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ARTICLE

The human first cell cycle: impact on implantation

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Abstract The morphology of fertilization events has been related to successful implantation by subjective criteria (pronuclei score, pronuclei symmetry and position). This work first described these events by time-lapse technology and then compared the timings of fertilization events (second polar body extrusion, first and second pronuclei appearance, abuttal and fading) in implanted versus nonimplanted embryos in a 2-year cohort retrospective study. A total of 1448 transferred embryos from 842 patients undergoing intracytoplasmic sperm injection with oocyte donation were monitored, 212 embryos from treatments where the number of gestational sacs matched the number of transferred embryos and 687 embryos from treatments no biochemical pregnancy was achieved. The timings at which second polar body extrusion (3.3–10.6 h), pronuclear fading (22.2–25.9 h) and length of S-phase (5.7–13.8 h) occurred were linked successfully to embryo implantation. The other parameters were apparently not related, as determined by image acquisition and time-lapse analysis.
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KEYWORDS: cell cycle, early fertilization events, known implantation, pronuclei, S-phase, time-lapse

Introduction

Identification and selection of embryos with the highest implantation potential while avoiding multiple pregnancies (Beuchat et al., 2008) is a major objective of IVF laboratories worldwide. Elective single transfer has been suggested as the most successful method of accomplishing this (Cutting et al., 2008).

Over the last 30 years, most laboratories have routinely selected embryos on the basis of morphological characteristics, which have been continuously refined by studying the pronuclear stage and morphology, the symmetry,

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fragmentation and multinucleation of the blastomeres and the ultimate morphology and in-vitro ability to progress to the blastocyst stage (Alikani et al., 2000; Baczkowski et al., 2004; Gardner et al., 2004; Neuber et al., 2003; Scott and Smith, 1998; Tesarik and Greco, 1999; Trounson et al., 1981).

Conventional embryo scoring depends on analysis of embryo morphology at predefined time points along the path of embryo development to obtain the most valuable information of embryo viability and implantation potential, but amongst these predetermined check points, there is a wealth of information that is not used. Time-lapse technology offers the possibility of observing the entire progression of embryo development, overcoming the limitations of the traditional periodical observations. In fact, morphokinetics has recently been proposed as an additional selection tool due to its strong relationship with other morphological parameters, and several studies support its link with in-vitro and in-vivo embryo viability (Chamayou et al., 2013; Ciray et al., 2006; Cruz et al., 2011, 2012; Hesters et al., 2008; Lemmen et al., 2008; Lundin et al., 2001; Meseguer et al., 2011a; Mio and Maeda, 2008; Pribenszky et al., 2010; Sakkas et al., 1998; Shoukir et al., 1997; Wong et al., 2010) and even with the ploidy of embryos more recently (Campbell et al., 2012, 2013; Chavez et al., 2012).

Fertilization includes the extrusion of the second polar body (PB), the appearance and fading of the pronuclei (PN) or the PN syngamy, events that frequently happen before or after the conventional first embryo observation performed at 16—22 h post insemination, frequently remaining unidentified when the assessment is performed at fixed time points. These events and their timings have previously been described by Payne et al. (1997). Following discrete zygote observation under light microscopy, fertilization was assessed, and pronuclear score also assisted embryo selection. The latter is a matter of controversy: some studies endorse the prognostic effect of pronuclear evaluation for embryo viability and chromosomal normalcy (Ebner et al., 2003; Garello et al., 1999; Gámiz et al., 2003; Montag and Van der Ven, 2001; Scott et al., 2000; Senn et al., 2006; Tesarik and Greco, 1999) while other studies have not been able to establish any correlation (Bar-Yoseph et al., 2011; James et al., 2006; Weitzman et al., 2010).

As far as is known, the present study provides the largest-ever description of the dynamics of the first cell cycle in human zygotes using time-lapse imaging. Moreover, the main fertilization events are related with the in-vivo developmental ability, by evaluating all transferred embryos with known implantation data and correlating the morphokinetic parameters of the fertilization events with implantation rate and ongoing pregnancy using a time-lapse system.

Materials and methods

The research was conducted at the Instituto Valenciano de Infertilidad (IVI) in Valencia and Vigo. All procedures and protocols were approved by the institutional review board in 2008, which regulates and approves clinical use of IVF procedures for research at IVI (ref. no. 0711-C-034-MM, approved 28 October 2008). The project complies with the Spanish law governing assisted reproductive technologies (14/2006).

The study was based on 1448 transferred embryos developed from normally fertilized oocytes supplied by 842 patients undergoing intracytoplasmic sperm injection (ICSI) cycles from the oocyte donation IVF programme between July 2009 and January 2012: 435 patients from IVI Vigo and 407 from IVI Valencia. Embryos were studied by time-lapse analysis, where the exact timing of the fertilization events cited below were evaluated in hours post insemination by ICSI.

Embryo implantation was confirmed by ultrasound scanning for gestational sacs with fetal heart beat after 7 weeks of pregnancy. Only embryos from treatments where the number of gestational sacs matched the number of transferred embryos (full implantation; $n = 212$) and embryos from treatments where no biochemical pregnancy was achieved (no implantation; $n = 687$) were included in the study; these embryos were defined as KID (known implantation data; Alikani et al., 1999). Treatments with partial implantation ($n = 549$ embryos) were excluded because it was not possible to determine which of the two transferred embryos implanted. It is still possible that certain embryos may split into two, in which case about 2% of the matches may be erroneous as reported (Knopman et al., 2010). This potential error must be taken into account in the KID analysis as a small bias.

Ovarian stimulation in oocyte donors

All donors were selected from the IVI oocyte donation programme. The donor selection criteria were as set out in Garrido et al. (2002) and in compliance with Spanish law (14/2006).

The mean age of the recipient was 38.4 years (range 24—50). All donors had normal menstrual cycles lasting between 26 to 34 days, body mass index 18—28 kg/m^2, no endocrine treatment (including gonadotrophins and oral contraception) for the 3 months preceding the study, normal uterus and ovaries at transvaginal ultrasound (no evidence of polycystic ovary syndrome) and an antral follicle count >20 on the first day of gonadotrophin administration after down-regulation with gonadotrophin-releasing hormone (GnRH) agonist (Meseguer et al., 2011b). Prior to the ovarian stimulation, the pituitary was down-regulated using GnRH agonist protocols (Melo et al., 2010).

Ovarian stimulation was carried out as previously described (Meseguer et al., 2008). Human chorionic gonadotrophin (HCG, Ovitrelle; Serono Laboratories, Madrid, Spain) was administered subcutaneously when at least eight leading follicles reached a mean diameter of ≥18 mm. Transvaginal oocyte retrieval was scheduled 36 h later. Protocol for endometrial preparation of recipients was performed according to Meseguer et al. (2008, 2011b).

Oocyte recovery, ICSI and embryo culture

Follicles were aspirated 36 h after HCG administration and oocytes were washed in Global w/HEPES (LifeGlobal, Canada) and cultured in Global for fertilization (LifeGlobal) at 37.0°C under a 6.0% CO_2 and 20.0% O_2 atmosphere for 3 h

before oocyte denudation. Then, the granulosa cells from oocytes were removed by mechanical pipetting in 1:1 Global hyaluronidase (80 IU/ml; LifeGlobal) and in Global w/HEPES prior to ICSI.

Cryptozoospermia (presenting <100,000 motile sperm cells in the ejaculate) and sperm samples coming from testicular biopsy were considered as an exclusion criteria.

ICSI was carried out in Global w/HEPES at ×400 magnification using an Olympus IX7 microscope 1 h later, that is 4 h after the egg retrieval. No more than six oocytes per dish were microinjected at a time. Approximately 1 min was spent per oocyte for ICSI. Lastly, inseminated oocytes were placed in pre-equilibrated culture slides (EmbryoSlide; UnisenseFertiliTech, Aarhus, Denmark) built with a central depression containing 12 straight—sided cylindrical wells, each of which was filled with a 20-μl droplet of Global culture medium. The depression containing the 12 wells was covered with an overlay of 1.4 ml LiteOil (LifeGlobal) to prevent evaporation.

EmbryoSlide culture dishes were prepared the day before the ICSI procedure and incubated overnight to pre-equilibrate in a conventional incubator at 37°C under a 6.0% CO_2 and 20.0% O_2 atmosphere. After pre-equilibration, all air bubbles were meticulously removed before the inseminated oocytes were individually transferred to the EmbryoSlide microwells and incubated in the time-lapse monitoring system at 37°C under 6.0% CO_2 until embryo transfer, which was performed approximately 72 h post ICSI.

The time-lapse system EmbryoScope (UnisenseFertiliTech, Aarhus, Denmark), is a tri-gas incubator with a built-in microscope to automatically acquire images of up to 72 individual embryos during development and is connected to a computer (EmbryoViewer). Images were acquired for each embryo at seven equidistant focal planes every 20 min and recorded as 1280 × 1024 digital images in the EmbryoViewer where they were analysed.

Embryo scoring and transfer

The success of fertilization was assessed 17—20 h after the ICSI procedure based on the digital images using the EmbryoViewer software. Embryo morphology was evaluated on day 2 (44—48 h post ICSI), and on day 3 (68—72 h post ICSI). Embryo selection for transfer or cryopreservation was performed by morphokinetics criteria as described in Meseguer et al. (2011a) on day 3.

The number of embryos transferred was in most cases two; single-embryo transfers were performed when applicable depending on treatment and in no instance were three embryos transferred in this study. Supernumerary embryos were cryopreserved for potential future transfer using vitrification on day 3 of embryo development (Cobo et al., 2010).

Clinical outcome determinations

βHCG was determined 13 days after embryo transfer and clinical pregnancy confirmed when a gestational sac with fetal heart beat was visible by ultrasound examination after 7 weeks of pregnancy.

Early fertilization events

The second PB extrusion, PN appearance, PN syngamy, PN movements within the cytoplasm, PN score, PN symmetry, PN fading, the length of the S-phase and the duration of the first cell cycle were evaluated retrospectively for transferred embryos using the EmbryoViewer image analysis software where the events and their timing were recorded in hours post ICSI. The timing of the events were defined as the instance of the first visible observation of each event. The location of the appearance and fading of these events was also annotated, so that the fertilization events were divided into timing events and categorical events.

Definition of fertilization timing events

2PB was defined as extrusion of the second polar body, the exact moment when the second polar body is extruded from the cytoplasm as initiation of the first cell cycle. 1PN was defined as when the first pronucleus is clearly formed. 2PN was defined as when both the male and female pronuclei are visible. Abuttal was defined as the male and female pronuclei are in contact. PN fading was defined as when both PN disappear. The length of the S-phase was defined as the time from pronuclear appearance (2PN) to pronuclear fading, while the zygote's DNA is replicated.

Definition of categorical fertilization events

PN Z-score was defined as the distribution of nuclear precursor bodies (NPB), in four score groups as assessed 17 ± 1 h after ICSI: Z1 = zygotes having 3—7 polarized NPB; Z2 = zygotes with homogeneously dispersed NPB; Z3 = all the zygotes with a NPB distribution not included in groups 1, 2 or 4 (i.e. >7 polarized NPB, one PN polarized and the other dispersed or both dispersed but not homogeneously); and Z4 = zygotes with only one or two NPB, as described by Gámiz et al. (2003).

PN symmetry was defined as the correspondence in size between pronuclei between female and male pronuclei comparison, where symmetry 1, 2 and 3 are defined as equal size, similar size or very different size, respectively (Sadowy et al., 1998). Embryos from zygotes with different pronuclear sizes (>4 μm difference) have been associated with a high rate of embryonic developmental arrest, a high rate of multinucleation on day 2 of development and a larger percentage of chromosomal abnormalities (Gámiz et al., 2003; Lawler et al., 2007; Munné and Cohen, 1998). The assessment of this categorical parameter was performed at the same time as the PN Z-score.

PN fading patterns was defined as PN movements through the cytoplasm and fading and were annotated into four categories, depending on where the PN move and fade out: central—central = movement in the centre of the cell and fading out in a central position; side—central = PN move towards the periphery of the zygote and then move back to a central position where they fade out; central—side = PN move in the centre of the cell and fade out in the periphery; side—side: both the movement and the fading out happen in the periphery of the zygote.

PN Z-score and abuttal are difficult to assess when the pronuclei are not in the same plane; in these circumstances this work used the flexibility provided by the time-lapse

software in allowing movements back and forth in time within the embryo images and between the seven layers of focus until both pronuclei are in the same focus and then performed a characterization. In the case of Z-score analysis, 870 of the 889 zygotes available were analysed; for PN abuttal, 747 of the 889 zygotes were verified.

Statistical analysis

The time recordings, in hours post ICSI microinjection, of fertilization events for implanted and nonimplanted transferred embryos were analysed using the Student's t-test as they were largely following a normal distribution. Chi-squared tests were used to compare the categorical fertilization events between implanted and nonimplanted embryos. With the intention of describing the distribution of the probabilities of implantation, timings (quantitative variable) were converted into a categorical variable, not with an arbitrary classification to set up these categories but with a system based on ordinations that gave four categories with an equal number of elements in each. Using this procedure, bias in the total number of embryos in each class was avoided. This work then assessed the percentage of embryos that implanted in each timing quartile to observe the temporal distribution of embryo implantation. The quartiles generated for the timings were transformed into binary variables to define optimal ranges.

The odds ratio (OR) of the effect of all binary variables generated on implantation were expressed in terms of 95% confidence interval (CI) and significance. By performing the logistic regression analysis, the effect of optimal ranges on implantation was quantified. Significance was calculated using the omnibus test (likelihood ratio) and the uncertainties uncovered by the model were evaluated by Negelkerke R^2, a coefficient that is analogous to the R^2 index of the linear regression analysis. Receiver operating characteristic (ROC) curves were employed to test the predictive value of all the variables included in the model with respect to implantation. ROC curve analysis provides the area under the curve, which are comprised between 0.5 and 1 and can be interpreted as a measurement of the global classification ability of the variables.

Statistical analysis was performed using Statistical Package for Social Sciences version 19 (SPSS, Chicago, IL, USA).

Results

The analysis comprised a total of 1448 fertilized and subsequently transferred embryos from 842 patients undergoing oocyte donation ICSI cycles. The mean age of the recipients was 38.4 years (range 24—50 years) and body mass index was 23.8 ± 4.5 kg/m^2. A total of 453 recipients became pregnant (53.8%, 95% CI 50.4—57.2%), with an average implantation rate of 32.6% (95% CI 30.7—34.5%).

The means of the timing events, described as the 2PB extrusion, 1PN and 2PN appearance, PN abuttal and fading and length of the S-phase are shown in Table 1. The simultaneous appearance of both pronuclei seems to be the norm in embryo development (96.4%), but in 52 instances one pronucleus appeared before the other.

The optimal ranges for 2PB, PN fading and the length of the S-phase were also calculated and are shown in Figure 1. There were no significant differences between the implanted and nonimplanted groups in the average length of time taken for 2PB to be extruded. However, the quartile analysis suggested that the implantation rate was higher (27.2%) for the eggs that took 3.3—10.6 h to extrude the 2PB than for those that took less time (1.0—3.2 h; 19.9%; $P = 0.022$).

For PN abuttal, the grouping into quartiles did not reveal any optimal range with higher implantation rates (data not shown).

Concerning PN fading, a higher implantation rate (27.7% versus 19.3%; $P = 0.009$) was observed in the eggs where pronuclei fading took 22.2—25.9 h, compared with those that took less or more time. Additionally, the length of the S-phase was affected; consequently, the implantation rate increased (27.2% versus 19.2%) at shorter phase lengths (5.7—13.8 h versus 13.9—26.5 h; Figure 1).

The variables presenting a relationship with implantation potential are given as box-plots to show the distribution of the variables (Figure 2). A logistic regression analysis was used to select and organize which observed timing events, expressed as binary variables inside or outside the optimal range. The model quantified and ordered the values considered as the most promising variables characterizing implanting embryos, as follows: the time of the second polar body extrusion (OR = 1.463, 95% CI 1.03—2.09) followed by the length of the S-phase (OR 0.628, 95% CI 0.439—0.897) and the time of PN fading (OR 1.558, 95% CI 1.0—3.58).

Using the previous variables, a logistic regression model was defined. A ROC curve analysis to determine the predictive properties of this model with respect to probability of implantation gave an area under the curve of 0.605 (95% CI 0.557—0.603).

The analysis of the categorical fertilization events gave the following results. The distribution of the four Z-scores (Z1, Z2, Z3, Z4) did not differ between implanted and nonimplanted embryos (Figure 3). The percentage of implanting KID embryos as it related to PN symmetry (1, 2, 3) is presented in Figure 4; differences were not significant. For the pronuclear fading patterns, over 90% of embryo pronuclei faded out in the central position and there was a tendency for the central—side pattern to be associated with a higher implantation rate, although differences were not significant (Figure 5).

Discussion

This study is, as far as is known, the largest retrospective study of early fertilization events and has involved the analysis of 1448 transferred embryos in relation to known implantation. PN development has previously been studied to predict embryo implantation potential or chromosomal abnormalities, but without a time-lapse incubator system only relatively few individual visual observations were possible during a 17—20-h period after oocyte insemination. Recent studies have shown the limitations of single observations and the advantages of time-lapse cinematography

Table 1 Exact timing of fertilization events analysed from transferred implanted and nonimplanted embryos.

	Implanted	*Nonimplanted*
2PB	3.37 (3.26–3.62)	3.21 (3.14–3.31)
1PN	7.54 (6.14–7.88)	5.80 (5.01–6.39)
2PN	9.55 (9.16–9.64)	9.42 (9.13–9.61)
Abuttal	13.36 (12.67–14.05)	13.87 (13.48–14.27)
PN fading	23.77 (23.42–24.12)	24.44 (23.17–24.70)
Length of S-phase	10.44 (9.77–11.11)	10.66 (9.91–11.26)

Values are mean hours (95% CI).
2PB = extrusion of the embryo second polar body; 1PN = when the first pronucleus is condensed and visible; 2PN = when both the male and female pronuclei are visible; abuttal = male and female pronuclei are in contact; PN fading = when both PN disappear; length of the S-phase = from PN fading to PN appearance.

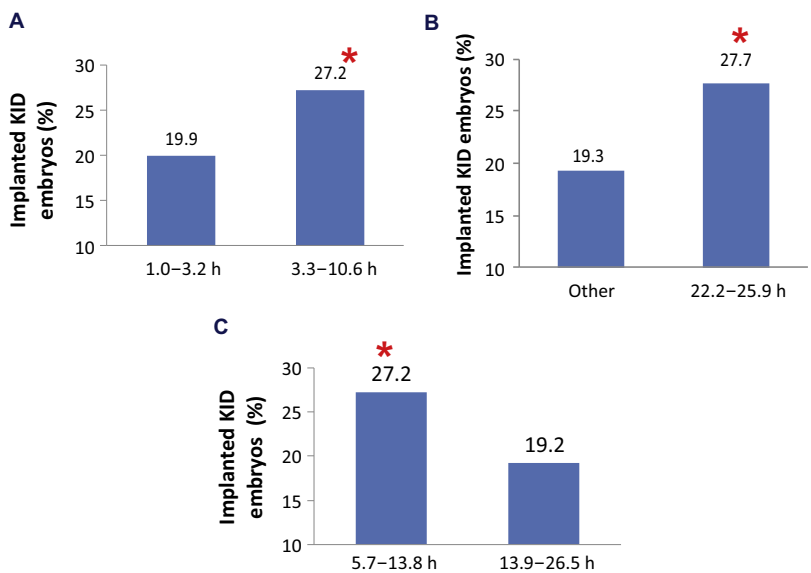

Figure 1 Implanting embryos according to fertilization timing events. Asterisks indicate optimal time range for each parameter (A) 2PB ($^{*}P = 0.022$); (B) PN fading ($^{*}P = 0.009$); (C) length of the S-phase ($^{*}P = 0.012$).

(Meseguer et al., 2011a). The current study aimed to identify temporal markers and optimal timings for the early fertilization events and assess their suitability as prognostic tools for embryo implantation success. The mid-point of the ICSI procedure was considered as the initial time ($t = 0$). Due to the time-lapse software characteristics used in this study, it was not possible to give a specific $t = 0$ for each oocyte, and therefore $t = 0$ was set as the moment where half the cohort had been microinjected. This is in agreement with the Istanbul Consensus Workshop recommendations, whereas all the timings of observations should be standardized and related to the time of insemination (ALPHA and ESHRE Special Interest Group, 2011a,b). It must be stated that the inclusion of delivery data in this manuscript would increase the clinical value of the findings here reported.

The mean time for the second polar body extrusion was very similar for the full implantation embryos and no implantation embryos (3.37 h and 3.21 h, respectively), with no significant differences. This is in agreement with recent results by Azzarello et al. (2012) of 3 h 47 min ± 0 h 17 min versus 3 h 37 min ± 0 h 11 min; for embryos resulting in live births and non live births respectively. However, when analysing the optimal time range for the 2PB, it seems that the oocytes extruding the second polar body 1.0–3.2 h post ICSI had a lower pregnancy rate than extrusions taking longer (**Figure 1**A). Previous observations by Payne et al. (1997) recorded 2.45 h as the average time for 2PB extrusion on good-morphology embryos and 3.23 h for poor-quality embryos; however, the number of zygotes studied in the current work is greater (899 embryos versus 38 embryos). None of the good-quality embryos were transferred by Payne et al. (1997) so no pregnancy information were available from this investigation, and also, the differences in Payne's laboratory and clinical conditions could be considered as a bias for the timing of key developmental events. These factors would explain the differences between the current findings and Payne's. The current results suggest that the timing of the 2PB extrusion is important for embryo implantation but that the position of the 2PB extrusion is not. Most of the embryos

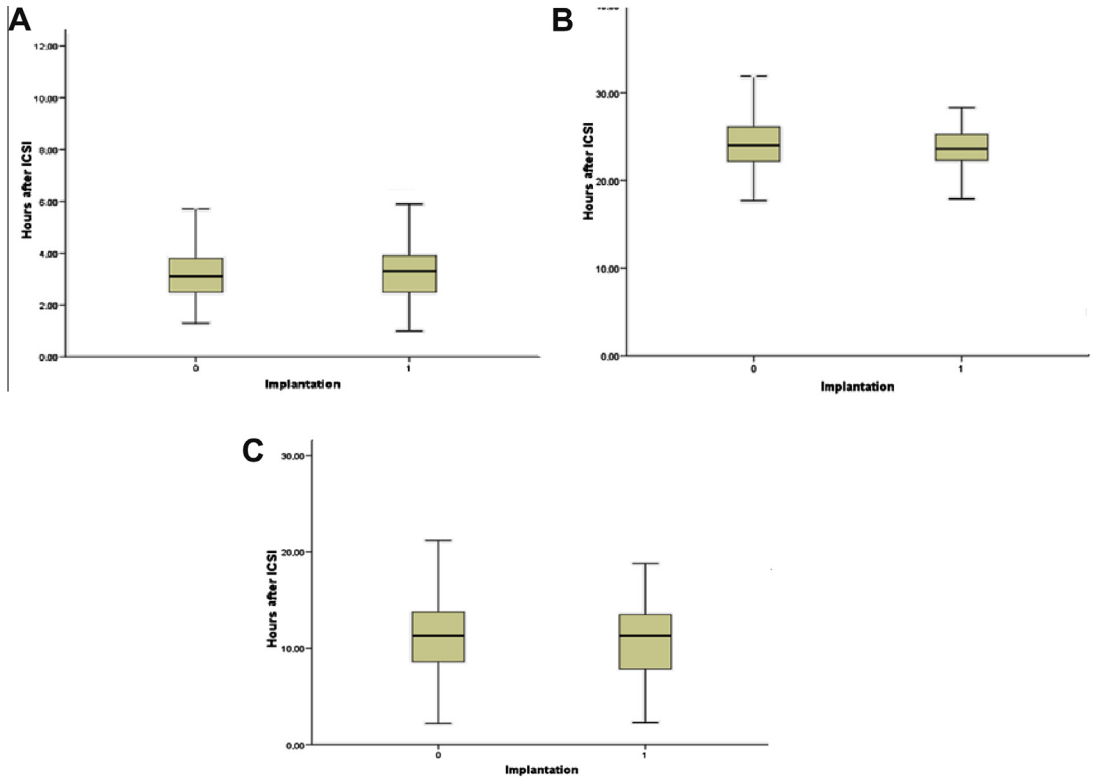

Figure 2 Timings of implanted and nonimplanted embryos according to fertilization timing events. (A) 2PB; (B) PN fading; (C) length of the S-phase.

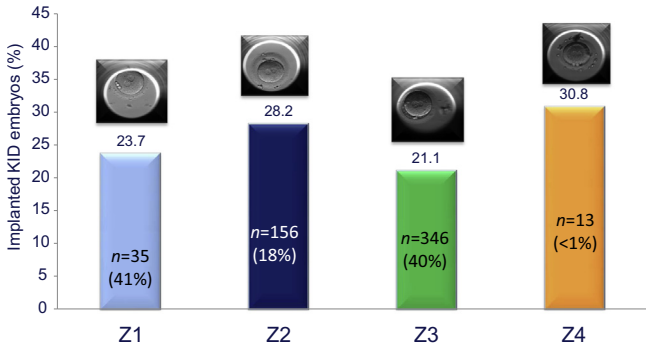

Figure 3 Implanting known implantation data (KID) embryos according to Z-score classification. Z1 = zygotes having 3–7 polarized NPB; Z2 = zygotes with homogeneously dispersed NPB; Z3 = all the zygotes with a NPB distribution not included in groups 1, 2 or 4 (i.e.>7 polarized NPB, one PN polarized and the other dispersed or both dispersed but not homogeneously); Z 4 = zygotes with only one or two NPB. Differences were not significant.

extruded the 2PB adjacent to the 1PB, although a small percentage (10%) extruded it on the opposite side, but this did not appear to have a significant relationship to embryo implantation (data not shown).

For implanting embryos, the appearance of the first PN took place 7.54 h post ICSI versus 5.80 h for those that did not implant. The simultaneous appearance of both pronuclei seems to be the norm in embryo development. However, it should be remembered that the time-lapse

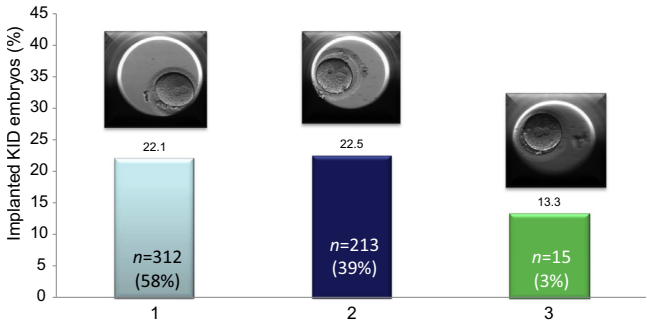

Figure 4 Percentage of implanting known implantation data (KID) embryos according to pattern of pronuclear appearance. Differences were not significant.

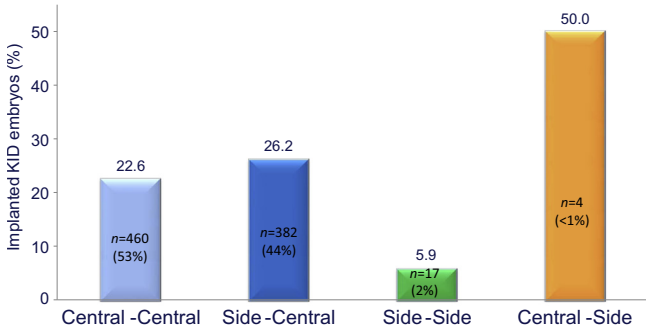

Figure 5 Percentage of implanting known implantation data (KID) embryos according to pronuclear fading patterns. Differences were not significant.

system took pictures every 20 min and, therefore, there is a lack of information for the intervening period, which could mask asynchronous appearances.

The timing of the simultaneous appearance of PN did not differ between fully implanted and nonimplanted embryos. Pronuclear appearance was recorded when pronuclear membrane and nucleoli were both first visible. The appearance of PN for embryos fully implanted was 9.55 h versus 9.42 h for nonimplanted. Earlier appearance of PN has previously been described (Payne et al., 1997). The effective resolution is limited by the pixel count in the camera, the optical limitations of the objective employed and also the periodicity of the picture capture. In this work, pictures were taken in seven equidistant focal planes every 20 min, which involves a lower imaging resolution and a possible retarded identification of the pronuclear appearance in the very early stages when they are only slightly perceptible, compared with the imaging system employed by Payne et al. (1997). However, the number of embryos analysed here was much larger and differences in the timing of pronuclear appearance must be expected. The position of the pronuclear appearance was also studied and related to embryo implantation. No differences were found between the three categories.

Once both pronuclei have appeared in the cytoplasm, the sperm centrosome organizes a microtubule array, the aster, which spreads through the oocyte cytoplasm, reaching the female pronucleus and drawing it towards the male one (Whitaker, 2005), subsequently moving both pronuclei within the cytoplasm. PN abuttal occurred at 13.36 h post ICSI, without any impact on the ability to implant.

Analysis of the embryo cohort showed that the most frequent Z-score was 1, followed by 3 and 2, with Z4 being the least frequent pronuclear pattern (1.2%). However, the Z-score had no effect on implantation. These results are in contrast with a previous study by Scott (2003), where the Z-score was proposed as a predictor of embryo selection, and with the previous study of Gámiz et al. (2003), where pronucleolar precursor body distribution was related with embryo morphology and chromosomal abnormalities; however, they agree with the findings of Azzarello et al. (2012). Considering all together, these observations may apparently obviate the Z-score evaluations, as according to the current data, it appears not to be related to embryo implantation. However, the evolution in pronuclear morphology is constant from first PN appearance until the fading out of the pronuclei (Payne et al., 1997; Scott et al., 2000; Tesarik and Kopecny, 1989) and may therefore give rise to bias in the scoring. So it seems appropriate to use the last image before pronuclear fade out as the best time to evaluate the Z-score, when the diameter of each PN is largest and pronuclear scoring could be assessed with greatest accuracy.

Pronuclear fading has been proven to have a significant effect on implantation where the timing of the fading for implanted embryos occurred earlier than for nonimplanted embryos. This parameter and the PN appearance define the length of the S-phase. Therefore it is obvious that the length of the S-phase also has an impact on implantation, as implanted embryos had a shorter S-phase than nonimplanted embryos. Both these parameters can be established as timing criteria relating to implantation as this study was performed on KID embryos.

Analysis of the optimal timing ranges for PN fading suggests that embryos where pronuclei fading took place at 22.2—25.9 h have greater probability of implanting than those showing earlier or later fading. These findings establish a range of time for embryos with higher implantation rate and complement the results from Azzarello et al. (2012), who found no live births in embryos whose PN fading happened before 20 h 45 min and a higher implantation rate in embryos with a slower PN fading, by narrowing the time of the pronuclear fading and giving a concrete period of time, from a wider population of embryos analysed.

This work also studied the movement of the pronuclei within the cytoplasm and the cytoplasmic location where the fading takes place. For >97% of the embryos, pronuclear fading took place at the central—central and side—central cytoplasmic position and had no effect on implantation, probably because of the homogeneity of this distribution. Less-frequent fading location patterns were side—side (<3%) and central—side (0.5%; **Figure 4**). Despite the latter having the highest implantation rate, its low frequency may reflect a simple statistical artefact.

Syngamy is defined as the moment of fusion between parental and maternal DNA (Whitaker, 2005), which this work considered as the time of PN fading. According to the data from this study and Meseguer et al. (2011a), the mean time taken from PN fading to the first embryo cleavage in KID embryos is about 4.04 h: this period includes the G2- and M-phases. Since PN is visible during a period from 5 to 26 h, PN morphokinetics is unlikely to be evaluated by just a single observation.

DNA replication occurs during the S-phase of the cell cycle. This study defined an average timing for the S-phase which was consistent with the duration reported by Masai et al. (2010), who determined that DNA genomic replication takes in the region of 7—8 h. This period will be determined by chromosome structures, nuclear positioning, patterns of histone modifications and transcriptional activity (Oda et al., 2012).

The embryos for which the S-phase lasted 5.7—13.8 h had a higher implantation rate than those with a longer S-phase ($P = 0.012$). This work was not able to strictly determine when the DNA is being replicated, but it seems that the embryos with a longer S-phase, and presumably a longer replication time, have a lower implantation rate than those with an S-phase lasting closer to 7—8 h.

It is possible that ICSI and IVF may differ in terms of duration of the S-phase, as reviewed by Hewitson et al. (2000). However, Cruz et al. (2013) reported no variation in the duration of the S-phase in the second or subsequent cell cycles according to the fertilization procedure used. Further cytogenetic investigation of this stage should be performed to better understand the S-phase during the first cell cycle and to narrow down the timings accurately, although, pursuant to the current data, the duration of the S-phase is significantly related to the embryo implantation rate.

The differences in the implantation rate of those embryos which were in the optimal time range for the fertilization events here analysed, compared with those which were out of optimal range, were smaller compared with

early cleavage morphokinetic parameters, such as time from microinjection to 5 cells (t_5), the duration of the second cell cycle (cc2) and the synchrony of divisions from the 2-cell to 4-cell stages (s2) (Meseguer et al., 2011a). PN evaluation by time lapse is more complex than t_5 or cc2 and s2 evaluation, as it depends on a greater list of issues, such as limitations of the time-lapse microscope resolution, frequency of images, and inaccessibility to roll the embryo when PN are overlapped, and also because establishing the moment when an embryo is cleaving is easier to identify than the concepts here analysed. Therefore, unless the embryos must be selected for transfer in the first cell cycle (for legal or technical reasons), it seems more useful to base embryo selection on t_5, cc2 and s2 parameters. However, prospective analyses for this latest purpose are still awaited to confirm the retrospective findings.

On the other hand, there are still a limited number of studies exploring fertilization and PN morphology in development, and more retrospective investigations are necessary to define which parameter is more suitable for embryo selection in the first cell cycle. Obviously, if this parameter or others are identified, their suitability must be confirmed by a prospective randomized trial.

In conclusion, this study offers new information on the timing of the main fertilization events and behaviour of PN fading patterns, and the retrospective analysis of the embryo morphokinetics indicates a relationship between the timing of pronuclear fading and implantation rate, which could be a useful tool in the selection of embryos with high implantation potential in the very early stages of embryo development. In contrast, the predictive value of the Z-score was found to be limited. A specific time point in embryo development should be established for checking pronuclear morphology and the Z-score, because pronuclear appearance and distribution are in constant development until the stage of pronuclear disappearance, with subsequent changes to the Z-score category depending on the time of the observation.

This study also introduces alternative parameters for the selection of embryos with higher implantation probabilities. Together, the timing of certain fertilization events seems to be related to embryo implantation potential. Observation of such events requires time-lapse monitoring of early embryo development.

Although this study is not a clinical trial, it does provide strong clinical value. Knowledge of the parameters potentially useful for selection is necessary before a clinical trial can be performed. Hence, a randomized controlled trial is not possible without a previous retrospective analysis to provide the hypothesis. This is the basis of any clinical study. To plan a future prospective investigation, further analysis is required to identify the morphokinetic variables that are potentially the most relevant. The real answer will come with a larger retrospective analysis that will combine all known morphokinetic variables; then a randomized controlled trial will be possible.

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References

Alikani, M., Cohen, J., Tomkin, G., Garrisi, G.J., Mack, C., Scott, R.T., 1999. Human embryo fragmentation in vitro and its implications for pregnancy and implantation. Fertil. Steril. 71, 836—842.

Alikani, M., Calderon, G., Tomkin, G., Garrisi, J., Kokot, M., Cohen, J., 2000. Cleavage anomalies in early human embryos and survival after prolonged culture *in vitro*. Hum. Reprod. 15, 2634—2643.

ALPHA Scientists in Reproductive MedicineESHRE Special Interest Group of Embryology, 2011a. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum. Reprod. 26, 1270—1283.

ALPHA Scientists in Reproductive MedicineESHRE Special Interest Group Embryology, 2011b. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Reprod. Biomed. Online 22, 632—646.

Azzarello, A., Hoest, T., Mikkelsen, A.L., 2012. The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture. Hum. Reprod. 27, 2649—2657.

Baczkowski, T., Kurzawa, R., Glabowski, W., 2004. Methods of embryo scoring in in-vitro fertilization. Reprod. Biol. 4, 5—22.

Bar-Yoseph, H., Levy, A., Sonin, Y., Alboteanu, S., Levitas, E., Lunenfeld, E., Har-Vardi, I., 2011. Morphological embryo assessment: reevaluation. Fertil. Steril. 95, 1624—1627.

Beuchat, A., Thevenaz, P., Unser, M., Ebner, T., Senn, A., Urner, F., Germond, M., Sorzano, C.O.S., 2008. Quantitative morphometrical characterization of human pronuclear zygotes. Hum. Reprod. 23, 1983—1992.

Campbell, A., Hickman, C.F.L., Duffy, S., Bowman, N., Gardner, K., Fishel, S., 2012. Morphokinetics of 'aneuploid' and 'euploid' human embryos inferred by polar body or trophectoderm biopsy. Hum. Reprod. 27, P-176.

Campbell, A., Fishel, S., Bowman, N., Duffy, S., Sedler, M., Hickman, C.F., 2013. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. Reprod. Biomed. Online 26, 477—485.

Chamayou, S., Pasquale, P., Storaci, G., Tomaselli, V., Alecci, C., Ragolia, C., Crescenzo, C., Guglielmino, A., 2013. The use of morphokinetic parameters to select all embryos with full capacity to implant. J. Assist. Reprod. Genet. 30, 703—710.

Chavez, S.L., Loewke, K.E., Han, J., Moussavi, F., Colls, P., Munne, S., Behr, B., Pera, R.A., 2012. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. Nat. Commun. 3, 1251.

Ciray, H.N., Ulug, U., Tosun, S., Erden, H.F., Bahceci, M., 2006. Outcome of 1114 ICSI and embryo transfer cycles of women 40 years of age and over. Reprod. Biomed. Online 13, 516—522.

Cobo, A., Meseguer, M., Remohi, J., Pellicer, A., 2010. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. Hum Reprod. 25, 2239—2246.

Cruz, M., Gadea, B., Garrido, N., Pedersen, K., Martinez, M., Perez-Cano, I., Munoz, M., Meseguer, M., 2011. Embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients whose embryos were monitored by time-lapse imaging. J. Assist. Reprod. Genet. 28, 569—573.

Cruz, M., Garrido, N., Herrero, J., Pérez-Cano, I., Muñoz, M., Meseguer, M., 2012. Timing of cell division in human

cleavage-stage embryos is linked with blastocyst formation and quality. Reprod. Biomed. Online 25, 371—381.
Cruz, M., Garrido, N., Gadea, B., Muñoz, M., Pérez-Cano, I., Meseguer, M., 2013. Oocyte insemination techniques are related to alterations of embryo developmental timing in an oocyte donation model. Reprod. Biomed. Online 27, 367—375.
Cutting, R., Morroll, D., Roberts, S.A., Pickering, S., Rutherford, A., 2008. Elective single embryo transfer: guidelines for practice British Fertility Society and Association of Clinical Embryologists. Hum. Fertil. 11, 131—146.
Ebner, T., Moser, M., Sommergruber, M., Gaiswinkler, U., Wiesinger, R., Puchner, M., Tews, G., 2003. Presence, but not type or degree of extension, of a cytoplasmic halo has a significant influence on preimplantation development and implantation behaviour. Hum. Reprod. 18, 2406—2412.
Gámiz, P., Rubio, C., de los Santos, M.J., Mercader, A., Simón, C., Remohí, J., Pellicer, A., 2003. The effect of pronuclear morphology on early development and chromosomal abnormalities in cleavage-stage embryos. Hum. Reprod. 18, 2413—2419.
Gardner, D.K., Surrey, E., Minjarez, D., Leitz, A., Stevens, J., Schoolcraft, W.B., 2004. Single blastocyst transfer: a prospective randomized trial. Fertil. Steril. 81, 551—555.
Garello, C., Baker, H., Rai, J., Montgomery, S., Wilson, P., Kennedy, C.R., Hartshorne, G.M., 1999. Pronuclear orientation, polar body placement, and embryo quality after intracytoplasmic sperm injection and in-vitro fertilization: further evidence for polarity in human oocytes? Hum. Reprod. 14, 2588—2595.
Garrido, N., Zuzuarregui, J.L., Meseguer, M., Simon, C., Remohi, J., Pellicer, A., 2002. Sperm and oocyte donor selection and management: experience of a 10 year follow-up of more than 2100 candidates. Hum. Reprod. 17, 3142—3148.
Hesters, L., Prisant, N., Fanchin, R., Mendez Lozano, D.H., Feyereisen, E., Frydman, R., Tachdjian, G., Frydman, N., 2008. Impact of early cleaved zygote morphology on embryo development and in-vitro fertilization-embryo transfer outcome: a prospective study. Fertil. Steril. 89, 1677—1684.
Hewitson, L., Simerly, C., Dominko, T., Schatten, G., 2000. Cellular and molecular events after in-vitro fertilization and intracytoplasmic sperm injection. Theriogenology 53, 95—104 (Review).
James, A.N., Hennessy, S., Reggio, B., Wiemer, K., Larsen, F., Cohen, J., 2006. The limited importance of pronuclear scoring of human zygotes. Hum. Reprod. 21, 1599—1604.
Knopman, J., Krey, L.C., Lee, J., Fino, M.E., Novetsky, A.P., Noyes, N., 2010. Monozygotic twinning: an eight-year experience at a large IVF center. Fertil. Steril. 94, 502—510.
Lawler, C., Naker, H.W.G., Edgar, D.H., 2007. Relationships between timing of syngamy, female age and implantation potential in human in-vitro-fertilised oocytes. Reprod. Fertil. Dev. 19, 482—487.
Lemmen, J.G., Agerholm, I., Ziebe, S., 2008. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. Reprod. Biomed. Online 17, 385—391.
Lundin, K., Bergh, C., Hardarson, T., 2001. Early embryo cleavage is a strong indicator of embryo quality in human IVF. Hum. Reprod. 16, 2652—2657.
Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N., Oda, M., 2010. Eukaryotic chromosome DNA replication: where, when, and how? Annu. Rev. Biochem. 79, 89—130.
Melo, M., Bellver, J., Garrido, N., Meseguer, M., Pellicer, A., Remohi, J., 2010. A prospective, randomized, controlled trial comparing three different gonadotropin regimens in oocyte donors: ovarian response, in-vitro fertilization outcome, and analysis of cost minimization. Fertil. Steril. 94, 958—964.
Meseguer, M., Martinez-Conejero, J.A., O'Connor, J.E., Pellicer, A., Remohi, J., Garrido, N., 2008. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. Fertil. Steril. 89, 1191—1199.
Meseguer, M., Herrero, J., Tejera, A., Hilligsoe, K.M., Ramsing, N.B., Remohi, J., 2011a. The use of morphokinetics as a predictor of embryo implantation. Hum. Reprod. 26, 2658—2671.
Meseguer, M., Santiso, R., Garrido, N., Garcia-Herrero, S., Remohi, J., Fernandez, J.L., 2011b. Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. Fertil. Steril. 95, 124—128.
Mio, Y., Maeda, K., 2008. Time-lapse cinematography of dynamic changes occurring during in-vitro development of human embryos. Am. J. Obstet. Gynecol. 199, 660.e1—660.e5.
Montag, M., Van der Ven, H., 2001. Evaluation of pronuclear morphology as the only selection criterion for further embryo culture and transfer: results of a prospective multicentre study. Hum. Reprod. 16, 2384—2389.
Munné, S., Cohen, J., 1998. Chromosome abnormalities in human embryos. Hum. Reprod. Update 4, 842—855.
Neuber, E., Rinaudo, P., Trimarchi, J.R., Sakkas, D., 2003. Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development. Hum. Reprod. 18, 1307—1312.
Oda, M., Kanoh, Y., Watanabe, Y., Masai, H., 2012. Regulation of DNA replication timing on human chromosome by a cell-type specific DNA binding protein SATB1. PLoS One 7, e42375.
Payne, D., Flaherty, S.P., Barry, M.F., Matthews, C.D., 1997. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. Hum. Reprod. 12, 532—541.
Pribenszky, C., Mátyás, S., Kovács, P., Losonczi, E., Zádori, J., Vajta, G., 2010. Pregnancy achieved by transfer of a single blastocyst selected by time-lapse monitoring. Reprod. Biomed. Online 21, 533—536.
Sadowy, S., Tomkin, G., Munné, S., Ferrara-Congedo, T., Cohen, J., 1998. Impaired development of zygotes with uneven pronuclear size. Zygote 6, 137—141.
Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, P.G., Campana, A., 1998. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum. Reprod. 13, 182—187.
Scott, L., 2003. Pronuclear scoring as a predictor of embryo development. Reprod. Biomed. Online 6, 201—214.
Scott, L.A., Smith, S., 1998. The successful use of pronuclear embryo transfers the day following oocyte retrieval. Hum.Reprod. 13, 1003—1013.
Scott, L., Alvero, R., Leondires, M., Miller, B., 2000. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum. Reprod. 15, 2394—2403.
Senn, A., Urner, F., Chanson, A., Primi, M.P., Wirthner, D., Germond, M., 2006. Morphological scoring of human pronuclear zygotes for prediction of pregnancy outcome. Hum. Reprod. 21, 234—239.
Shoukir, Y., Campana, A., Farley, T., Sakkas, D., 1997. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Hum. Reprod. 12, 1531—1536.
Tesarik, J., Kopecny, V., 1989. Development of human male pronucleus: ultrastructure and timing. Gamete Res. 24, 135—149.
Tesarik, J., Greco, E., 1999. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum. Reprod. 14, 1318—1323.
Trounson, A.O., Leeton, J.F., Wood, C., Webb, J., Wood, J., 1981. Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. Science 212, 681—682.

Weitzman, V.N., Schnee-Riesz, J., Benadiva, C., Nulsen, J., Siano, L., Maier, D., 2010. Predictive value of embryo grading for embryos with known outcomes. Fertil. Steril. 93, 658—662.

Whitaker, M. (Ed.), 2005. Syngamy and cell cycle control, second ed.Meyers, Robert A. (Ed.). Encyclopedia of Molecular Cell Biology and Molecular Medicine, VCH Verlag GmbH and Co. KGaA, Weinheim.

Wong, C.C., Loewke, K.E., Bossert, N.L., Behr, B., De Jonge, C.J., Baer, T.M., ReijoPera, R.A., 2010. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat. Biotechnol. 28, 1115—1124.

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