Live birth rate and number of blastomeres on day 2 transfer

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Abstract

Purpose To investigate whether the presence of large fragment (LF) and abnormal cell divisions (ACDs) has influenced the correlation between live birth rate and number of blastomeres detected on day 2 by conventional scoring.

Methods This study included 578 embryos cultured in time lapse and selected for transfer by conventional scoring on day 2. By time-lapse recordings, embryos were reassessed to identify ACDs and/or LFs mistaken as blastomeres. The latter identifications were used to recalculate fragmentation rate and the number of blastomeres. Live birth rate according to number of blastomeres was compared in (a) embryos selected by conventional scoring and (b) embryos reassessed by time lapse.

Results After conventional scoring, embryos with four cells had a significantly higher pregnancy rate than embryos with less than four cells and embryos with more than four cells. By time-lapse assessment, ACDs and/or recalculated fragmentation >25 % was recognized in 106/578 (18.3 %) of transferred embryos. None of them resulted in a live birth. After exclusion of these embryos, the number of blastomeres on the day of transfer did not have any impact on live birth rate.

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Capsule Recognition of ACDs and fragmentation >25 % is more predictive of live birth than number of blastomeres.

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Conclusion Conventional scoring on day 2 did not detect ACDs and LFs mistaken as blastomeres. LFs can lead to a recalculated fragmentation rate to >25 %. No significant correlation between live birth rate and number of blastomeres in day 2 embryos was observed when embryos with ACDs and fragmentation >25 % were excluded. Recognition of ACDs and fragmentation >25 % is more predictive of live birth than number of blastomeres.

Keywords Time lapse · Live birth · Number of blastomeres · Embryo · Fragmentation rate

Background

Conventional scoring of embryos on day 2 is primarily based on single observation of the number of blastomeres and the fragmentation rate prior to the transfer [1]. Embryos scored as four-cell stage and <25 % of fragmentation are suggested to have higher implantation and pregnancy rate [1].

Time-lapse imaging in cell culture introduced a continuous registration of the embryo development. Recent time-lapse studies of cell division dynamics have demonstrated the occurrence of abnormal cytokinesis, e.g., direct cleavage [2–5], which has been correlated to poorer embryo development [5] and lower embryo implantation [3].

Fragmentation is a dynamic event [6] which is difficult to be assessed by conventional scoring. Large fragments (LFs), which represent 5 % of the total counted blastomeres [7], can be distinguished from blastomeres only by the presence of the nucleus. Unfortunately, the detection of the nucleus is not always possible; e.g., the nucleus is disassembled during the mitotic phase [7]. Incorrect distinction between LFs and blastomeres affects the embryo scoring [8], since the number of cells results overestimated and fragmentation rate

underestimated. To minimize this flaw, it has been recommended to classify the cytoplasmic structures with diameters <45 and >45 μm as fragments and blastomeres, respectively [7, 8].

In this retrospective study, we have investigated whether the presence of LF and abnormal cell divisions (ACDs) has influenced the correlation between live birth rate and number of blastomeres detected on day 2 by conventional scoring. We included all the embryos selected for transfer by conventional scoring on day 2 with ≥ 2 cells and fragmentation ≤ 25 %. By time-lapse recordings, we recognized ACDs and LFs mistaken as blastomeres. The sensitivity of time-lapse assessment of the nuclei was confirmed by nuclear staining of surplus embryos.

Methods

Study population

This prospective cohort study included 434 couples undergoing intracytoplasmic sperm injection (ICSI) at Holbaek Fertility Clinic (Holbaek Hospital, Denmark) from 20 May 2010 until 16 February 2013. All embryos were included with a known destiny, live birth, or no live birth. Therefore, transfers of two embryos resulting in single live birth were excluded. Live birth was defined as a live born baby after gestational age of 24 weeks.

In total, 578 transferred embryos resulted in 119 live births, i.e., 93 from 290 single transfers and 26 from 144 double transfers. No monozygotic twins were observed. All embryos were cultured in time-lapse incubation system; time-lapse recordings were not used in embryo selection for transfer. Embryos were selected for transfer by conventional scoring on day 2. In addition, 34 embryos excluded from transfer were used to perform a nuclear assessment control test.

The National Ethical Committee of Medical Science of Denmark approved the study (Number: SJ-250). Informed consent was obtained from all individual participants included in the study including donation of embryos excluded from transfer to research.

All procedures including ovarian stimulation, aspiration, and ICSI were performed as described in the supplemental methods (Suppl. methods) [9].

Conventional embryo selection

Five embryologists performed the embryo selection for transfer by conventional scoring at the three pre-set times: At 18 h post-fertilization (PF), the fertilization, e.g., presence of two pronuclei (2PN) and the second polar body, was assessed; at 25 h PF, early cleavage defined as the presence of two cells/blastomeres was assessed. Finally, at 45 h PF, the

fragmentation rate and the number of blastomeres were scored. Only embryos with second polar body, 2PN, and at least the first cleavage completed were selected for transfer. The embryo ranking was as follows: Embryos scored as four cells had first priority; embryos scored as >4 cells were second, and embryos scored as <4 cells third. Only embryos with fragmentation rate <25 % were used for transfer [10, 11]. Cytoplasmic structures with a diameter ≥ 45 μm were scored as blastomeres [7].

Time-lapse reassessment

Two operators reassessed time-lapse recordings of all the transferred embryos. These two were not involved in the embryo selection for transfer. The operators registered the appearance of (i) ACDs and (ii) presence of LFs.

The criterion to recognize the beginning of a cell division was the disassembling of the nucleus (Fig. 1a). The term of the cell division was recognized by the reassembling of the nuclei in the resulting daughter cells. Only large cytoplasmic structures divided by membranes and presenting a clear nucleus were defined as blastomeres (Fig. 1b).

Two groups of ACDs were reported according to the number of blastomeres produced (Fig. 1b). Production of more than two blastomeres per cell division was defined as multi cell division; production of less than two blastomeres per cell division was defined as failed cell division. It was not reported whether the ACD occurred at the first cell division (first cleavage) or second and third cell division (second cleavage).

LFs were distinguished by the absence of a nucleus and a diameter larger than 45 μm (Fig. 1c). LFs were counted at 45 h PF. The volume of LFs was added to the estimated volume of small fragments. This sum of fragments was designated the recalculated total fragmentation, and it was reported when >25 % (Fig. 1c). The number of blastomeres was then recalculated.

Live birth rate

All embryos selected for transfer by conventional scoring were divided into three groups according to the number of blastomeres assessed: first, <4 cells; second, 4 cells; and third, >4 cells. Life birth rates in each of these groups were reported.

After time-lapse reassessment, embryos with ACD and fragments >25 % were excluded ($n = 106$). The remaining 472 embryos were divided again according to the recalculated number of blastomeres in three previously described groups. Then, the life birth rate in each group was reported.

Nuclear assessment control test

The ability of the two operators to recognize the nuclei was validated by DNA staining tests. They evaluated the time-

a NORMAL CELL DIVISION **b** ABNORMAL CELL DIVISIONS

Multi cell division

Failed cell division

c FRAGMENTATION (at 45 hours post fertilization) **Fig. 1** Examples of **a** normal cell division, **b** abnormal cell divisions, and **c** recalculation of fragmentation

lapse recordings of 34 embryos not selected for transfer. The number of nucleated blastomeres assessed by time lapse was compared to the number of nuclei observed by staining of the DNA with DAPI (Suppl. methods).

Statistical analyses

Life birth rates according to number of blastomeres were compared by χ^2 table contingency tests.

The correlation between (i) age and live birth rate and (ii) age and ACD and fragmentation >25 % was performed by Student's *t* test and reported as mean $\pm$ SEM.

All results were computed using statistic software R-Studio™ (RStudio, Inc.), and $P < 0.05$ was considered significant.

Results

After reassessment, either ACD and/or recalculated fragmentation >25 % occurred in 106 of 578 transferred embryos

(18.3 %). Embryos presenting ACD and fragmentation >25 % were less frequent in embryos scored as four cells (36/429; 8.4 %) than in embryos with less than four cells (18/35; 51.4 %) and more than four cells (62/114; 45.6 %). None of these 106 embryos resulted in a live birth.

Multi cell divisions and failed cell divisions, when reported as independent events, were observed in 44 (7.6 %) and 30 (5.2 %) embryos, respectively.

LFs were recognized in 103 embryos (17.8 %). Once the total fragmentation was recalculated, summing together the volume of the small fragments recognized by conventional scoring and the volume of LF recognized by time lapse, 52 embryos (9.0 %) resulted to have a fragmentation rate >25 %.

Live birth rate according to number of blastomeres evaluated by conventional scoring showed that embryos at four-cell stage had higher live birth rate (embryos scored as 4 cells 102/429, 23.8 %; <4 cells 3/35, 8.6 %; >4 cells 14/114, 12.3 %; $p > 0.05$) (Fig. 2, in black). According to reassessment of the number of blastomeres and the exclusion of embryos with ACD and fragmentation >25 %, the number of blastomeres did not affect the live birth rate (embryos scored as 4 cells 109/430, 25.3 %; <4 cells 4/19, 21.1 %; >4 cells 6/23, 26.1 %; $p = \text{NS}$) (Fig. 2, in gray).

Average women ages were not different between embryos presenting ACDs and fragmentation >25 % and the others ($p = \text{NS}$).

Women with live birth were significantly younger than women with no live birth (30.7 ± 0.4 vs. 32.2 ± 0.2 years old) ($p < 0.05$).

Detection of nuclei by DNA staining

All the nuclei recognized by time lapse in the 34 control test embryos were detected by DNA staining. The embryo

Fig. 2 Live birth rate (%) according to number of blastomeres evaluated by conventional scoring (black) and after excluding embryos presenting abnormal cleavage and >25 % fragmentation (gray). Bars show SEM. * $P < 0.05$

Fig. 3 Example of staining and time-lapse images of an embryo. The four pictures represent the very same embryo. **a, b** Same stained embryo observed in two different focus planes. The nuclei are recognizable in *blue* and the cytoplasm and cell membrane in *red*. Four nucleated blastomeres (*B*) and one large anucleated fragment (*F*). **c, d** Same embryo observed in time lapse at two different focus planes

reassessment by time-lapse monitoring at the fixation time resulted in 167 nucleated blastomeres and 28 LFs (Fig. 3).

Discussion

In this study, we revised the correlation between live birth and the number of blastomeres in embryos scored on day 2. The number of blastomeres has been one of the most relevant selection parameters applied by conventional scoring on cleavage-stage embryos [1]. By time-lapse assessment, we could observe the nuclei and identify ACD and large anucleated fragments, not seen by conventional scoring.

Previous studies have suggested that embryos at four-cell stage on day 2 have higher blastocyst rate, implantation rate, and live birth [12, 13]. Embryos cleaving slower and faster have lower implantation rate, and they have more frequently

abnormalities [1]. In this study, we have observed similar results, since embryos scored as four cells by conventional scoring showed a higher live birth rate.

By time-lapse reassessment, we could observe that half of the transferred embryos in our clinic, which were assessed by conventional scoring as less and more than four cells, presented ACDs and fragmentation >25 % (respectively, 51.4 and 45.6 %). Only a minority of the embryos scored as four cells presented such patterns (8.4 %). Once we excluded embryos with ACDs and fragmentation >25 %, the number of blastomeres did not predict the live birth potential. This result suggests that ACDs and fragmentation >25 % are one of the main reasons of the lower live birth rate of embryos with less and more than four cells. The transfer of four-cell embryos was thus an effective approach to avoid the transfer of embryos with ACDs and fragmentation >25 %, since these two irregularities could not be detected by conventional scoring.

By time-lapse assessment, recognition of ACDs and fragmentation >25 % resulted more relevant than selection by number of blastomeres in predicting live birth.

Nonetheless, the sample of embryos with less and more than four cells was limited in this study, since embryos with four cells were transferred first. It cannot be excluded a higher live birth rate among the four-cell embryos when a larger sample is evaluated. Moreover, embryos assessed by conventional scoring with fragmentation >25 % were excluded from transfer, and thus, our sample did not represent all embryos. However, we have no reason to expect a lower rate of ACDs and LFs in embryos with a higher rate of small fragments.

LFs mistaken as blastomeres affected the scoring of a large number of embryos. Once we recalculated the total fragmentation including LF volumes, 9 % of the embryos showed fragmentation >25 %. None of these embryos resulted in a live birth, confirming the detrimental effect of high fragmentation on embryo potential. Previous studies based on fixed and stained embryos had suggested the presence of LFs mistaken as blastomeres [7, 8]. Due to the limitation in recognizing the nuclei in every blastomere by conventional scoring, they recommended to distinguish LFs from blastomeres by diameter cutoff. This approach can be considered outdated when the embryo scoring is performed by time-lapse assessment. Our nuclear assessment control test validated the time-lapse assessment as sensitive in assessing the fragmentation

Fig. 4 Example of failed cell division at the second cleavage. Arrows indicate the nuclei. **a** Blastomere at two-cell stage. **b** Disassembling of the nucleus. **c** Reassembling of two nuclei

rate and the number of blastomeres in comparison to the conventional scoring (Fig. 3). Nevertheless, we should acknowledge that, in a few cases, it was difficult to assess the number of blastomeres and fragmentation rate, despite the DNA marker. A higher-resolution monitoring system could prevent these difficulties.

We observed two main failed cell division patterns. The first pattern showed the reassembling of the nucleus without cytokinesis or the fusion of the two just-cleaved blastomeres. This pattern has been investigated previously, but no mention of the nucleation status was described [14]. The second pattern showed a cytokinetic activity producing only small or large anucleated fragments. This pattern has not been investigated previously. Eventually, both the pattern produced a binucleated blastomere (Fig. 4). In our study, no live birth was observed after the transfer of embryos with ACD. We know from previous studies that embryos presenting the first cleavage producing more than two blastomeres have a poor outcome [3].

Although we did not find any live birth in these groups, we cannot exclude seldom live births in a larger dataset. Recently, Almagoor [15] has showed three live births from the transfer of blastocysts presenting a cell division producing more than two blastomeres at the second cleavage.

In our experience, the most effective approach to recognize ACDs and LFs is the assessment of the cell division as a cytokinesis synchronized to the disassembly and reassembling of the nuclei.

The disassembling of the nucleus is the start of the mitotic phase, and the reassembling in daughter cells is the end of the mitotic phase. Eukaryotic cells require an open mitosis, i.e., a disassembly of the nuclear membrane [16, 17], to achieve correct chromosome segregation. Nuclear envelope breakdown occurs during the prophase and reassembled during the telophase [18], while the cytokinesis initiation is activated during the anaphase and completed during the telophase [19]. Therefore, a cytokinesis occurring without disassembly and reassembly of the nuclei cannot be defined as a cell division.

The assessment of the nuclei has been reported to be difficult by conventional scoring [7]. By time-lapse incubation, we had access to larger time span, which made easier to avoid the observation of the nucleus at the moment that it was disassembled, i.e., during the mitotic phase [7].

So far, time-lapse technology has been used mainly to analyze the timing of early embryo events [2, 20–24] and the timing of the fifth cell division has been suggested to be predictive of implantation [20]. However, we should be careful in the interpretation of these timing parameters, since cell divisions were observed solely as cytokinetic events and the observation of the nuclei was not clearly defined. In our study, embryos assessed as five cells represented the largest group with ACDs and LFs. We assume that the timing of cell divisions of abnormal embryos and LF release have probably impaired the prediction potential of these timing parameters.

Recently, Athayde Wirka [5] has published several patterns of abnormal embryo cleavages recognizable only by time-lapse assessment. The technology used in that study is based on dark-field microscopy, which can visualize the cellular membranes, but it challenges the assessment for presence of the nuclei. Our study, which is based on light microscopy, offers a different point of view, since it focuses on the assessment of the nuclei more easily detected with light microscopy.

Conclusions

Conventional scoring on day 2 did not detect ACDs and LFs mistaken as blastomeres. LFs can lead to recalculate fragmentation rate to >25 %. No significant correlation between live birth rate and number of blastomeres in day 2 embryos was observed when embryos with ACDs and fragmentation >25 % were excluded. Recognition of ACDs and fragmentation >25 % is more predictive of live birth than number of blastomeres.

Compliance with ethical standards The National Ethical Committee of Medical Science of Denmark approved the study (Number: SJ-250). Informed consent was obtained from all individual participants included in the study including donation of embryos excluded from transfer to research.

Conflict of interest The authors declare that they have no conflict of interest.

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