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MATERNAL-FETAL MEDICINE

Oocyte morphology on day 0 correlates with aneuploidy as detected by polar body biopsy and FISH

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Abstract
Purpose For better selection of oocytes and embryos, preimplantation genetic screening (PGS) was introduced. As from the beginning of IVF, morphology was used as selection criteria; we investigated the combination of both. If there was a correlation between phenotype and genotype, invasive PGS might be replaced.
Method Therefore, 104 cycles with PGS were done by biopsy of the first polar body and FISH with five chromosomes. Morphology of the oocyte was recorded digitally and noted for 12 categories in 4–13 values; evaluation of the chromosomes was noted for five chromosomes in five values. Morphology and genetics were correlated to each other.
Result Correlations between morphology and genetics for day 0 were found: oocytes with an irregular or dark zona are less probable to have a normal chromosome 13 (80 vs. 53 %, $p = 0.001$). A medium amount of detritus in the perivitelline space makes it more probable to have a normal chromosome 18 (94 vs. 78 %, $p = 0.001$). A halo in the cytoplasm makes it less probable to be euploid for chromosome 22 (56 vs. 75 %, $p = 0.018$). For day 1, pattern "1, 2, 3 and fine" in the pronuclei makes it more probable to be euploid for chromosome 22 (78 vs. 63 %, $p = 0.002$).
Conclusion There are correlations between the oocyte genome and its morphology also on day 0. These correlations are not sufficient to replace PGS.

Keywords Oocyte · Morphology · Genome · PGS · Polar body biopsy · FISH

Introduction

The result of artificial reproductive techniques (ART) in the human species is limited. Thinkable approaches for optimization are: better quality, in the clinical or laboratory work, and better selection of gametes or embryos. For better selection, the "omics" were introduced in ART: preimplantation screening of oocytes and (pre-) embryos by genomics, proteomics and metabolomics. The principle of this idea is not new: from the beginning of IVF, morphology of oocytes and embryos was used as selection criteria. The problem is: to what extent is there a correlation between phenotype and genotype in oocytes and embryos at that early stage, in vitro? If this extent is only low, any selection criteria other than PGS can only lead to limited success. Ideal selection criteria other than PGS should exclude a maximum of aneuploidies.

Morphology is an easy-to-detect phenotypical selection tool. But criteria for its description have to be standardized and evaluated. Furthermore, morphology changes over time, so the point in time for observation, matters. Last but not least, it is not clear as to which point in time correlates best with the outcome. Also the best time for a genetic analysis of the embryo is not clear for various reasons. Morphology checks in practice are normally done daily

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when other works on oocytes and embryos are carried out. These times differ, and morphologic criteria are not unanimously accepted. Investigations of the morphologic development in relation to a time scheme with time lapse ("embryo scope") might deliver an additional selection marker. As many randomized controlled trials (RCT) found no benefit of PGS [1] and only now first significant results were presented [2–4], we wanted to check if PGS could be replaced by morphologic observations. As embryo selection is not allowed in our country, we opted for polar body biopsy. So we related the morphology of the oocyte to the result of its genetic investigation.

Morphology of oocytes and embryos can be checked by stereo light microscopy, on a routine basis, at lower magnification (80×), or more detailed at the stage of the microscope of the micromanipulator (400×). It is also possible to record the picture by photograph or film. For the genetic part, FISH and aCGH are thinkable. For scientific morphologic investigations of oocytes or embryos, 400× magnification and photography are most common. For PGS of oocytes first or first and second polar bodies are analysed. For genetic analysis, FISH with 5–9 probes are most common. With the introduction of PGS in our program [5] by laser biopsy we opted for digital imaging of all oocytes. In order to reduce possible harm to the oocyte by two distinct micromanipulations (ICSI and biopsy) within a day we biopsied only the first polar body. As we did not charge the patients for PGS we limited FISH to five probes.

Methods

PGS was offered to all patients with eight or more oocytes. If there were more than about 12 we offered "splitting" to the patients: as cryopreservation of biopsied oocytes is not very successful, we proposed to perform PGS on 8–10 oocytes and freeze the rest. We used all established stimulation protocols, adapted to the patient, with a first line therapy with the long GnRH protocol with oral contraceptives (Valette, Ethinylestradiol 0.03 mg and Dienogest 2 mg) and nasal Spay (Synarela, Nafarelin 0.8 mg/d).

After hyaluronidase treatment, the amount of mature oocytes was detected. Together with the patient, it was decided if a splitting between PGS and cryopreservation should be done. For hybridization the standard set of probes from Vysis was used: chromosomes 13, 16, 18, 21 and 22. The digital registration of the oocyte was performed with the Octax system (MTG, Bruckberg, Germany). The FISH results were evaluated by two investigators following preset standards. If their decision was not unanimous, a senior third decided. The results were noted for each chromosome of the five chromosomes in 5 values from 0 to 4, depending on the number of signals detected (Table 1). As each chromosome in the first polar body has got two chromatids, two signals signified one whole chromosome in the polar body and one chromosome in the oocyte, which signified the only normal value.

The morphology was noted at the time of biopsy and on the following day (Table 2). Primarily five distinct microanatomic parts of the oocyte were described: first polar body, pronuclei, cytoplasm, perivitelline space and zona pellucida. These categories were ranked in preset values for each category (see Table 2). Secondly any other special morphologic phenomena were noted (Table 2, # 8, 10, 12). The morphologic and genetic categories were than correlated with each other (Table 3). This can be done in an analysis from fine to coarse: (1) single categories versus single categories, with all variables or divided in only two ("good" and "bad"); (2) single versus all categories as a whole, again with all categories as variables or only two (genetics or morphology of the whole oocyte) and vice versa (3); (4) all genetic categories as a whole versus all morphologic categories as a whole, again with all categories as variables or only two.

The study was registered and approved by the Ethics Commission of the Medical Faculty of the Christian-

Table 1 Chromosomes

5 genetic categories: chromosomes	5 genetic variables: chromatids	Genetic variables: explanations
Chromosome 13	0	No chromatid in polar body: 2 chromosomes in oocyte = abnormal (aneuploidy)
Chromosome 16	1	1 chromatid in polar body: 1 chromosome and 1 chromatid in oocyte = abnormal (aneuploidy)
Chromosome 18	**2**	1 chromosome in polar body: 1 chromosome in oocyte = **normal (euploid)**
Chromosome 21	3	3 chromatids in polar body: 1 chromatid in oocyte = abnormal (aneuploidy)
Chromosome 22	4	2 chromosomes in polar body: no chromosome in oocyte = abnormal (aneuploidy)

The 5 variables apply to each chromosome. Bold signifies the normal variable

Table 2 Morphology

#	12 morphologic categories	4–13 morphologic variables	Numbers variables	Oocytes
1	Polar body 1	None, round, fragmented, big	4	182
2	Polar body 2	None, round, fragmented, oval	4	78
3	Pronuclei	None, 1, 2, 3	4	932
4	Pattern pronuclei	0, 1, 2, 3, 4, 5, fine granular	7	567
5	Number polar bodies	0, 1, 2, 3	4	663
6	Polar body 2	Fragments 0, 2, 3, 4, 5, 6, 7	7	133
7	Cytoplasm	Very good, good, bad, degenerating	4	853
8	Cytoplasm specialties	Halo, oval, unshaped, dark, degenerated, cytoplasm out of zona, nose, centered, vacuole, oil, fragmented, in perivitelline space, coarsely granular	13	298
9	Perivitelline space	Granulation degree 0, 1, 2, 3	4	354
10	Perivitelline space specialties	Small, normal, big, oil	4	204
11	Zona	Small, normal, thick, irregular, dark, no zona, marked-off	7	444
12	Zona specialties	Big hole, vitreous, porous, porous dark, frazzled, fringed	6	41

All observations on day 0, except numbers 3, 4, 6, on day 1

Table 3 Possible correlations between genetics and morphology

Number of investigation	Genetics	Morphology
I. Single/single	Single genetic categories (five chromosomes with 5 variables)	Single morphologic categories (12 categories with 4–13 variables)
1	Variable (a)	Variable (e)
2	Variable (a)	Dichotomous (f)
3	Dichotomous (b)	Variable (e)
4	Dichotomous (b)	Dichotomous (f)
II. Single/whole	Single genetic categories (five chromosomes with 5 variables)	Whole morphologic status (12 categories together)
5	Variable (a)	Variable (g)
6	Variable (a)	Dichotomous (h)
7	Dichotomous (b)	Variable (g)
8	Dichotomous (b)	Dichotomous (h)
III. Whole/single	Whole genetic status (five chromosomes together)	Single morphologic categories (12 categories with 4–10 variables)
9	Variable (c)	Variable (e)
10	Variable (c)	Dichotomous (f)
11	Dichotomous (d)	Variable (e)
12	Dichotomous (d)	Dichotomous (f)
IV. Whole/whole	Whole genetic status (five chromosomes together)	Whole morphologic status (12 categories together)
13	Variable (c)	Variable (g)
14	Variable (c)	Dichotomous (h)
15	Dichotomous (d)	Variable (g)
16	Dichotomous (d)	Dichotomous (h)

a one chromosome, five variables; **b** one chromosome, two variables: normal or abnormal; **c** all chromosomes, some normal; **d** all chromosomes, all normal or abnormal; **e** one category, 4–13 variables; **f** one category, two variables: normal, abnormal; **g** all categories, some normal; **h** all categories, all normal or abnormal

Table 4 Significant correlations between genetics and morphology

Genetics Chromosome	Morphology Morphologic category	Correlations Chromosome/morphologic category	Oocytes	Significances p
13	Zona	(1.) variable/variable	201	0.027
		(2.) dichotomous/variable		0.005
		(3.) dichotomous/dichotomous: normal vs. rest		0.033
		(81 vs. 68 %, 80/99 vs. 69/102)		
		(4.) dichotomous/dichotomous: small, normal, thick vs. irregular, dark		0.001
		(80 vs. 53 %, 126/158 vs. 23/43)		
16	Zona	(5.) variable/variable	293	0.024
18	Perivitelline space	(6.) variable/variable	242	0.008
		(7.) dichotomous/variable		0.007
		(8.) dichotomous/dichotomous: medium detritus vs. rest		0.001
		(94 vs. 78 %, 101/108 vs. 104/134)		
21	Zona specialties	(9.) dichotomous/variable	26	0.05
22	Cytoplasm specialties	(10.) variable/dichotomous	167	0.011
		(11.) dichotomous/dichotomous: halo vs. rest		0.018
		(56 vs. 75 %, 24/43 vs. 93/124)		
	Pattern pronuclei	(12.) dichotomous/variable	347	0.025
		(13.) dichotomous/dichotomous: 1,2,5, fine vs. rest		0.002
		(78 vs. 63 %, 123/157 vs. 119/190)		
	Zona specialties	(14.) dichotomous/variable	24	0.001

Albrechts-University, Kiel. Statistic tests were performed with SPSS. Significant correlations were accepted if the Chi-square test (Pearson) showed an asymptotic significance (2-sided) of ≤ 0.05.

Results

From 104 PGS cycles we generated 1,181 mature oocytes; 893 were biopsied, and from 621 we got genetic results. Morphologic phenomena were noted in detail with a maximum of 932 for pronuclei (Table 2). Oocytes were not biopsied because of "splitting", attributing part of the oocyte pool to cryopreservation. Polar bodies could not be biopsied due to biological or technical reasons. Oocytes were not classified morphologically due to omissions in the laboratory. Genetic results were not achieved due to problems with fixation, hybridisation or analysis.

Significant correlations could be found for all four different comparisons of the first step of analysis (Table 3 I 1–4.), i.e., for single chromosomes versus single morphologic categories. These comprised four of the five major entities: for day 0 zona, perivitelline space, cytoplasm but not polar bodies, and for day 1 pronuclei (Table 4). Further steps found a single correlation (Table 3, III 11, "whole vs. single") for euploid oocytes versus zona specialties ($p = 0.028$). The zona correlates with chromosomes 13, 16, 21 and 22. A normal zona correlates with a normal chromosome 13, especially oocytes with an irregular or dark zona are less probable to have a normal chromosome 13 (80 vs. 53 %, $p = 0.001$). The perivitelline space correlates with chromosome 18, a medium amount of detritus makes it more probable to have a normal chromosome 18 (94 vs. 78 %, $p = 0.001$). Cytoplasm specialties and the pattern of the pronuclei correlate with chromosome 22. A halo makes it less probable to be euploid for chromosome 22 (56 vs. 75 %, $p = 0.018$). Pattern "1, 2, 3 and fine" make it more probable to be euploid for chromosome 22 (78 vs. 63 %, $p = 0.002$).

Discussion

From 621 oocytes we got chromosomal results and from up to 932 oocytes morphological descriptions. These are sufficient data to correlate morphology and genetics. Significant correlations were found for zona, perivitelline space, cytoplasm and pronuclei. There is only little literature

concerning a relation between oocyte morphology and aneuploidy for day 0. Bad cytoplasm was related to a seemingly increased proportion of aneuploidy, by conventional karyogram [6]. For day 1 pronuclei scores were found related to aneuploidy with blastomere biopsy day 3 and FISH [7–10], as well as development in vitro with time lapse [11]. First analysis in the beginning of our trial found correlations of oocyte aneuploidy with zona, polar body and perivitelline space [12, 13].

Correlations between oocyte morphology and development in vitro and in vivo were often looked for and mostly found:

- For the zona (yes: [14–21]; no: [22, 23]);
- For the polar body (yes: [24–29]; no: [30–32]);
- For the perivitelline space (yes: [24, 29, 33]; no: [22, 34]);
- For the cytoplasm (yes: [24, 29, 35–37]; also for spindles [38]; no: [22, 23, 26, 32, 39]);
- For pronuclei (yes: [7–10, 28, 40, 41]).

So it is not astonishing that most oocyte features also for day 0 can be also related to genetics. Only the correlations found unfortunately are not big enough to have a clinical relevance. The differences are just between 15 and 27 percent points and only relate to single chromosomes. aCGH might bring a higher efficiency towards a relation between oocyte morphology and genome. For the aim of the study, to find possibly a morphologic replacement for PGS, one can say that this was not found. Even if there is a correlation between a normal zona and a normal chromosome 13, the discrimination is not big enough and cannot lead to a clinical relevance. So for the time being PGS cannot be replaced by morphology, but a future broader application of aCGH with an analysis of all chromosomes might change that.

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Conflict of interest All authors declare no conflict of interest.

Ethical standards The PGS study was registered and approved by the Ethics Commission of the Medical Faculty of the Christian-Albrechts-University Kiel.

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