Improving embryo quality in assisted reproduction
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CHAPTER 2

Temporal and developmental-stage variation in the occurrence of mitotic errors in tripronuclear human preimplantation embryos

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Fulco van der Veen
Sjoerd Repping
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Abstract

Mitotic errors during early development of human preimplantation embryos are common, rendering a large proportion of embryos chromosomally mosaic. It is also known that the percentage of diploid cells in human diploid-aneuploid mosaic embryos is higher at the blastocyst than at the cleavage stage. In this study, we examined whether there is temporal and/or developmental-stage variation in the occurrence of mitotic errors in human preimplantation embryos from the first day of development onwards using mitotically stable digynic tripronuclear human embryos as a model system. All the cells of the 114 digynic tripronuclear human preimplantation embryos included were analyzed by fluorescence in situ hybridization for chromosomes 1, 13, 16, 17, 18, 21, X and Y. Embryos were grouped according to day of development (1 - 6) and developmental stage (2-cell to blastocyst stage). The possibility of a mitotic error was highest in the first and second mitotic divisions. The percentage of cells with mitotic errors increased during preimplantation development and was highest at the 9 - 16 cell stage (76%, $P=0.027$). Thereafter, the percentage of cells with mitotic errors decreased to 64% at the morula and 56% at the blastocyst stage. The pattern found correlates with the activation of the embryonic genome at the 8 - 16 cell stage. A better insight in the timing of occurrence of mitotic errors in human preimplantation embryos could help in understanding and prevention of these errors and is relevant in the context of PGS.
**Introduction**

Chromosomal mosaicism, the phenomenon that cells in an embryo differ in chromosomal constitution, is common in human preimplantation embryos. A recent systematic review on the prevalence of mosaicism in spare human embryos showed that 73% of all human preimplantation embryos are mosaic [1]. The observed high incidence of mosaicism suggests that human preimplantation embryos are prone to errors during the first mitotic divisions after fertilization. Moreover, it was shown that the percentage of diploid cells in human diploid-aneuploid mosaic embryos was higher at the blastocyst stage (74%) than at the cleavage stage (62%) [1-3]. This suggests that there is a temporal and/or developmental-stage variation in the occurrence of mitotic errors in human preimplantation embryos.

Studies on the occurrence of mitotic errors in human preimplantation embryos are hampered by the fact that only spare embryos, that is, embryos of lower quality that will not be transferred or cryopreserved, can be examined. Another problem is that the examination of such embryos can only start on the day of embryo transfer (usually day 3 after injection or insemination) or later. Therefore, little is known on the occurrence of mitotic errors during the first cleavages of human preimplantation development. The first cleavages can be critical for further embryo development because the embryonic genome is not active yet and the embryo is depended on prestored maternal mRNA and proteins [4].

Here we use human tripronuclear (3PN) embryos to study the occurrence of mitotic errors during the first cleavages of human development. Tripronuclear embryos are embryos that show three pronuclei the first day after insemination and occur with an incidence of 2% - 9% in in vitro fertilization (IVF) cycles [5, 6] and 3% - 6% in intracytoplasmic sperm injection (ICSI) cycles [5-7]. In clinical practice, these embryos are never transferred into the uterus as these embryos are considered to be triploid.

Tripronuclear embryos can be divided in dispermic 3PN embryos and digynic 3PN embryos [8, 9]. Dispermic 3PN embryos are most often found in IVF, originating from oocytes that correctly excreted two polar bodies after fertilization but were fertilized by two spermatozoa. Dispermic embryos do not show bipolar spindles, presumably because there is an additional set of centrioles from the second spermatozoon, and as a consequence, they become mosaic from first cleavage onwards [10, 11]. Thus, dispermic 3PN embryos are not a good model for studying the temporal/developmental incidence of mosaicism.

Digynic 3PN embryos are most often found in ICSI, originating from oocytes that were fertilized by a single spermatozoon but failed to extrude the second polar body following fertilization. Digynic 3PN embryos contain only a single bipolar spindle, undergo regular chromosome segregation [12], and have a low rate of mosaicism [11, 12]. In fact, the removal of one pronucleus restores the embryo to normal diploid state [11]. Furthermore, the existence of stable triploid cell lines [13] and the capability of triploid embryos to
develop into blastocysts [14, 15] and even to live births [16] let us assume that these cells are not necessarily mitotically unstable.

In this study, we aimed at determining the difference in susceptibility to mitotic errors at different time points or developmental stages, during human preimplantation development. A better understanding of the developmental and temporal occurrence of mitotic errors will aid in evaluation of the consequences of mosaicism on preimplantation genetic screening (PGS), help determine the optimal developmental stage to perform PGS and, ultimately, help in attempting to prevent these errors in order to increase overall embryo quality and success rates in assisted reproductive technologies (ART).

**Materials and Methods**

**Samples**

The study was approved by the Dutch Central Committee on Research involving Human Subjects (CCMO). Only ICSI-derived (digynic) 3PN embryos were used for this study. Couples undergoing ICSI treatment at the Academic Medical Center (Amsterdam, the Netherlands) and University Medical Center Groningen (Groningen, the Netherlands) were asked to participate in this research, and written informed consent was obtained in all cases. A total of 114 fresh 3PN embryos were donated for research.

Donated embryos were randomly fixated on glass slides at six different time points, that is, Day 1 - 6 after fertilization. Embryos were transferred into 0.1% poloxoxyethylene (20) sorbitan monolaurate (Tween 20) in 0.01 N HCl to remove the zona pellucida, to dissociate the blastomeres, and to separate the individual nuclei. Fixation was performed by adding methanol:acetic acid (3:1) followed by dehydration in an increasing ethanol series [17, 18].

**Fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) was performed in two rounds for the analysis of chromosomes 1, 13, 16, 17, 18, 21, X and Y as previously described [19]. In the first round, all the available nuclei were analyzed for chromosomes 1, 16 and 17 using chromosome enumeration probes (Abbott Molecular); for the second round, the same nuclei were analyzed for the other five chromosomes with MyltiVysion PGT probes (Abbott Molecular). In case of abundant cytoplasm, the slides were pretreated with 0.0025% pepsin to optimize hybridization results. The following scoring criteria were used for the assessment of chromosome copy numbers: 1) two signals represented two chromosomes if at least one signal fitted in between these two signals, 2) if there was contact between two signals, the signal was judged as one, and 3) in case there was doubt about a signal, then the copy number of that signal was judged as unknown [20].
Assessment of aneuploidy

The chromosomal makeup of each blastomere of an embryo was used to reconstruct the ploidy status of that embryo at the zygote stage (Figure 1). To assess whether an embryo has acquired mitotic errors, we compared its chromosomal content to the presumed chromosomal content of the zygote. When the two did not differ, we assumed no mitotic errors had occurred. When the two chromosomal contents differed, blastomeres with altered content were assumed to have acquired mitotic errors. The probability that an individual blastomere acquires mitotic errors at a given cell division was calculated using the following formula: \( P_d = \frac{((\Phi_{d+1} - \Phi_d) / (1 - \Phi_d)) / N}{N} \), where \( \Phi_d \) is the observed incidence of chromosome aneuploidy among all embryos at the initiating cell stage and \( N \) is the number of cells at the initiating cell stage [21].

Statistical Analyses

All embryos were grouped according to time point (Day 1 to Day 6), and in a second, separate analysis, the same embryos were grouped according to developmental stage (2-cell, 4-cell, 8-cell, 16-cell, morula or blastocyst stage). Embryos with 3 - 4 cells were grouped at the 4-cell stage, embryos with 5 - 8 cells at the 8-cell stage and embryos with 9 - 16 cells at the 16-cell stage. Because part of the analyzed embryos were fixated at Day 1 (2-cell stage), the grouping of the embryos to the different ploidy statuses was based on a minimum of two blastomeres. For the analysis of the data, we assumed that all blastomeres,

<table>
<thead>
<tr>
<th>Blastomeres within embryo</th>
<th>FISH round 1</th>
<th>FISH round 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#16</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Presumed zygote makeup</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

**Figure 1:** An example of assessing the presumed zygote makeup from the individual blastomeres of a 4-cell embryo. For every chromosome (for example chromosome 1) the number of copies that appeared most often (for example 3) was presumed to be the number of chromosome copies present at the zygote stage.
whether diploid or aneuploid, divided at a similar rate during early embryo development [21]. To determine whether there was a temporal and developmental window during which mitotic errors occur more frequently, we plotted the percentages of blastomeres with mitotic errors against the developmental stage (number of blastomeres present in an embryo) or day of development. To investigate whether the occurrence of mitotic errors followed a certain pattern over time, we used regression analysis. The data were fitted to three different regression models (following linear, quadratic, and cubic patterns), and the $P$ value for each model was calculated to select the pattern that described our data most accurately.

**Results**

Clinical parameters like maternal age, gravidity, parity, duration and causes of infertility of the women that donated the 3PN embryos are provided in Table 1. The average maternal age of all women was 35 yr of age. The embryos that were analyzed on Day 6 of development were provided from women of an average of 38 yr of age, but this difference was not significant.

**Chromosomal makeup of 3PN embryos**

Seven hundred and fifty-seven blastomeres from 114 tripronuclear embryos were analyzed. The precise findings of all the embryos are shown in Table 2. In total, we found that at the time of analysis two embryos (2%) were uniformly diploid, six (5%) were uniformly aneuploid, and 106 (93%) were mosaic. Upon reconstruction of the chromosomal makeup of the zygote, 21 (18%) were diploid, 25 (22%) were triploid, six (5%) haploid and 62 (54%) were aneuploid (Table 2).
### Table 2: The chromosomal makeup of 3PN human preimplantation embryos (n=114) at the assumed zygote stage and at the time of analysis.

<table>
<thead>
<tr>
<th>Chromosomal makeup</th>
<th>Criteria</th>
<th>Analysis per developmental stage</th>
<th>A assumed zygote stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (n=114)</td>
<td>2-cell stage (n=7)</td>
</tr>
<tr>
<td>Diploid</td>
<td>All blastomeres contain two chromosomes for each chromosome pair tested</td>
<td>2 (2)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Mosaic</td>
<td>106 (93)</td>
<td>4 (57)</td>
<td>32 (89)</td>
</tr>
<tr>
<td>Diploid-aneuploid mosaic</td>
<td>20 (18)</td>
<td>2 (29)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Diploid-triploid mosaic</td>
<td>3 (3)</td>
<td>0 (0)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Triploid-aneuploid mosaic</td>
<td>26 (23)</td>
<td>1 (14)</td>
<td>7 (19)</td>
</tr>
<tr>
<td>Aneuploid mosaic</td>
<td>57 (50)</td>
<td>1 (14)</td>
<td>22 (61)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>6 (5)</td>
<td>2 (29)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Haploid</td>
<td>All blastomeres contain one chromosome for each chromosome pair tested</td>
<td>2 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Triploid</td>
<td>3 (3)</td>
<td>2 (29)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Polyploid</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Complex abnormal</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
Occurrence of mitotic errors

A high percentage of blastomeres with mitotic errors was observed at all days and at all stages of human preimplantation development. The percentage of blastomeres with mitotic errors increased during the first divisions, showing the highest percentage (67%) at Day 5 (Figure 2). The percentage of blastomeres with mitotic errors decreased to 61% at Day 6. When the embryos were analyzed according to developmental stage, the same pattern was observed, with an increase in the percentage of blastomeres with mitotic errors up to the 9 - 16 cell stage (76%) and then a decrease to 64% at the morula stage and 56% at the blastocyst stage (Figure 3).

Regression analysis showed that the data could follow all three regression models tested, but the data followed the quadratic model best (linear model: $P=0.018$ and $P=0.041$ according to day of development and developmental stage, respectively; quadratic model: $P=0.010$ and $P=0.001$ according to day of development and developmental stage, respectively; and cubic model: $P=0.027$ and $P=0.002$ according to day of development and developmental stage, respectively) (Figure 4).

Using the approach described from Bean et al. [21], we calculated the probability that a specific cell acquired mitotic errors at a given cell division. The results of this analysis showed that the probability of a specific cell to acquire mitotic errors decreased with each division and leveled out at the morula and blastocyst stage (Figure 5). At the morula stage, the possibility of a given cell to malsegregate has a negative value as the incidence of the observed occurrence of aneuploidy at the morula stage is lower than the incidence of the observed occurrence of aneuploidy in the developmental stage before that (16-cell stage).

**Figure 2:** Percentage of blastomeres with mitotic errors in human preimplantation 3PN embryos at different days of development. The number of embryos (and blastomeres) analyzed per group is indicated above each bar.
Discussion

Our results showed that the possibility of any given cell to malsegregate is highest in the first two cleavages after fertilization. These results are in accordance with previous studies on mouse and human embryos that showed that nondisjunction events predominantly pertain to the earliest cleavage divisions [21, 22]. The percentage of cells with mitotic errors increased during early development, peaking at the 9-16 cell stage and decreasing thereafter. This pattern resembles normally fertilized (2PN) human preimplantation embryos where the percentage of diploid cells in blastocysts was found to be higher than in cleavage embryos [1].

<table>
<thead>
<tr>
<th>Regression model</th>
<th>Formula</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per day</td>
</tr>
<tr>
<td>Linear</td>
<td>( y = \alpha + \beta x )</td>
<td>0.018</td>
</tr>
<tr>
<td>Quadratic</td>
<td>( y = \alpha + \beta x^2 + \gamma x )</td>
<td>0.010</td>
</tr>
<tr>
<td>Cubic</td>
<td>( y = \alpha + \beta x^3 + \gamma x^2 + \delta x )</td>
<td>0.027</td>
</tr>
</tbody>
</table>

\( \alpha, \beta, \gamma, \delta \) constants and \( \beta \neq 0 \)

Figure 3: Percentage of blastomeres with mitotic errors in human preimplantation 3PN embryos at different developmental stages. The number of embryos (and blastomeres) analyzed per group is indicated above each bar.

Figure 4: The obtained \( P \) values per regression model used.
Limitations of the study

In this study, we used the most commonly used technique, FISH to define the chromosomal status of 3PN embryos. We were therefore unable to detect embryos that were aneuploid for the chromosomes that we did not test. Moreover, FISH does not allow the detection of partial or segmental aneuploidy since the FISH probes hybridize to a specific locus or the centromere and provide information only about that segment of the chromosomes [23]. Another technical limitation of FISH is that scoring errors can potentially occur due to loss or damage of nuclear material, overlapping of signals, split signals, diffused signals, hybridization failure, and probe inefficiency. FISH analysis has 92% - 99% accuracy per probe, so when using a multiprobe panel on one cell, the risk of misdiagnosis is indeed significant [24-26]. Nonetheless, FISH can be considered a reliable technique for aneuploidy determination for research purposes [24]. Because the focus of this study is not to report the exact rate of aneuploidy at the different developmental stages, which could be affected by scoring errors, but rather to study the pattern of its occurrence, and FISH scoring errors are not likely to occur more in one developmental stage compared to the other, we believe that the use of FISH did not affect our results.

Chromosomal makeup of 3PN embryos

In our study the percentage of 3PN embryos at the cleavage stage that were fully triploid was 3%. This percentage is in contrast to a study that reported a percentage of 55.7% of fully triploid 3PN embryos derived after ICSI [12]. This difference might be due to the fact that the other study analyzed only three chromosomes (18, X and Y) while we analyzed eight. The effect of the number of analyzed chromosomes on the percentage of mosaicism reported was also described in a recent review [1]. Also, we reported a slightly higher rate of triploid embryos at the cleavage stage (38% Days 2 - 4) in comparison to a study that found 32% of the ICSI-derived tripronuclear embryos to be triploid [14]. In contrast to our study, the study by Grau et al. analyzed five chromosomes in one hybridization round and only one blastomere per embryo.
When we reconstructed the chromosomal makeup of the zygotes using the chromosomal composition of the analyzed blastomeres, we found that 25 (22%) of the embryos were presumably triploid at the zygote stage. Our findings on the percentage of 3PN zygotes that are fully triploid are in contrast to those of other studies that showed a percentage of 7% - 88% of the 3PN zygotes to be triploid [5, 27]. This difference might be explained by the different criteria of assessing triploidy of zygotes. We used the analysis of individual blastomeres to reconstruct the presumed chromosomal makeup of the zygote while the other studies analyzed 3PN zygotes that were arrested before the first cleavage division. In addition, we strictly followed the rule that the zygotes had to show three copies of all analyzed chromosomes, while one study assessed zygotes as triploid if the majority of chromosomes were present in three copies [5].

Occurrence of mitotic errors

We found high rates of mitotic errors in the first days after fertilization that are reduced after the 9 - 16 cell stage. The first mitotic divisions are almost completely dependent on stored mRNA and proteins in the oocyte because there is no transcription from the embryonic genome in this early stage of development [4, 28, 29]. The quality of human oocytes, their proteins, and gene transcripts could diminish over time because of factors such as the accumulation of radiation or toxic agents, oxidative stress [30], compromised mitochondria [31], or telomere shortening [32]. This compromised environment could then result in absence or reduced stringency of cell-cycle checkpoint mechanisms during the earliest stages of human preimplantation development, thereby facilitating chromosome segregation errors, especially in women of advanced maternal age [29, 33, 34]. Once the embryonic genome is activated, cell-cycle checkpoints could become functional again, resulting in relatively less aneuploid cells afterwards. It could therefore be hypothesized that the incidence of mosaicism will be highest in human preimplantation embryos around the 8-cell stage or Day 3 after fertilization, just before the embryonic genome is activated [35].

Apart from reinstating the mitotic machinery, the activation of the embryonic genome potentially initiates other phenomena that could explain the lower rate of aneuploidies at later stages of development. First, embryonic genome activation could induce the correction of aneuploidies via mechanisms such as anaphase lagging, corrective non-disjunction or chromosome fragmentation [36]. Second, it could induce preferential proliferation of diploid cells and induce arrest or apoptosis of aneuploid cells [37]. Indeed, data indicate that apoptosis does not become activated in preimplantation embryos until the morula stage [38]. Third, it could induce preferential allocation of diploid cells to the inner cell mass or embryo proper [35]. Confined placental mosaicism, in which placental tissue was trisomic whereas the fetus was diploid, has been reported as a result of the loss of trisomic chromosomes in the embryonic tissue [39]. However, further research on the fate of the abnormal blastomeres within an embryo is necessary to confirm this hypothesis [40-42].
In conclusion, our data indicate that there is clear temporal and developmental-stage variation in the occurrence of mitotic errors in human preimplantation embryos. As mitotic errors are most likely to occur during the first three cleavages, more research should be conducted into which elements (either biological or technical) control their occurrence during this stage of development. Possibly, improved ovarian stimulation or in vitro culture systems will limit the rate of mitotic errors and thereby increase overall embryo quality and, with that, treatment success in ART. The high prevalence of mosaicism at all stages of preimplantation development causes a serious diagnostic dilemma to PGS during which the selection of embryos for transfer into the uterus is based on the chromosomal content of a single blastomere that is removed from each embryo. Embryo biopsy for PGS has often been performed at the third day of embryo development, and our data indicate that this is in fact the time when the percentage of blastomeres with mitotic errors is highest.

References


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Occurrence of mitotic errors in 3PN human embryos


