. 2*a, b*) and considered as 'collapse event'. We then correlated pregnancy outcomes according with these two events (collapse versus contractions).

Pregnancy test and clinical outcome

Chemical pregnancy was assessed based on serum β-HCG levels 9 days after the embryo transfer. At 7 weeks, a scan was performed to confirm the presence of fetal heart activity or gestational sac formation. The implantation rate (IR) was defined as the number of gestational sacs at the seven-week scan. A clinical ongoing pregnancy rate (OPR) was defined as a pregnancy with a fetal heart.

Statistical analysis and definition of outcomes

An analysis of variance was run to test for any significant differences in the mean of any continuous variable compared. Chi-squared tests were carried out to compare categorical data. The analysis included the transferred blastocysts. A logistic regression analysis was performed using the binary response parameter as: presence of a gestational sac with fetal heartbeat '1' (implanted blastocyst) or no gestational sac '0'. Embryos from EFREC were re-categorized according to ASEBIR criteria to be able to include the same variable related to morphology in all the categories. A further analysis was performed by combining the embryo morphological classification according to ASEBIR and blastocyst collapse. In the additional regression analysis, we included four potential bias variables [oocyte age (oocyte donors and autologous patients), type of cycle (donors and autologous), the clinic in which the study was performed (EFREC and IVI) and the fertilization method (IVF and ICSI)], which may interfere with the relationship between blastocysts collapse, morphology and implantation potential.

Results

On day 5, morphological analysis of blastocyst development was performed between 110 and 120 h post insemination. The collapse episodes were annotated retrospectively following completion of embryo transfer. Subsequently, the annotation was compared with the clinical outcome extracted from the unit's database. Therefore, the analysis and categorization of the degree of collapse of each transferred blastocyst was carried out 'blind', in that the embryologist were not aware of the clinical outcome. In total, 1297 cycles were analyzed and included in this study, all of which included elective single blastocyst transfer. Here, 259 blastocysts presented at least one collapse episode during development (19.9%) and the remaining 1038 either contracted minimally or had not collapsed

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Table 1. Pregnancy outcome of those patients which presented a collapsed blastocyst transferred compared with those which do not. Note: Unless otherwise indicated, values are means and 95% CI (between parentheses)

	Not collapsed (1038 patients)	Collapsed (259 patients)	*P*-value
Pregnancy + test rate (%)	61.0 (58.0–64.0)	45.2 (39.1–51.3)	<0.001
Miscarriage rate (%)	19.5 (16.4–22.6)	28.0 (19.9–36.1)	NS
Ongoing pregnancy rate (%)	51.9 (48.9–59.9)	37.5 (31.6–43.4)	<0.001
EFREC	56.3	17.5	<0.001
IVI	50.5	41.1	0.014
Incidence of collapse %			
EFREC	86.3	13.7	
IVI	78.2	21.8	0.002

EFREC, Edinburgh Fertility and Reproductive Endocrine Centre; IVI, IVIRMA Zaragoza, IVIRMA Barcelona, IVIRMA Valencia, Valencia, Spain; NS: not significant.

Table 2. Logistic regression analysis of ongoing (presence of gestational sac and fetal heart beat) as affected by blastocyst collapse, type of cycle, age (patients and donors) and blastocyst morphology

Model effect	Values	OR	*P*-value
Blastocyst contraction	Not versus Yes	1.99 (1.43–2.75)	<0.001
Type of cycle	Donation versus autologous	1.67 (1.15–2.44)	0.008
Age	Years	per year 0.99 (0.97–1.02)	NS
Blastocyst morphology	A versus D	2.31 (1.16–4.61)	0.001
Blastocyst morphology	B versus D	1.41 (0.71–2.77)	NS
Blastocyst morphology	C versus D	0.92 (0.43–1.94)	NS
Clinic	EFREC versus IVI	0.87 (0.45–1.79)	NS
Fertilization method	ICSI versus IVF	1.16 (0.60–2.23)	NS

EFREC, Edinburgh Fertility and Reproductive Endocrine Centre; ICSI, intracytoplasmic sperm injection; IVI, IVIRMA Zaragoza, IVIRMA Barcelona, IVIRMA Valencia, Valencia, Spain; IVF, *in vitro* fertilization; NS, not significant; OR, odds ratio with 95% confidence intervals in parentheses.

(80.1%). A small percentage of embryos showed multiple collapse events (two or three) as previously described (Marcos *et al.*, 2015). There were no differences in the characteristics of patients who had a collapsed blastocyst transferred compared with those who had transfer of a non-collapsed blastocyst. None of the parameters, including age and aetiology of infertility was significant, except that more women who received embryos that had exhibited collapse used donor eggs (61.0% versus 43.1%). According to a previous study (Marcos *et al.*, 2015), we noticed that the average timings of collapse were not different between implanted and not implanted embryos. Furthermore, the duration of the collapse event was not different between the two groups (implanted and not implanted) and was not associated with the embryo quality (Marcos *et al.*, 2015). The results described in Table 1 compare the clinical outcome between patients who had transfer of a collapsed blastocyst versus a non-collapsed blastocyst. A significantly higher positive pregnancy test rate of 61.0% (95% CI 58.0–64.0%) was observed when blastocysts that had not collapsed during development were replaced compared with cycles in which collapsed blastocyst were transferred 45.2% (95% CI 39.1–51.3%). In addition, a significantly higher OPR of 51.9% (95% CI 48.9–59.9%) was observed when blastocysts without any collapse were transferred compared with blastocysts which exhibited a collapse event during their development 37.5% (95% CI 31.6–43.4%). Although it did not reach statistical significance, there was a trend towards a higher miscarriage rates of 28.0% (95% CI 19.9–36.1%) in those cycles in which collapsed blastocysts were replaced compared with non-collapsed group 19.5% (95% CI 16.4– 22.6%; Table 1). The incidence of collapse reported in IVI clinics was significantly higher compared with EFREC clinic, although in both clinical settings ongoing pregnancy was significantly lower in patients who had had a collapsed blastocyst replaced (Table 1). IVF insemination cycles were only performed at EFREC clinic, and no difference was noted for blastocyst collapse events in relation to the insemination technique adopted (15.6% in ICSI versus 10.6% in IVF, $P > 0.005$, NS). A logistic regression analysis was performed on OPR to account for the effect of the possible confounding factors (Table 2). Blastocyst collapse events had a significant effect on the results, the odds ratio (OR) for the implantation of blastocysts in the collapse group versus the non-collapsed group was 1.99 (95% CI 1.43–2.75). Comparison between IR and morphological features of blastocysts transferred according to whether or not they had exhibited collapse is reported in Fig. 3. The distribution of IR was significantly different between collapsed and not collapsed blastocysts. We calculated the incidence of blastocyst collapse in each one of the morphology categories. Interestingly we observed that it was significantly higher in the poor morphology group compared with good morphology blastocysts [respectively C morphology (32.9%), B morphology (21.9%) and A (16.2%); $P < 0.001$].

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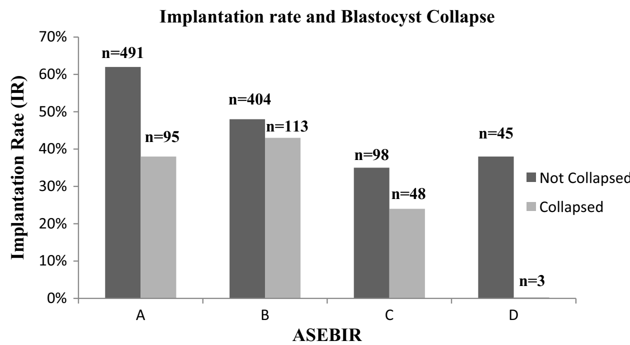

Figure 3. Implantation rate (IR) depending on blastocyst morphology following Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) criteria. The distributions of IR were significantly different between collapsed and not collapsed embryos when the blastocyst presented A morphology. Number of blastocyst in each category is presented. ASEBIR criteria considered expanded blastocysts to be the time when the zona pellucida starts to thin, so that the blastocoel is as full as possible. In addition it subdivided blastocysts into four categories from A to D described here: A: tightly packed many cells (compact); B: loosely grouped several cells (non-compact); C: very few cells; and D: degenerate or non-existent. The trophectoderm was assessed as follows: A: many cell forming a cohesive epithelium; B: homogenous but few cells; C: very few cells; and D: degenerate/degenerating. ASEBIR blastocysts were scored as follows. Score A is equivalent to 1Aa, 2Aa, 3Aa, 4Aa, 5Aa, 6Aa (Gardner's score adopted by the Edinburgh Fertility and Reproductive Endocrine Centre (EFREC) clinic). Score B is equivalent to 1Ba, 2Ba, 3Ba, 4Ba, 5Ba, 6Ba, 1Ab, 2Ab, 3Ab, 4Ab, 5Ab, 6Ab (Gardner's score). Scores C and D are equivalent to 1Cc, 2Cc, 3Cc, 4Cc, 5Cc, 6Cc, 1cC, 2cC, 3cC, 4cC, 5cC, 6cC.

Discussion

Previous studies in humans have demonstrated that, during *in vitro* embryo culture, spontaneous blastocyst collapse may adversely affect implantation and pregnancy outcome (Marcos *et al.*, 2015; Bodri *et al.*, 2016). The authors recommended against transferring such blastocysts if any other alternative was available. Accordingly, the aim of this multicentre study was to evaluate, in a large trial, if blastocyst collapse could be a predictive negative marker for embryo implantation. The molecular mechanisms associated with this process are still unclear. Massip and Mulnard reported that a moderate pulsatile contraction is not incompatible with normal hatching in cow blastocysts. Conversely, when blastocyst expansion is repeatedly interrupted by several collapses, hatching does not occur even when the ZP is broken (Massip and Mulnard, 1980; Massip *et al.*, 1982). Another study, published by Massip in the cow model observed a similar trend. In their analysis, 18.5% of blastocysts did not hatch at all, their expansion was interrupted by several rapid collapse events and re-expansions until they finally collapsed and degenerated (Massip *et al.*, 1982). In the mouse model, Niimura (2003) reported that blastocysts showing consecutive weak contractions reached, mostly, the stage of hatching, while those suffering strong collapse(s) failed to hatch. The author suggested that weak contractions (less than 20% volume reduction) might be normal and play an important role in the hatching process, whereas strong contractions (20% or more) may inhibit hatching. In addition, with the use of electron microscopy, the formation of intercellular ridges between trophectoderm cells on the surface of expanded blastocysts was described. Interestingly, after the collapse event such intercellular ridges did not appear. The author concluded that collapse events may be used to evaluate the developmental ability of mouse embryos (Niimura, 2003). Similar results were also reported by Iwata and colleagues, analyzing human embryos cultured *in vitro* using TLM. This study showed that some blastocysts underwent several collapse episodes and then finally degenerated. The number of collapses was significantly higher in unhatched blastocysts compared with those that hatched successfully (Iwata *et al.*, 2014). In the present study, we adopted a threshold value of 50% for blastocyst collapse for the following reason: an obvious collapse event (>50% volume reduction) could be easily identified and less prone to subjective evaluation. Additionally, if any reproductive consequence was produced by this behaviour it could be more noticeable. This multicentre study was carried out using different culture media between units. EFREC used VitroLife single step medium (G-TL^{TM} VitroLife, Sweden) and the IVI units adopted the sequential approach proposed by Cook (Cook^{TM}, Sydney, Australia). All units performed *in vitro* embryo culture with the same gas mixture concentrations (6.0% CO_2, 5.0% O_2, 89.0% N_2 at 37°C). Although the exact formulations of the two media used are not made public by the manufacturers, there are likely to be differences in energy substrates, phosphate and other micronutrients. A previous study has demonstrated with the use of TLM that the kinetics of embryo growth is similar for two distinct types of culture medium (Basile *et al.*, 2013). There is still a significant debate over the merits of these two culture media (single or sequential). However, the published literature suggests that these distinct approaches to metabolic support during preimplantation development produce comparable clinical outcomes (Lane and Gardner, 2007; Biggers and Summers, 2008). Based on the latter, we believe that it is unlikely that different culture media had a direct effect on the spontaneous blastocyst collapse observed, although this caveat cannot be ruled out. In addition, the average OPR in EFREC was 51.0% and 48.5% in IVI and this difference was not significant (P = 0.439). Therefore, in this study analyzing transfers of single blastocysts, culture media with differences in composition resulted in similar pregnancy outcome between clinics. Our data are in agreement with a study presented by Harton *et al.* (2016) at the ESHRE. The authors examined the relationship between blastocyst collapse patterns and euploid human embryos resulting in live birth. Fifty-five patients with 85 transferred euploid blastocysts from fresh/frozen cycles were analyzed. Results showed that euploid blastocysts that resulted in live births had statistically significantly lower frequencies of collapse episodes. Therefore, blastocysts that experienced a higher number and frequent collapses were less likely to achieve live births. A study published by Togashi *et al.* (2015), investigated by TLM the role of gap junctions in *in vitro* mouse embryo development. Particularly, they examined the role of gap junction inhibitors such as oleamide and heptanol. Severe collapse events were significantly more frequent in embryos cultured with oleamide or heptanol in comparison with control embryos. The authors reported that gap junction inhibition could delay the development to the blastocyst stage and induce collapse events (Togashi *et al.*, 2015). Gap junctions are fundamentally important during early preimplantation development, not only for intracellular communication but also in the maintenance of cellular homeostasis (Houghton, 2005). In line with that, although we cannot directly demonstrate that dysfunction of gap junctions is the cause of the collapse events observed, we hypothesize that there might be a connection between these phenomena, and that disruption of gap junctions may negatively affect implantation potential in human blastocysts. As previously described, blastocyst expansion is dependent on several mechanisms for the accumulation and retention of fluid in the blastocoele cavity (Ducibella *et al.*, 1975; Hastings and Enders, 1975; Ducibella, 1977; Baltz *et al.*, 1997; Marikawa and Alarcon, 2012). When blastocyst development displays several collapse

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and re-expansion events, this could lead to loss of water from the embryo, possibly due to damage between cell junctions. Therefore, strong blastocyst collapse may be associated with a functional and structural anomaly of the trophectoderm cells. Furthermore, the re-expansion process of collapsed blastocysts requires energy, the depletion of which may have a detrimental long-term effect. Renard *et al.* (1980), reported differences in metabolic activity, particularly concerning glucose uptake, between bovine blastocysts. In particular, they found that blastocysts that failed to expand after a 20 h culture period did not take up glucose from the medium. As in the mouse model, glucose is a necessary factor *in vitro* for blastocyst development and hatching, the authors hypothesized that strong collapse might detrimentally influence energy and metabolic process and therefore negatively affect pregnancy outcome (Wordinger and Brinster, 1976; Renard *et al.*, 1980). In recent years, the analysis of morphokinetic parameters related to embryonic development has become an exciting area in ART research. Several published studies have demonstrated advantages in *in vitro* embryo culture using this approach, and algorithms for embryo selection have been established and published by several authors (Wong *et al.*, 2010; Meseguer *et al.*, 2011b; Dal Canto *et al.*, 2012; Campbell *et al.*, 2013; Basile *et al.*, 2014; Fishel *et al.*, 2017). Advances in embryo culture has enabled Assisted Conception Units to move towards a policy of eSET to reduce the incidence of multiple pregnancies (Vilska *et al.*, 1999; Gerris *et al.*, 2002; van Montfoort *et al.*, 2005; Johnston *et al.*, 2014). Single embryo replacement has presented a challenge to embryologists, who must try to optimize embryo culture and selection to maintain success rates, while also reducing the overall number of embryos transferred. Therefore, uninterrupted *in vitro* embryo culture by TLM provides not only images and continuous assessment of human embryos, with minimal disturbance to the controlled culture environment (Lemmen *et al.*, 2008; Gardner and Kelley, 2017), but also beneficial dynamic information related to embryonic development that can be used for embryo selection (Meseguer *et al.*, 2011b; Basile *et al.*, 2014; Campbell, 2014).

In conclusion, although this is a retrospective analysis with associated limitation this is, to our knowledge, the first multicentre study looking at blastocyst collapse as a predictor of implantation and pregnancy outcome. The major strengths of this study are the multicentre nature and the large sample size. Our study may be useful for future research to investigate if there is any relationship between collapse event and chromosomal abnormalities and aneuploidy, or error on mitotic chromosome segregation. It is evident that chromosomal content plays an essential role in early embryogenesis and would be important to determine if there is any connection between the euploid state of an embryo and the collapse episode. Analysis of blastocyst collapse could be used as a tool to improve embryo selection, especially when there are large numbers of blastocysts available for embryo transfer. Ideally, prospective randomized controlled studies are needed, but considering that collapse events have already been demonstrated to be a negative marker of implantation (Marcos *et al.*, 2015; Bodri *et al.*, 2016) it would have been ethically challenging to propose such a kind of study. In this multicentre study, performed in four IVF units, we established the negative relationship between blastocyst collapse and implantation potential in embryos cultured *in vitro* with the use of TLM. Based on our findings, we suggest that observations of blastocyst collapse events may improve the evaluation of human embryo assessment, and may be used as a relevant feature to further improve IVF/ICSI outcome following elective single blastocyst transfer. Finally, further studies including live birth rates should be investigated to see if there is a difference between the two groups.

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References

Alikani M, Calderon G, Tomkin G, Garrisi J, Kokot M and Cohen J (2000) Cleavage anomalies in early human embryos and survival after prolonged culture *in-vitro*. *Hum Reprod* **15**, 2634–43.

Aparicio-Ruiz B, Basile N, Perez-Albala S, Bronet F, Remohí J and Meseguer M (2016) Automatic time-lapse instrument is superior to singlepoint morphology observation for selecting viable embryos: retrospective study in oocyte donation. *Fertil Steril* **106**, 1379, 1385.e10.

Baltz JM, Smith SS, Biggers JD and Lechene C (1997) Intracellular ion concentrations and their maintenance by Na^+/K^+-ATPase in preimplantation mouse embryos. *Zygote* **5**, 1–9.

Basile N, Morbeck D, García-Velasco J, Bronet F and Meseguer M (2013) Type of culture media does not affect embryo kinetics: a time-lapse analysis of sibling oocytes. *Hum Reprod* **28**, 634–41.

Basile N, Nogales Mdel C, Bronet F, Florensa M, Riqueiros M, Rodrigo L, Garcia-Velasco J and Meseguer M (2014) Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertil Steril* **101**, 699–704.

Bellver J, Ayllon Y, Ferrando M, Melo M, Goyri E, Pellicer A, Remohí J and Meseguer M (2010) Female obesity impairs *in vitro* fertilization outcome without affecting embryo quality. *Fertil Steril* **93**, 447–54.

Biggers JD (1998) Reflections on the culture of the preimplantation embryo. *Int J Dev Biol* **42**, 879–84.

Biggers JD and Summers MC (2008) Choosing a culture medium: making informed choices. *Fertil Steril* **90**, 473–83.

Biggers JD, Bell JE and Benos DJ (1988) Mammalian blastocyst: transport functions in a developing epithelium. *Am J Physiol* **255**, C419–32.

Blake D, Farquhar CN, Johnson N and Proctor M (2007) Cleavage stage versus blastocyst stage embryo transfer in assisted conception. *Cochrane Database Syst Rev* **4**, CD002118.

Bodri D, Sugimoto T, Yao Serna J, Kawachiya S, Kato R and Matsumoto T (2016) Blastocyst collapse is not an independent predictor of reduced live birth: a time-lapse study. *Fertil Steril* **105**, 1476–83.

Bourne H, Edgar DH and Baker HWG (2004) Sperm preparation techniques. In: Gardner DK, Weissman A, Howles CM and Shoham Z (eds). *Textbook of Assisted Reproductive Techniques: Laboratory and Clinical Perspectives* 2nd edn. USA: Informa Healthcare, pp. 79–91.

Braude P (2013) Selecting the 'best' embryos: prospects for improvement. *Reprod Biomed Online* **27**, 644–53.

Campbell A (2014) Non-invasive techniques: embryo selection by time-lapse imaging. In: Montag, M. (ed.), *A Practical Guide to Selecting Gametes and Embryos*. CRC Press, Boca Raton, FL, USA, pp. 177–89.

Campbell A, Fishel S, Bowman N, Duffy S, Sedler M and Hickman CF (2013) Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reprod Biomed Online* **26**, 477–85.

Cole RJ (1967) Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *J Embryol Exp Morphol* **17**, 481–90.

Cruz M, Garrido N, Herrero J, Perez-Cano I, Munoz M and Meseguer M (2012) Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reprod Biomed Online* **25**, 371–81.

Cutting R, Morroll D, Roberts SA, Pickering SJ, Rutherford A; BFS and ACE (2008) Elective single embryo transfer: guidelines for practice. British Fertility Society and Association of Clinical Embryologists. *Hum Fertil (Camb.)* **11**, 131–46.

https://doi.org/10.1017/S0967199419000819 Published online by Cambridge University Press

Dal Canto M, Coticchio G, Mignini-Renzini M, De Ponti E, Novara PV, Brambillasca F, Comi R and Fadini R (2012) Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. *Reprod Biomed Online* **25**, 474–80.

De Vos A, Van Landuyt L, Santos-Ribeiro S, Camus M, Van de Velde H, Tournaye H and Verheyen G (2016) Cumulative live birth rates after fresh and vitrified cleavage-stage versus blastocyst-stage embryo transfer in the first treatment cycle. *Hum Reprod* **31**, 2442–9.

Ducibella T (1977) Surface changes of the developing trophoblast cell. In *Development in Mammals* (ed. MH Johnson), pp. 5–31. Amsterdam, New York, Oxford: North-Holland Publishing Co.

Ducibella T, Albertini DF, Anderson E and Biggers JD (1975) The preimplantation mammalian embryo: characterization of intercellular junctions and their appearance during development. *Dev Biol* **45**, 231–50.

Eckert JJ and Fleming TP (2008) Tight junction biogenesis during early development. *Biochim Biophys Acta* **1778**, 717–28.

Erbach GT, Biggers JD, Manning PC and Nowak RA (2013) Localization of parathyroid hormone-related protein in the preimplantation mouse embryo is associated with events of blastocyst hatching. *J Assist Reprod Genet* **30**, 1009–15.

Fishel S, Campbell A, Montgomery S, Smith R, Nice L, Duffy S, Jenner L, Berrisford K, Kellam L, Smith R, D'Cruz I *et al* (2017) Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis. *Reprod Biomed Online* **35**, 407–16.

Fleming TP, Sheth B and Fesenko I. (2001) Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis. *Front Biosci* **6**, D1000–7.

Gardner DK and Kelley RL (2017) Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis* **8**, 418–35.

Gardner DK and Schoolcraft WB (1999) Culture and transfer of human blastocysts. *Curr Opin Obstet Gynecol* **11**, 307–11.

Gerris J, de Neubourg D, Mangelschots K, van Royen E, Vercruyssen M, Barudy-Vasquez J, Valkenburg M and Ryckaert G (2002) Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme. *Hum Reprod* **17**, 2626–31.

Glass RH, Lin TP and Florence J (1973) Mouse blastocyst re-expansion following puncture and treatment with inhibitors. *J Reprod Fertil* **35**, 533–6.

Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR and Blake D (2016) Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* **6**, CD002118.

Gonzales DS and Bavister BD (1995) Zona pellucida escape by hamster blastocysts *in vitro* is delayed and morphologically different compared with zona escape *in vivo*. *Biol Reprod* **52**, 470–80.

Gonzales DS, Jones JM, Pinyopummintr T, Carnevale EM, Ginther OJ, Shapiro SS and Bavister BD (1996) Trophectoderm projections: a potential means for locomotion, attachment and implantation of bovine, equine, and human blastocysts. *Hum Reprod* **11**, 2739–45.

Harton G, Tan L, Chian M, Suraj V, Shen S and Larman MG (2016) Euploid blastocysts that experience more collapses are less likely to result in live birth: a quantitative and automated analysis of time-lapse cinematography. Abstract ESHRE 2016 O-222.

Hastings RA and Enders AC (1975) Junctional complexes in the preimplantation rabbit embryo. *Anatom Rec* **181**, 17–34.

Houghton FD (2005) Role of gap junctions during early embryo development. *Reproduction* **129**, 129–35.

Huang TT, Chinn K, Kosasa T, Ahn HJ and Kessel B (2016) Morphokinetics of human blastocyst expansion *in vitro*. *Reprod Biomed Online* **33**, 659–67.

Huang ZP, Yu H, Yang ZM, Shen WX, Wang J and Shen QX (2004) Uterine expression of implantation serine proteinase 2 during the implantation period and *in vivo* inhibitory effect of its antibody on embryo implantation in mice. *Reprod Fertil Dev* **16**, 379–84.

Iwata K, Yumoto K, Sugishima M, Mizoguchi C, Kai Y, Iba Y and Mio Y (2014) Analysis of compaction initiation in human embryos by using time-lapse cinematography. *J Assist Reprod Genet* **31**, 421–6.

Johnston J, Gusmano MK and Patrizio P (2014) Preterm births, multiples, and fertility treatment: recommendations for changes to policy and clinical practices. *Fertil Steril* **102**, 36–9.

Lane M and Gardner DK (2007) Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* **21**, 83–100.

Lemmen JG, Agerholm I and Ziebe S (2008) Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. *Reprod Biomed Online* **17**, 385–91.

Lewis WH and Gregory PW (1929) Cinematographs of living developing rabbit eggs. *Science* **69**, 226–9.

Marcos J, Perez-Albala S, Mifsud A, Molla M, Landeras J and Meseguer M (2015) Collapse of blastocysts is strongly related to lower implantation success: a time-lapse study. *Hum Reprod* **30**, 2501–8.

Marikawa Y and Alarcon VB (2012) Creation of trophectoderm, the first epithelium, in mouse preimplantation development. *Results Probl Cell Differ* **55**, 165–84.

Massip A and Mulnard J (1980) Time-lapse cinematographic analysis of hatching of normal and frozen–thawed cow blastocysts. *J Reprod Fertil* **58**, 475–8.

Massip A, Mulnard J, Vanderzwalmen P, Hanzen C and Ectors F (1982) The behaviour of cow blastocyst *in vitro*: cinematographic and morphometric analysis. *J Anat* **134**(Pt 2), 399–405.

Meseguer M, Santiso R, Garrido N, García-Herrero S, Remohí J and Fernandez JL (2011a) Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertil Steril* **95**, 124–8.

Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB and Remohí J (2011b) The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* **26**, 2658–71.

Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohí J and Meseguer M (2016) Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril* **105**, 376–84.

Niimura S (2003) Time-lapse videomicrographic analyses of contractions in mouse blastocysts. *J Reprod Dev* **49**, 413–23.

O'Sullivan CM, Liu SY, Rancourt SL and Rancourt DE (2001) Regulation of the trypsin-related proteinase ISP2 by progesterone in endometrial gland epithelium during implantation in mice. *Reproduction* **122**, 235–44.

Renard JP, Philippson A and Menezo Y (1980) *In-vitro* uptake of glucose by bovine blastocysts. *J Reprod Fertil* **58**, 161–4.

Sciorio R, Thong JK and Pickering SJ (2018) Comparison of the development of human embryos cultured in either an EmbryoScope or benchtop incubator. *J Assist Reprod Genet* **35**, 515–22.

Seshagiri PB, Sen Roy S, Sireesha G and Rao RP (2009) Cellular and molecular regulation of mammalian blastocyst hatching. *J Reprod Immun* **83**, 79–84.

Sharma N, Liu S, Tang L, Irwin J, Meng G and Rancourt DE (2006) Implantation serine proteinases heterodimerize and are critical in hatching and implantation. *BMC Dev Biol* **6**, 61.

Sharma N, Fahr J, Renaux B, Saifeddine M, Kumar R, Nishikawa S, Mihara K, Ramachandran R, Hollenberg MD and Rancourt DE (2013) Implantation serine proteinase 2 is a monomeric enzyme with mixed serine proteolytic activity and can silence signalling via proteinase activated receptors. *Biochem Cell Biol* **91**, 487–97.

Sireesha GV, Mason RW, Hassanein M, Tonack S, Navarrete Santos A, Fischer B and Seshagiri PB (2008) Role of cathepsins in blastocyst hatching in the golden hamster. *Mol Hum Reprod* **14**, 337–46.

Steptoe P and Edwards RG (1978) Birth after the reimplantation of a human embryo. *Lancet* **2**, 366.

Sun ZG, Shi HJ, Gu Z, Wang J and Shen QX (2007) A single intrauterine injection of the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride reversibly inhibits embryo implantation in mice. *Contraception* **76**, 250–5.

Togashi K, Kumagai J, Sato E, Shirasawa H, Shimoda Y, Makino K, Sato W, Kumazawa Y, Omori Y and Terada Y (2015) Dysfunction in gap junction intercellular communication induces aberrant behavior of the inner cell mass and frequent collapses of expanded blastocysts in mouse embryos. *J Assist Reprod Genet* **32**, 969–76.

https://doi.org/10.1017/S0967199419000819 Published online by Cambridge University Press

van Montfoort AP, Dumoulin JC, Land JA, Coonen E, Derhaag JG and Evers JL (2005) Elective single embryo transfer (eSET) policy in the first three IVF/ICSI treatment cycles. *Hum Reprod* **20**, 433–6.

Vilska S, Tiitinen A, Hyden-Granskog C and Hovatta O (1999) Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. *Hum Reprod* **14**, 2392–5.

Wang SX (2011) The past, present and future of embryo selection in in vitro fertilization: Frontiers in Reproductive Conference. *Yale J Biol Med* **84**, 487–90.

Watson AJ, Natale DR and Barcroft LC (2004) Molecular regulation of blastocyst formation. *Anim Reprod Sci* 82–83, 583–92.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM and Reijo Pera RA (2010) Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* **28**, 1115–21.

Wordinger RJ and Brinster RL (1976) Influence of reduced glucose levels on the *in vitro* hatching, attachment and trophoblast outgrowth of the mouse blastocyst. *Dev Biol* **53**, 294–6.

Yamanaka Y, Ralston A, Stephenson RO and Rossant J (2006) Cell and molecular regulation of the mouse blastocyst. *Dev Dyn* **235**, 2301–14.

Zhang JQ, Li XL, Peng Y, Guo X, Heng BC and Tong GQ (2010) Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. *Reprod Biomed Online* **20**, 510–5.

Zhao Y, Brezina P, Hsu CC, Garcia J, Brinsden PR and Wallach E (2011) *In vitro* fertilization: four decades of reflections and promises. *Biochim Biophys Acta* **1810**, 843–52.

https://doi.org/10.1017/S0967199419000819 Published online by Cambridge University Press