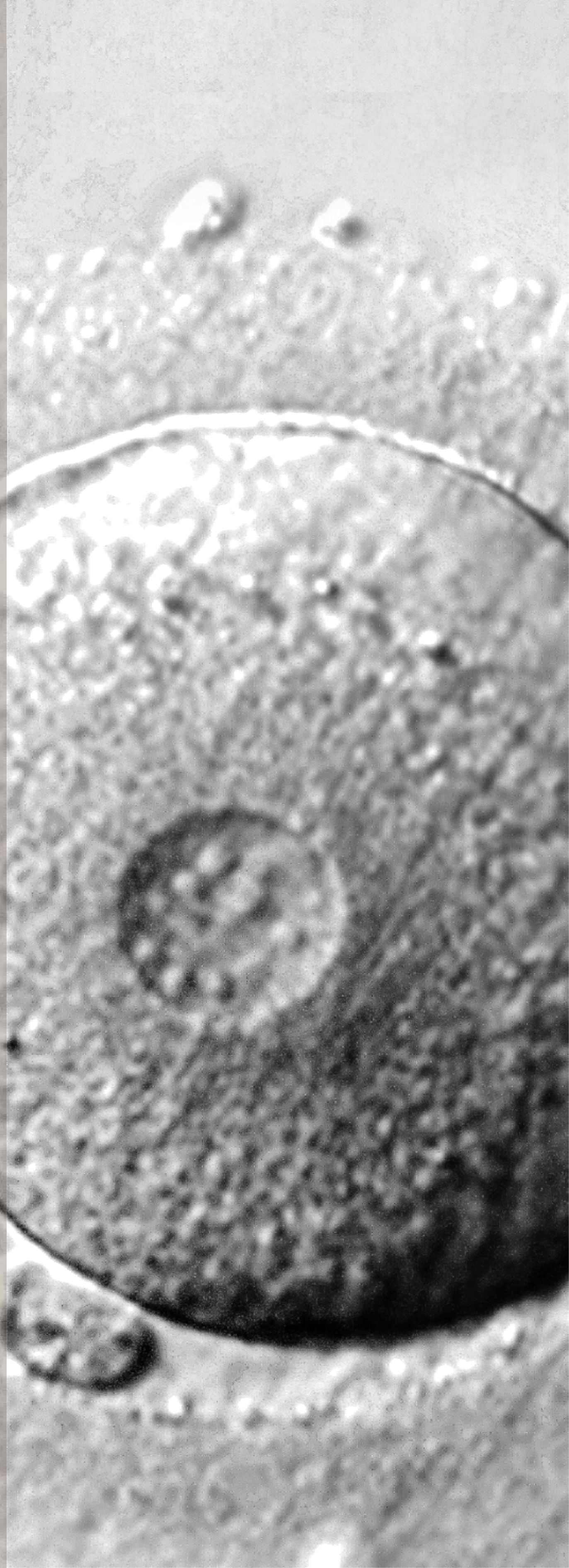
Zigots monopronucleats: origen, desenvolupament i constitució cromosòmica

Sílvia Mateo Cuadros



Tesi Doctoral

Novembre 2017

CAPÍTOL 3

OBJECTIUS

1- Analitzar, mitjançant hibridació in situ fluorescent (FISH), les característiques cromosòmiques d'embrions derivats de zigots monopronucleats provinents d'ICSI i correlacionar els resultats amb la seva capacitat de desenvolupament *in vitro*.

2- Analitzar, mitjançant hibridació genòmica comparada (aCGH), la constitució cromosòmica d'embrions derivats de zigots monopronucleats provinents d'ICSI i avaluar el seu potencial d'implantació.

3- Establir la millor estratègia, respecte l'estadi embrionari i la metodologia diagnòstica, per a l'anàlisi dels embrions provinents de zigots monopronucleats obtinguts mitjançant ICSI.

4- Analitzar els paràmetres morfocinètics del desenvolupament *in vitro* de blastocists derivats de zigots monopronucleats provinents d'ICSI i comparar-los amb els dels embrions provinents de zigots amb dos pronuclis i dos corpuscles polars.

CAPÍTOL 4

RESULTATS

4.1. Article 1

Títol: *In vitro development and chromosome constitution of embryos derived from monopronucleated zygotes after intracytoplasmic sperm injection*.

Autors: Sílvia Mateo, Mònica Parriego, Montserrat Boada, Francesca Vidal, Buenaventura Coroleu i Anna Veiga.

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In vitro development and chromosome constitution of embryos derived from monopronucleated zygotes after intracytoplasmic sperm injection

Sílvia Mateo, M.Sc.,[a] Mònica Parriego, M.Sc.,[a] Montserrat Boada, Ph.D.,[a] Francesca Vidal, Ph.D.,[b] Buenaventura Coroleu, M.D.,[a] and Anna Veiga, Ph.D.[a,c]

[a] Reproductive Medicine Service, Department of Obstetrics, Gynecology and Reproduction, Institut Universitari Dexeus; [b] Cell Biology Unit, Faculty of Biosciences, Universitat Autònoma de Barcelona; and [c] Stem Cell Bank, Center for Regenerative Medicine, Barcelona, Spain

Objective: To study the chromosome constitution and in vitro development of embryos derived from monopronucleated (1PN) zygotes after intracytoplasmic sperm injection (ICSI).
Design: Prospective study.
Setting: Private assisted reproduction center.
Patient(s): Fifty-four 1PN 2PB zygotes obtained from 48 ICSI cycles.
Intervention(s): Pronuclear diameter measured 18 $\pm$ 2 hours after ICSI and embryonic in vitro development assessed until the blastocyst stage or arrest; embryos disaggregated and all cells analyzed for chromosomes 13, 18, 21, X, and Y by fluorescent in situ hybridization (FISH).
Main Outcome Measure(s): Pronuclear size, in vitro embryo development, and cytogenetic characteristics.
Result(s): All the embryos were chromosomally abnormal (7.4% diploid mosaic, 16.7% haploid mosaics, 5.5% aneuploid mosaic, and 70.4% chaotic mosaic), and 35.2% showed a Y chromosome. No difference in the pronuclear size was observed according to the type of abnormality. Only 7.4% of the embryos resulted in blastocysts with good morphologic features.
Conclusion(s): Despite showing only one pronucleus, most of the zygotes resulted from fertilization. Embryos derived from 1PN ICSI zygotes are chromosomally abnormal. Neither the pronuclear size nor the in vitro developmental ability of these embryos can be predictive of their chromosomal constitution. According to our results, embryos that develop from 1PN ICSI zygotes should be discarded for any reproductive purpose. (Fertil Steril® 2013;99:897–902. ©2013 by American Society for Reproductive Medicine.)
Key Words: Chromosome constitution, FISH, intracytoplasmic sperm injection, in vitro development, monopronucleated zygotes

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Zygotes showing a single pronucleus are sometimes considered acceptable for clinical use when no two pronuclei (2PN) and two polar bodies (2PB) zygotes are available. It has been reported that monopronucleated (1PN) zygotes obtained from conventional in vitro fertilization (IVF) are mainly diploid (1, 2) and could be used for reproductive purposes; however, 1PN zygotes resulting from intracytoplasmic sperm injection (ICSI) are usually discarded because they have been associated with chromosomal abnormalities. Several studies have addressed the chromosomal status of embryos derived from 1PN ICSI zygotes, but no concordant results have been reported (3–5). Discrepancies between these studies can be related to their methodologies, the number of cells analyzed, or the developmental stage when the analyses were

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Reprint requests: Sílvia Mateo, M.Sc., Service of Reproductive Medicine, Institut Universitari Dexeus, Gran Via Carles III, 71–75, 08028 Barcelona, Spain (E-mail: silmat@dexeus.com).

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performed. Furthermore, few studies have evaluated the correlation between the morphologic characteristics of these embryos and the chromosomal status (1, 6).

Three mechanisms have been proposed as possible origins of 1PN zygotes. The first one is based on the asynchrony during pronuclear formation (1) and the second is the occurrence of syngamy (7). In these two cases, fertilization has occurred, and the resulting embryos could be diploid. The third mechanism refers to oocyte parthenogenetic activation and would lead to a haploid embryo. The use of time-lapse equipment will likely shed light on the occurrence of these events, although limited information is available at present.

Thus, there is still an important lack of information about embryos derived from 1PN ICSI zygotes; because some of them develop properly in vitro, it is necessary to determine whether it is appropriate to use them for reproductive purposes. We evaluated the chromosome constitution of these embryos and investigated any possible relation between pronuclear size, cytogenetic characteristics, and in vitro developmental ability. We also determined whether embryos derived from 1PN ICSI zygotes could be suitable for reproductive purposes, either for transfer or cryopreservation.

MATERIALS AND METHODS

Pronuclear Morphometric Analysis and Embryo Development

A total of 54 zygotes from 48 cycles, all 1PN and 2PB at 18 $\pm$ 2 hours after ICSI, were included in this study. Written informed consent to donate the embryos was obtained from the patients, and was approved by the review board of the center; the study was performed under the auspices of “Càtedra d’Investigació en Obstetrícia i Ginecologia” of the Department of Obstetrics, Gynecology and Reproduction, Institut Universitari Dexeus-Universitat Autònoma de Barcelona.

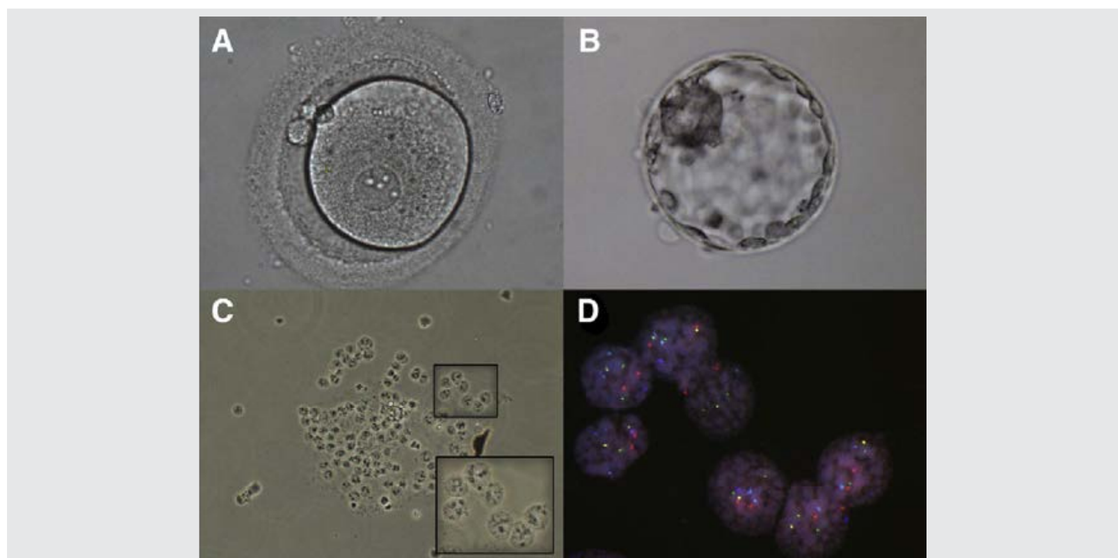
Images of all the zygotes were recorded on OCTAX Eye-WareTM Olympus system when fertilization was checked (Fig. 1). Measurements of the two maximum perpendicular diameters at the middle plane of the pronucleus were taken. For statistical analysis, the mean of the two diameters and the area ($A = \pi.r_1 \cdot r_2$) of the pronucleus were considered. Zygotes were cultured in vitro (Vitrolife G5 series), and embryo evaluation was performed daily until fixation (days 3–7).

As a control group, images of 28 ICSI zygotes showing 2PN and 2PB 18 $\pm$ 2 hours after ICSI were recorded, and the diameters of the pronuclei were measured with the same criteria and methodology as in the study group to have a pronuclear diameter value of normal fertilized zygotes.

Chromosomal Analysis

All 54 embryos selected for the study were individually processed for cytogenetic analysis when in vitro developmental arrest was observed or when reaching the blastocyst stage. Embryos were fixed as previously described by Clouston et al. (8) with minor modifications. Briefly, cleavage embryos, morulas, and blastocysts were entirely placed for 5 minutes in a phosphate-buffered saline (PBS) solution (6% human serum albumin), then for 1 minute in a Carnoy’s solution (methanol-acetic acid, 3:1); they were then transferred

FIGURE 1



(A) Monopronuclear (1PN) zygote, 16 to 20 hours after ICSI. (B) Blastocyst image from a 1PN ICSI zygote (D+5). (C) Fixation of 5th day blastocyst (magnification: ×10). In the smaller image, nuclei detail (magnification: ×20). (D) Same image processed by FISH showing to XY diploid constitution: two chromosomes 13 (*red*), two 18 (*aqua*), two 21 (*green*), one X (*blue*), and one Y (*gold*).

Mateo. Chromosome analysis of 1PN ICSI zygotes. Fertil Steril 2013.

for 1 minute to a methanol-acetic acid-water solution (3:3:1), and finally they were fixed on a slide by adding Carnoy's solution. The last step of the fixation procedure was performed under an inverted microscope and was adapted to the characteristics of the embryos for an accurate cell spreading. After fixation, the slides were left to dry, and they stored at −4°C until processed for fluorescent in situ hybridization (FISH) analysis.

The slides were processed for FISH using a commercial probe panel, specific for chromosomes 13, 18, 21, X, and Y (MultiVysion PGT Multi-color Probe; Vysis, AbbotMolecular) according to the manufacturers' protocols. Interphase nuclei were evaluated using an Olympus BX-61 fluorescent microscope (Olympus Barcelona) equipped with specific filters for FITC, Cy3, and Aqua and a multiband pass filter (DAPI/FITC/Texas Red). Overlapped nuclei, metaphase figures, and chromatin fragments were discarded from the analysis. Every informative nucleus from each embryo was analyzed. Embryos were further classified according to their chromosomal constitution following the criteria reported by Delhanty et al. (9) with slight modifications (Fig. 2).

Statistical Analysis

Comparisons between quantitative variables performed by Kruskal-Wallis test, and qualitative variables were analyzed by chi-square test. All tests were bilateral, and $\alpha = 0.05$ was considered statistically significant. Statistical Package for the Social Sciences 20.0 (SPSS) was used to perform the statistical analysis.

RESULTS

Pronuclear Morphometric Analysis and Embryo Characteristics

Morphometric measurements were obtained from 54 1PN ICSI zygotes. The mean pronucleus diameter was 29 μm (standard deviation [SD] $\pm$ 3.1; range: 17.9–34.6 μm). The mean area of the pronuclei was 668 μm^2 (SD $\pm$ 134.9; range: 250.5–938.6 μm^2). No correlation between the pronuclear size and the time of observation after ICSI was found (Rho Spearman coefficient: $r = 0.046$) (P=.743; mean: 18.4 hours; SD $\pm$ 1.2) (Supplemental Table 1, available online). In the control group, the mean pronuclear size was 24.92 μm (SD $\pm$ 1.96; range: 20.31–28.60 μm). The size of the pronucleus was statistically significantly larger in 1PN ICSI zygotes than in the 2PN ICSI zygotes ($P<.0001$).

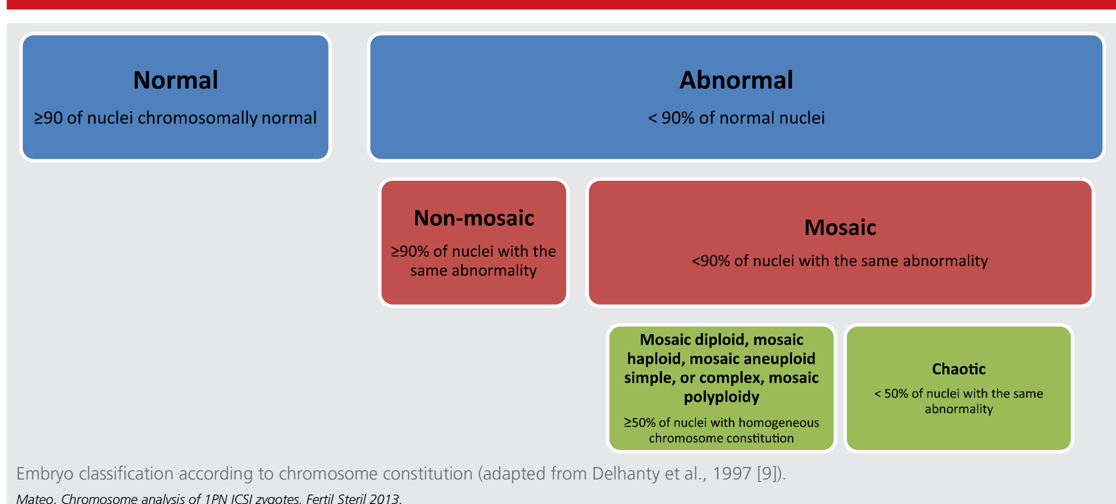
Embryo evaluation on day 3 showed that 26 (48.1%) of the 54 embryos presented with at least 6 cells and a maximum of 20% fragmentation. It was also observed that 8 (14.8%) of the 54 embryos had reached the blastocyst stage (Table 1), although only 4 (7.4%) of the 54 resulted blastocysts with good morphologic features, with well-formed inner-cell mass and trophectoderm.

Chromosomal Analysis

Cytogenetic results were obtained in the 54 embryos analyzed (36 cleavage stage embryos, 10 morulas, and 8 blastocyts). A high variability was observed with the number of cells suitable for diagnosis. The mean number of cells analyzed per embryo was 16.56 $\pm$ 25.4 (range: 2–133), with a total of 894 nuclei evaluated. In embryos at early cleavage stages, a mean of 68.47% cells were diagnosed (range: 42.8% to 100%). In morulas and blastocysts, the high number of cells and the presence of some overlapped nuclei after fixation made it difficult to determine the percentage of diagnosed cells per embryo.

All the embryos were chromosomally abnormal: 7.4% diploid mosaic, 16.7% haploid mosaic, 5.5% aneuploid mosaic, and 70.4% chaotic mosaic (Table 1). Of the embryos, 24 (44%) of 54 showed diploid cells in variable percentages (14.3% to 83%). The presence of a Y chromosome was observed in 19 (35.2%) of 54 embryos. In four embryos, all the cells were analyzed and turned out to be chaotic mosaics.

FIGURE 2



Embryo classification according to chromosome constitution (adapted from Delhanty et al., 1997 [9]).

Mateo. Chromosome analysis of 1PN ICSI zygotes. Fertil Steril 2013.

TABLE 1

Embryo development of monopronucleated (1PN) two polar body (2PB) ICSI zygotes according to its chromosomal constitution (embryos ≥2 diagnosed nuclei).

	Normal	Abnormal					
		Non-mosaic	Mosaic				
			Diploid	Haploid	Aneuploid	Chaotic	Total
n	0	0	4	9	3	38	54
Arrested at cleavage stage, n (%)	0	0	0	7 (77.8%)	2 (66.7%)	27 (71.1%)	36 (66.7%)
Arrested at morula, n (%)	0	0	1 (25%)	1 (11.1%)	1 (33.3%)	7 (18.4%)	10 (18.5%)
Blastocyst, n (%)	0	0	3 (75%)	1 (11.1%)	0	4 (10.5%)	8 (14.8%)

Note: P= .028.

Mateo. Chromosome analysis of 1PN ICSI zygotes. Fertil Steril 2013.

The relation between pronuclear size and chromosomal constitution was evaluated (Table 2). No differences were obtained when comparing pronuclei diameters and pronuclear area with the cytogenetic characteristic of the embryo (P=.306 and P=.299, respectively).

A correlation between in vitro development and the chromosomal constitution of the embryo was found (P=.028) (Table 1) showing that most of the diploid mosaic embryos (75%) reached the blastocyst stage. A single haploid mosaic embryo (11.1%) developed to a blastocyst of poor morphologic features. Seventy-one percent of the chaotic embryos arrested at early stages of development, and 10.5% resulted in blastocysts, even though only one had good morphology. This single good blastocyst showed three cell lines (haploid X, diploid XX, and diploid XY lines) (Fig. 1).

DISCUSSION

In this study, we analyzed the chromosome constitution, the pronuclear size, and capability to develop to the blastocyst stage of embryos derived from 1PN ICSI zygotes to evaluate their possible origin and to provide information about the decision to use them clinically for reproductive purposes. One of the most relevant observations was the evidence of a Y chromosome in 35.2% of the embryos analyzed, which permits the estimation that up to 70% of the 1PN ICSI zygotes had been fertilized. This finding is different from those reported by other studies (10%) (10). As a result, and contrary to those previous reports, most of 1PN zygotes we obtained after ICSI did not derive from a parthenogenetic activation of the oocyte but resulted from the formation of a single pronucleus combining maternal and paternal genomes, from an asynchronic 2PN formation, or from a pronuclear fusion.

Our data concerning pronuclear size (29 μm) are in good accordance with the results of Otsu et al. (6), who studied the chromosome constitution of 1PN conventional IVF-derived embryos. They reported that zygotes with a pronuclear diameter ≥29 μm resulted mostly in diploid embryos. The larger PN size observed in 1PN ICSI zygotes in comparison with 2PN ICSI zygotes does not support the hypothesis of asynchrony. Furthermore, the few cases reported that used early time-lapse monitoring (11) do not describe the occurrence of pronuclei fusion. Moreover, preliminary time-lapse data from 21 1PN ICSI zygotes obtained in our center have shown that a single pronucleus is always observed (unpublished observations) and there is no morphometric evidence to support the occurrence of PN fusion. According to all the information obtained, it can be postulated that the origin of 1PN ICSI zygotes is the formation of a single PN with maternal and paternal genomes combined with an irregular membrane formation surrounding both genomes.

Another strength of our results is that all the embryos analyzed were chromosomally abnormal and thus should not be considered for embryo transfer. The percentage of abnormal embryos found in our study was higher than results previously published elsewhere, where 10% to 28% of diploidy was reported in embryos derived from monopronuclear zygotes (3, 4). Concerning chromosome constitution, the incidence of haploidy was lower than described by others (3, 4, 12). Furthermore, most embryos in our data turned out

TABLE 2

Pronucleus morphometric characteristics of monopronucleated (1PN) two polar body (2PB) ICSI-zygotes according to its chromosomal constitution (embryos ≥2 diagnosed nuclei).

	Normal	Abnormal						
		Non-mosaic	Mosaic					
			Diploid	Haploid	Aneuploid	Chaotic	Total	*P* value
n (%)	0	0	4 (7.4%)	9 (16.7%)	3 (5.5%)	38 (70.4%)	54	
Diameter PN (μm)	–	–	30.5 (±2)	27.9 (±2.7)	29.9 (±0.9)	29 (±3.4)	29 (±3.1)	.306
Area PN (μm^2)	–	–	732.8 (±92.9)	616 (±112.5)	698.9 (±38)	671.2 (±146.5)	668 (±134.9)	.299

Mateo. Chromosome analysis of 1PN ICSI zygotes. Fertil Steril 2013.

to be chaotic (70.4%), which reinforces the abnormality of the 1PN ICSI zygotes when compared with 2PN embryos donated for research which comprised only 2% of chaotic embryos (13). It is possible that the presence of a single PN with maternal and paternal genome could lead to anomalies in the first mitotic division leading to an abnormal chromosomal segregation, which may contribute to rising incidence of chaotic embryos. Although we have observed some mosaic diploid embryos with a predominance of euploid cells, it must be pointed out that a normal chromosomal constitution and correct development are uncertain. Although a negative selection against aneuploid cells has been suggested by some investigators (13), and it has been postulated that a certain percentage of mosaicism might not be detrimental for embryo development (14), these rescue mechanisms have not been confirmed.

Differences reported in the literature on the rate of abnormal embryos derived from 1PN ICSI zygotes can be explained by different methodologic aspects: the number of analyzed cells, differences in the developmental stage of the embryos studied, number of chromosomes tested, and the criteria used to classify the embryos according to their chromosomal constitution. Concerning the number of cells analyzed per embryo, large variation is observed in the literature. Although some studies report data obtained from the analysis of only one cell per embryo (12), our study evaluated a high number and percentage of cells per embryo. Taking into account that 44.4% of the embryos had at least one diploid cell, these embryos could have been classified wrongly if only one cell had been studied.

The comprehensive embryo analysis performed in our study can explain the absence of euploid embryos when compared with others. Another explanation for the discrepancies between studies (3, 4) is that the analysis was not always performed on the same day of embryo development and the extended in vitro culture of 1PN zygotes could lead to higher percentages of chaotic embryos. Furthermore, we focused our cytogenetic study on 5 chromosomes, but only three chromosomes were tested by others (4). This may contribute to the detection of a higher number of chromosomal abnormalities, thus leading to an increase in the total number of abnormal embryos. It seems likely that if all the chromosomes were tested, more chromosomal abnormalities would have been detected and a lower proportion of diploid cells would be obtained, decreasing the total amount of diploid mosaics. Even then, our results concerning the normality of the embryos would not have changed: all were abnormal.

Other possible causes for the differences observed are the criteria and the number of cells used to classify embryos based on their chromosome constitution. In our study, we basically followed the classification of Delhanty et al. (9), slightly modified; we considered the FISH error rate as 10% (15), and the cutoff as 50% for mosaic embryos, but others have used less restrictive criteria (14).

Regarding in vitro embryo development, we observed that embryos derived from 1PN ICSI zygotes could be morphologically competent on day 3 (48.1%), and a low percentage can reach the blastocyst stage (14.8%). This is in good agreement with previous work (12), although other investigators have reported that none of the cultured embryos reached blastocyst stage (6).

A direct correlation between development and chromosomal constitution was found, just as other investigators have described (16). In our series, the higher proportion of 1PN ICSI zygotes diagnosed as diploid mosaics were those able to reach the blastocyst stage with good morphology. If their pronuclear status had not been taken into account, some of these blastocysts might have been used either for transfer or for cryopreservation in a reproduction treatment. Although centers used to discard embryos derived from 1PN ICSI zygotes, even the most recent guidelines do not highlight that they should be considered nonviable embryos (17).

It can be concluded from our study that most of the embryos derived from 1PN ICSI zygotes come from fertilized oocytes and are chromosomally abnormal. The observation of a single pronucleus may reflect an error in PN formation during the fertilization process. This could lead to abnormal mitosis and give rise to chromosomal abnormalities. Neither the pronuclear size nor the ability to reach the blastocyst stage is a predictor of the chromosomal constitution in these embryos. The results of our work clearly recommend discarding embryos derived from 1PN zygotes after ICSI, rather than using them for transfer or cryopreservation, even though some of them may develop properly in vitro up to the blastocyst stage.

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SUPPLEMENTAL TABLE 1

Pronuclei diameters (μm) respect the time of observation.

Time of observation (h after ICSI)	N	Mean	SD	Minimum	Maximum
16	4	27.5725	1.01415	26.52	28.56
17	7	30.7700	2.15639	27.92	34.61
18	17	29.1300	3.05058	21.57	34.05
19	16	28.0787	3.95368	17.87	32.67
20	10	29.6467	2.81141	23.63	32.79
Total	54	29.0100	3.14918	17.87	34.61

Note: ICSI = intracytoplasmic sperm injection; SD = standard deviation.

Mateo. Chromosome analysis of 1PN ICSI zygotes. Fertil Steril 2013.

4.2. Article 2

Títol: *Could monopronucleated ICSI zygotes be considered for transfer? Analysis through time-lapse monitoring and PGS.*

Autors: Sílvia Mateo, Francesca Vidal, Mònica Parriego, Ignacio Rodríguez, Víctor Montalvo, Anna Veiga i Montserrat Boada.

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ASSISTED REPRODUCTION TECHNOLOGIES

Could monopronucleated ICSI zygotes be considered for transfer? Analysis through time-lapse monitoring and PGS

S. Mateo[1] · F. Vidal[2] · M. Parriego[1] · I. Rodríguez[3] · V. Montalvo[1] · A. Veiga[1,4] · M. Boada[1]

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Abstract
Purpose The purpose of this study was to investigate the chromosomal constitution and the developmental potential of intracytoplasmic sperm injection (ICSI) deriving embryos displaying a single pronucleus at the zygote stage.
Methods Eighty-eight embryos from single pronucleus (1PN) two polar bodies (2PB) ICSI zygotes from 64 preimplantational genetic screening (PGS) cycles (October 2012–December 2014), were retrospectively analyzed. Zygotes were cultured in a time-lapse incubator. Embryo biopsy was performed on day 3 and genetic analysis approached by array comparative genomic hybridization.
Results Chromosomal analysis revealed that 17% (15/88) of embryos derived from 1PN 2PB zygotes were diagnosed as euploid. After blastomere biopsy at day 3, the blastocyst rate at day 5 was 3.4% (3/88). Only 2.3% (2/88) euploid blastocysts were obtained. In two couples and after counseling and patient agreement, the transfer of a euploid blastocyst from a 1PN 2PB ICSI zygote was performed resulting in the birth of a healthy child.
Conclusions These results open the possibility to consider embryos coming from 1PN 2PB ICSI zygotes for transfer when no other embryos from 2PN 2PB ICSI zygotes are available and if a PGS diagnosis of euploidy is obtained. Confirmation of biparental inheritance is strongly recommended.

Keywords aCGH · Monopronucleated zygotes · 1PN · PGS · Time-lapse

Introduction

The most important goal of assisted reproduction techniques (ART) is the delivery of a healthy child. In the last years, two technologies, preimplantational genetic screening (PGS) with comprehensive chromosome screening (CCS) and time-lapse embryo monitoring, have been used to investigate parameters that could contribute to assess the implantation potential of embryos.

The application of PGS in embryos from ART cycles aims to improve the implantation rates (raising the birth rate and decreasing the miscarriage rate), avoiding the replacement of aneuploid embryos [1–3]. Furthermore, it has been reported that poor-prognosis patients (advanced maternal age, implantation failure, male factor, recurrent miscarriage) produce embryos with high aneuploidy rates and may especially benefit from this technology [3–5]. Moreover, the optimization of incubators, combining stable culture conditions and high-resolution time-lapse image capturing, allows a continuous monitorized analysis of embryo development until the blastocyst stage. As it has been reported [6–9], the information obtained by these systems may help to select embryos with the highest implantation potential. PGS and time-lapse monitoring used in combination

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✉ S. Mateo
silmat@dexeus.com

[1] Reproductive Medicine Service, Department of Obstetrics, Gynecology and Reproduction, Women's Health Dexeus, Gran Via Carles III, 71-75, 08028 Barcelona, Spain

[2] Cell Biology Unit, Faculty of Biosciences, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

[3] Unit of Biostatistics, Department of Obstetrics, Gynecology and Reproduction, Women's Health Dexeus, Gran Via Carles III, 71-75, 08028 Barcelona, Spain

[4] Stem Cell Bank, Centre for Regenerative Medicine, Dr. Aiguader, 88, 08003 Barcelona, Spain

can generate valuable information on the decision of transferring or discarding specific embryos, as both the chromosomal constitution and the dynamics of embryo development can be taken into account [10].

In certain patients, the only embryos available arise from abnormal fertilization, abnormal cleavage and/or blastomere multinucleation. In these situations, there is a general consensus about discarding zygotes showing three pronuclei, and this decision is also adopted for most single pronucleus (1PN)-derived embryos. It has been reported that monopronucleated zygotes obtained from conventional IVF (cIVF) are mainly diploid [11, 12] and can be used for reproductive purposes. Embryos arising from 1PN intracytoplasmic sperm injection (ICSI) zygotes are not usually considered for transfer as there are concerns about their chromosomal constitution and are usually discarded. However, because in certain cases there are no other embryos available, new information would be valuable to help in the decision about transferring or discarding them.

Several articles investigating the possible origin of 1PN zygotes and reporting data about the chromosome constitution of the resulting embryos have been published [13–15]. These reports have shown that certain embryos originating from 1PN two polar bodies (2PB) zygotes could have a normal chromosomal content.

In this work, we have retrospectively analyzed by array comparative genomic hybridization (aCGH) the chromosomal constitution of embryos arising from 1PN 2PB ICSI zygotes obtained from patients submitted to PGS cycles. Although we are aware of the limitations of day 3 biopsy and the use of aCGH for embryo ploidy detection, we have considered that the information gained through the results could be of benefit for the discussion about the possible use of these embryos for reproductive purposes.

Material and methods

PGS cycles performed from October 2012 to December 2014 were retrospectively analyzed. During this period, 385 cycles were accomplished, and 64 cycles meet the criteria of the study: containing at least one embryo where a 1PN and 2PB at the zygote stage were observed, embryos being cultured in time-lapse incubator after ICSI, embryo biopsy done on day 3, and genetic analysis performed by aCGH.

From the 64 PGS cycles analyzed, the mean maternal age was 38.6 years (±3.9) and the PGS indications were as follows: recurrent miscarriage (20.3%), advanced maternal age (25%), male factor (23.4%), implantation failure (26.6%), and previous affected pregnancy (4.7%).

A total of 115 embryos from 1PN 2PB zygotes were recorded, and 88 of them fulfill the criteria for embryo biopsy on day 3 and constituted the study population.

Intracytoplasmic sperm injection was performed 40 h after HCG administration and zygotes were cultured in LifeGlobal total® media (LifeGlobal®) in an EmbryoScope® (Vitrolife) capturing images every 15 min in five focal planes. Continued sequential observation performed by dynamic monitoring allowed excluding sporadic cases of asynchronous 2PN formation or 2PN fusion. Monopronucleated zygotes without extrusion of the 2PB were also excluded from the study. Zygotes were cultured until day 3 when biopsy was performed. Embryos showing five or more blastomeres, and a maximum of 25% of cytoplasmic fragmentation, were biopsied. A single cell per embryo was biopsied. Blastomeres were analyzed by aCGH using 24 sure kit and fluorescent labeling system (Illumina®) according to manufacturer's protocols. After biopsy, embryos were placed in fresh LifeGlobal total® media (LifeGlobal®) and cultured until day 5 when transfer was performed.

During the period analyzed, the PGS policy of our center was to perform embryo biopsy on day 3 and to select for transfer the embryos reported to be euploid that reached the blastocyst stage on day 5.

The chromosomal status of embryos arising from 1PN 2PB ICSI zygotes was analyzed by aCGH and embryos were classified as euploid, aneuploid, or with non-conclusive results. In the group of aneuploid embryos, two subgroups were established according to the classification described by Johnson et al. [16]: simple aneuploidy (1–2 chromosome abnormalities) or complex aneuploidy (≥3 chromosome abnormalities). Embryos without diagnosis were classified into two subgroups depending on whether there has been DNA amplification but non-conclusive results were obtained or no DNA amplification was observed.

According to morphological analysis and developmental outcome on day 5, embryos were classified as blastocysts (early blastocysts, expanded and hatching blastocysts), morulae, and arrested or degenerated embryos.

Euploid embryos from 2PN 2PB zygotes were always first selected for transfer. Exceptionally, in two cycles from two different patients where no euploid embryos from 2PN zygotes were available, patients were informed about the possibility to transfer one embryo from 1PN 2PB ICSI zygote diagnosed as euploid. This option was extensively discussed with the couples, including the limitations of the diagnosis and the possible risks. Thus, after patient agreement, two single embryo transfers of euploid blastocysts derived from 1PN 2PB ICSI zygotes were performed.

Euploidy and blastocyst rate were calculated and the association between the chromosomal constitution and the developmental stage was analyzed using chi-square test. All tests were bilateral with a significance set to 0.05. The clinical outcome of the cases where embryos coming from 1PN 2PB ICSI zygotes were replaced were analyzed.

The project was approved by the institutional review board of the center.

Results

The mean number of inseminated oocytes in the 64 cycles studied was 14.14 (±4.16), with a mean number of 10 (±3.41) oocytes fertilized, and a mean number of biopsied embryos of 9.75 (±3.02).

The median time of second polar body appearance was 3.53 h post insemination (hpi) (range 1.43–5.13) and the median time of pronucleus appearance was 8.2 hpi (range 5.95–12.63).

The chromosomal analysis of 88 embryos coming from 1PN 2PB ICSI zygotes showed that 17% (15/88) were euploid and 60.2% (53/88) aneuploid. No diagnosis was obtained in the remaining 22.7% embryos (20/88) due to an absence of DNA amplification (13.6%; 12/88) or to non-conclusive results (9.1%; 8/88). According to the aCGH results 28.4% (25/88) showed a simple aneuploidy and 31.8% (28/88) a complex aneuploidy. The presence of a Y chromosome was evidenced in 10.3% of the diagnosed embryos (7/68).

Embryo development analysis showed that 3.4% (3/88) of the biopsied embryos from 1PN 2PB ICSI zygotes reached the blastocyst stage, 36.4% (32/88) arrested at the morula stage and 60.2% arrested their development at earlier stages (53/88).

The relationship between the developmental stage, after blastomere biopsy, and the chromosomal constitution is detailed in Table 1. Of the 15 embryos diagnosed as euploid, only 2 (13.3%) formed a blastocyst whereas none of the aneuploid embryos reached this stage. The majority of aneuploid embryos (69.8%; 37/53) arrested their development at early stages. Among embryos without diagnosis, one reached the blastocyst stage (5%; 1/20) and the remaining ones arrested at the morula stage (35%; 7/20) or at earlier stages (60%; 12/20). In total, only 2.3% (2/88) euploid blastocysts were obtained.

In two patients, all the embryos coming from 2PN zygotes were diagnosed as aneuploid. In one patient, 12 embryos were biopsied with 11 being aneuploid and the only one diagnosed as euploid came from 1PN 2PB ICSI zygote. The other patient had 9 embryos biopsied, with 8 being aneuploid and the remaining embryo, from 1PN 2PB ICSI zygote, diagnosed as euploid. In these two patients, and because no euploid embryos from 2PN 2PB zygotes were available, single embryo transfer of a blastocyst from a 1PN 2PB ICSI zygote diagnosed as euploid was performed on day 5 (Figs. 1 and 2) (Video 1, Online Resource). One pregnancy was achieved resulting in the birth of a healthy child. No prenatal diagnosis was performed. The patient delivered at 40 weeks of gestation a healthy female of 3136 g and 47 cm. Child development has been followed up since birth and, at the moment of preparing this manuscript, the baby was 3 years old and no alterations were observed in her development according to ordinary pediatric check-up and parent's communication.

Discussion

The use of dynamic monitoring (time-lapse) during this study allowed a detailed observation of second polar body extrusion and pronuclear formation. Unlike previous studies, where traditional culture systems and observations at specific check points were used [13, 17, 18], our approach unequivocally guarantees that all the zygotes included in the study displayed only a single pronucleus and had extruded the second polar body (Fig. 1). Asynchronous pronuclear formation or pronuclear fusion, as causes for the appearance of a single PN, can be ruled out as PN formation was accurately ascertained through time-lapse imaging and were clearly established as exclusion criteria. No debris or fragments in the perivitelin space were present that could make the observation of PN formation difficult. The presence of 2PB was also clearly established to avoid the inclusion of diploid embryos resulting from the non-extrusion of the second polar body. Timings for second PB extrusion and PN appearance are similar to those observed in 2PN zygotes [19]. According to the images obtained, our hypothesis is that the origin of the monopronucleated zygotes could be due to either a parthenogenetic oocyte activation or to an abnormal formation of the nuclear envelope. This last could result from the assembly of the two genetic materials in a single PN or from the failure to organize a nuclear envelope around one of the parental genomes [20].

Table 1 Relationship between developmental stage in day 5, after biopsy on day 3, and chromosomal constitution of embryos derived from 1PN 2PB ICSI zygotes

Chromosomal constitution		Developmental stage D5			
		Blastocyst	Morulae	Arrested	Total
Euploid		2 (13.3%)	9 (60.0%)	4 (26.7%)	15
Aneuploid		0 (0.0%)	16 (30.2%)	37 (69.8%)	53
Non-diagnosed	Without conclusive diagnosis	1 (12.5%)	5 (62.5%)	2 (25%)	8
	No amplification	0	2 (16.7%)	10 (83.3%)	12
	TOTAL				88

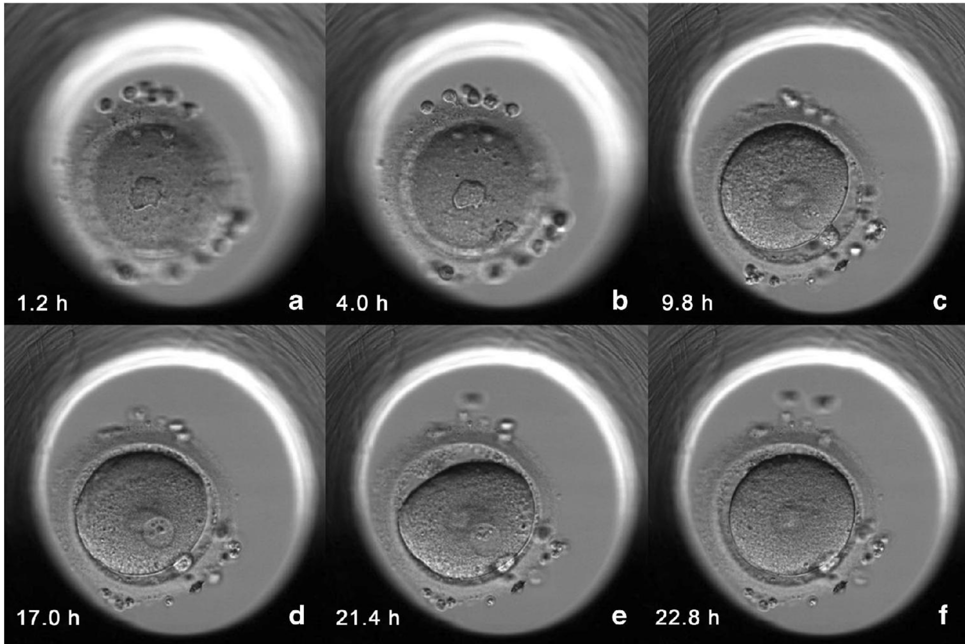

Fig. 1 Time-lapse imaging of second polar body (PB) extrusion and pronuclear (PN) formation and fading of the 1PN 2PB ICSI zygote that produced an ongoing pregnancy and the delivery of a healthy child. The time of image captures (hours post ICSI) is shown at the *bottom left* of each image. **a**. First PB. **b** Extrusion of the second PB. **c**–**e** Formation of 1PN. **f** Fading of PN

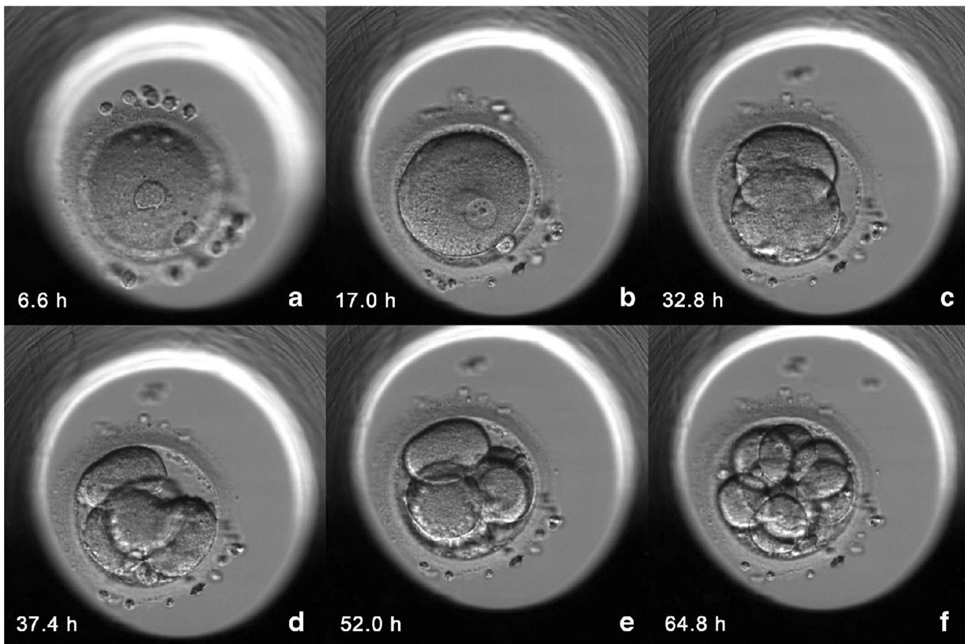

Fig. 2 Time-lapse imaging of in vitro development until day 3 of 1PN 2PB ICSI zygote that produced an ongoing pregnancy and the delivery of a healthy child. The time of image captures (hours post ICSI) is showed at the *bottom left* of each image. **a** First and second PB. **b** 1PN 2PB. **c**–**f** 2-, 4-, 5-, and 8-cell embryo

The results obtained after blastomere biopsy and aCGH analysis of embryos from 1PN ICSI zygotes reported 22.7% of undiagnosed embryos. This figure is higher than that in the 2PN embryos from our PGS program, which is around 10% (PGS program unpublished data), and is mainly due to the high number of DNA amplification failures in 1PN embryos (13.6%) in comparison to 2PN embryos (5.4%, PGS program unpublished data). We hypothesize that the high percentage of amplification failures observed can be due to DNA fragmentation or degeneration that occurs in embryos from 1PN ICSI zygotes prior to embryo development arrest. This explanation is in harmony with the observation of a high rate of embryo developmental arrest after day 3 in 1PN embryos (60.2%). Furthermore, it can also be presumed that the low ability of biopsied embryos from 1PN ICSI zygotes to reach the blastocyst stage can be attributed to the fact that some of them are probably haploid and, as has been previously described, haploid embryos derived from 1PN zygotes can be negatively selected during in vitro culture [21, 22]. Even so, it cannot be ruled out that biopsy on day 3 may negatively affect the development capability.

The euploidy rate observed in embryos from 1PN 2PB ICSI zygotes (17%) is lower than that reported in early publications (27.9 and 44.4% of diploid embryos) [17, 23]. The discrepancies could be due to differences between the method of pronuclear assessment (time-lapse vs punctual observations), different diagnostic techniques used (aCGH vs fluorescent in situ hybridization (FISH)) and patient population (PGS patients vs IVF patients).

In a previous report, using five-chromosome FISH analysis, no euploid embryos were found among embryos from 1PN 2PB ICSI zygotes [13]. The differences in the euploidy rate observed between the two studies could be attributed to the experimental designs. In Mateo et al. [13], all embryos, irrespective of their developmental stage (arrested or blastocyst) and morphology, were included, and all the cells were analyzed. In the present study, only embryos that fulfill the criteria for embryo biopsy on day 3 (≥5 cells and ≤25% of fragmentation) were analyzed and arrested or fragmented embryos were not considered. As it has been described [24, 25], a high percentage of cytoplasmic fragmentation or developmental arrest is associated with high levels of chromosome abnormalities. Furthermore, the higher euploidy rate observed in the present study compared with the previous one [13], can be due to the underestimation of mosaicism, a common phenomenon in preimplantation embryos [23, 26, 27].

As previously mentioned, it is important to point out that aCGH has inherent limitations for the detection of ploidy alterations [28] and this represents an important limitation of the results obtained. Thus, the euploidy rate reported in our study has to be taken with caution and further studies of 1PN ICSI deriving embryos should be encouraged.

Considering embryo development, biopsied embryos from 1PN 2PB ICSI zygotes showed a blastocyst rate at day 5 of 3.4% which is similar to rates previously reported [13, 29, 30]. However, this percentage is lower than in 2PN embryos also biopsied on day 3 (24.3%, PGS program unpublished data).The low blastocyst rate obtained could probably be due to the haploid status or other chromosomal or genetic abnormalities that hinder the development to the blastocyst stage in 1PN ICSI zygotes. This would be in accordance with the estimation of diploidy rate inferred from the 10.3% of male embryos observed. The expected balanced XY versus XX embryo rate makes it possible to infer that only around 20.6% of the embryos were diploid.

The clinical use of 1PN 2PB zygotes for transfer or cryopreservation is rare as it has been described that most of them are chromosomally abnormal [13, 15]. As a consequence, data about the implantation potential of embryos from 1PN 2PB zygotes are very scarce. Very few pregnancies and births have been reported after the transfer of embryos from 1PN 2PN zygotes obtained after cIVF [11, 29–31]. After ICSI, there is only one publication reporting a livebirth [18]. In all these publications, although accurate observations after insemination were made, time-lapse was not used. Asynchronous pronuclear formation, pronuclear fusion, or even an abnormal timing of PN appearance/fading, which cannot be detected at the usual check points, could not be completely excluded.

In this work, we report two single embryo transfers of euploid blastocysts from 1PN 2PB ICSI zygotes, assessed with time-lapse technology thus ensuring the status of the single pronucleated zygote, resulting in a livebirth of a healthy female in one of them.

Our results indicate that euploid embryos arising from 1PN 2PB ICSI zygotes could be considered for transfer when there are no euploid embryos from 2PN 2PB ICSI zygotes available. Nevertheless, we strongly recommend DNA fingerprinting analysis before the reproductive use of these embryos. Patients have to be widely informed and counseled and sign a specific informed consent form.

It has to be taken into account that the incidence of 1PN 2PB embryos is low (3.1% unpublished data), and as a consequence, a limited number of embryos from 1PN 2PB ICSI zygotes will be potentially usable. Furthermore, extending embryo culture until blastocyst stage would reduce the number of embryos to be analyzed. Moreover, the analysis of trophectoderm cells would allow better diagnosis approaches and avoid unnecessary day 3 biopsies of embryos that could have initiated a degenerative process [28, 32].

In summary, when no euploid 2PN 2PB embryos are available and after appropriate patient counseling, we suggest that embryos coming from 1PN 2PB ICSI zygotes diagnosed as euploid and after DNA fingerprinting analyses, could be considered for reproductive purposes. Furthermore, in order to

optimize the cost-benefit of our proposal, we suggest approaching PGS by trophectoderm biopsy.

Finally, we would like to emphasize that the aim of the present paper was not to encourage the transfer of 1PN 2PB ICSI-derived embryos as a general practice but to gain knowledge about their chromosomal constitution and give elements to promote the debate about the possible reproductive use of these embryos when no other ones are available.

chi-square test showed association between both variables ($p = 0.012$).

Compliance with ethical standards The project was approved by the institutional review board of the center.

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

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4.3. Article 3

Títol: *Chromosomal analysis of blastocysts derived from monopronucleated ICSI zygotes: approach by double trophectoderm biopsy.*

Autors: Sílvia Mateo, Francesca Vidal, Lluc Coll, Anna Veiga i Montserrat Boada.

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Original Article

Chromosomal analysis of blastocyst derived from monopronucleated ICSI zygotes: approach by double trophectoderm biopsy

Silvia Mateo[1], Francesca Vidal[2], Lluc Coll[1], Anna Veiga[1,3], Montserrat Boada[1]

[1] Reproductive Medicine Service, Department of Obstetrics, Gynaecology and Reproduction, Women's Health Dexeus, Barcelona, Spain.
[2] Cell Biology Unit, Faculty of Biosciences, Universitat Autònoma de Barcelona, Barcelona, Spain.
[3] Stem Cell Bank, Centre for Regenerative Medicine, Barcelona, Spain.

ABSTRACT

Objective: This study aims to increase the knowledge about monopronucleated ICSI-derived blastocysts, analyzing trophectoderm biopsies by aCGH and FISH to evaluate their chromosome constitution.

Methods: Fifteen monopronucleated ICSI-derived blastocysts were studied. Double trophectoderm biopsy was performed and analyzed by FISH and aCGH. The blastocysts were classified according to chromosome constitution. Disagreements between the two techniques were assessed.

Results: Results obtained after FISH and aCGH analyses showed the following: 20% (3/15) and 60% (9/15) diploid females, respectively; 26.7% (4/15) and 26.7% (4/15) diploid males, respectively; and 53.3% (8/15) and 13.3% (2/15) mosaics, respectively. No mosaic male embryos were found using FISH or aCGH. There were disagreements in 40% (6/15) of the cases due to the higher detection of mosaicism by FISH compared to aCGH.

Conclusions: The combination of FISH and aCGH has been shown to be a suitable approach to increase the knowledge about monopronucleated ICSI-derived embryos. FISH analysis of blastocysts derived from monopronucleated ICSI zygotes enabled us to conclude that aCGH underestimates haploidy. Some diploid embryos diagnosed by aCGH are in fact mosaic. In cases where these embryos would be used for reproductive purposes, extra analysis of parental genome origin is recommended.

Keywords: monopronucleated zygote, trophectoderm biopsy, aCGH, FISH

INTRODUCTION

The finding of a unique pronucleus is evidence that some error has occurred in the fertilization process. The questions are: why does this happen, how do the resulting embryos appear, and can they be considered for reproductive purposes? Many authors contributed to this research to expand the knowledge about monopronucleated zygotes (1PN) (Munné *et al.*, 1993; Staessen *et al.*, 1993; Levron *et al.*, 1995; Sultan *et al.*, 1995; Staessen & Van Steirteghem, 1997; Otsu *et al.*, 2004; Van Der Heijden *et al.*, 2009; Mateo *et al.*, 2013; 2017; Azevedo *et al.*, 2014; Rosenbusch, 2014).

The genetic composition of monopronucleated ICSI zygotes may have different parental origin, and the mechanisms leading their formation can be diverse:

i) Gynogenetic embryos derived from 1PN ICSI zygotes could be the result of a parthenogenetic activation, without the participation of the paternal genome. This could be due to the extrusion of spermatozoa to the perivitelline space, the absence of decondensation of the paternal nucleus, or premature paternal chromosome condensation (Flaherty *et al.*, 1998). If only one polar body (PB) is present, the embryo would be diploid. When two PB are found, the embryo will be haploid, or diploid if endoreduplication has occurred.

ii) Monopronucleated zygotes can also originate androgenetic embryos when there is correct formation of the male pronucleus, avoiding the formation of the female pronucleus. This could be due to the complete extrusion of maternal genome in the second polar body, or due to the maintenance of the meiotic spindle of the oocyte (Azevedo *et al.*, 2014; Kai *et al.*, 2015).

iii) Monopronucleated zygotes with biparental origin could arise from the formation of a unique pronucleus, including maternal and paternal genomes (Levron *et al.*, 1995; Van Der Heijden *et al.*, 2009; Kai *et al.*, 2015). In this case, the union of the maternal and paternal genetic materials could be produced prior to the membrane formation due to the tight proximity of the spermatozoa and the oocyte spindle, or due to the formation of two pronuclei and a subsequent fusion in one pronucleus (Levron *et al.*, 1995; Flaherty *et al.*, 1998; Meseguer, 2016). The finding of an asynchronous pronuclei has also been reported before, and could be another reason for finding a unique pronucleus (Staessen *et al.*, 1993) when time-lapse methodology is not used.

Concerning the possible reproductive use of these embryos, there are different considerations depending on whether they arise from conventional IVF (cIVF) or intracytoplasmic sperm injection (ICSI). While the former is often accepted for clinical use, embryos from 1PN ICSI zygotes are usually discarded due to the reported high incidence of chromosomal abnormalities.

A recently published paper, reporting that some of these zygotes can reach the blastocyst stage, being euploid and resulting in the birth of a healthy child, suggests that they could be used for reproductive purposes in certain cases (Mateo *et al.*, 2017).

A diagnosis of euploidy must be mandatory to consider any 1PN ICSI-derived embryo for transfer. Currently, the Pre-implantation Genetic Screening (PGS) approach used in most centers is addressed to a Comprehensive Chromosomal Screening (CCS) by array Comparative Genome Hybridization (aCGH) in biopsied trophectoderm cells. However, the main limitation of aCGH is its suitability to ascertain the ploidy status of the studied embryos (Gutiérrez-Mateo *et al.*, 2011; Scriven, 2013), and this is an important limitation when there is risk of having haploid embryos in the cohort studied. Fluorescent *in situ* hybridization (FISH), although being set aside by most groups since the implementation of aCGH, could easily provide information about embryo ploidy.

The objective of this study is to analyze trophectoderm biopsies by aCGH and FISH, to assess to which extend the use of aCGH may lead to an underestimation of haploidy, and to gain knowledge about the chromosome content of embryos coming from 1PN ICSI zygotes.

MATERIAL AND METHODS

Fifteen blastocysts from monopronucleated (1PN) zygotes with two polar bodies (2PB) obtained after

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ICSI were analyzed. Patients agreed to donate vitrified blastocysts from 1PN 2PB ICSI zygotes, after being informed that these embryos were considered not suitable for reproduction, because of their reported high incidence of chromosomal abnormalities. Patients signed the corresponding written informed consent. In all cases, embryos from 2PN 2PB zygotes were available for transfer. The study was approved by the institutional review board of the center.

ICSI was performed at 40h post HCG administration. After ICSI, zygotes were cultured in LifeGlobal total® media (LifeGlobal®) and were placed in a time-lapse incubator (EmbryoScope®-Vitrolife). Images of 5 focal planes were acquired at every 15 minutes. Dynamic monitoring allowed the presence of only a single PN to be confirmed, and either asynchronous 2PN formation or 2PN fusion to be excluded.

Monopronucleated ICSI zygotes were maintained in culture until blastocyst formation. Blastocysts were vitrified and, after being rejected for reproductive purposes, analyzed for research.

Vitrification and warming

Kitazato media were used for vitrification/warming according to the manufacturer's protocol and cryotop® (Kitazato®) was used as support. After warming, all blastocysts were cultured in LifeGlobal total® media for 30 minutes.

Blastocyst biopsy

For zona drilling, the embryos were moved to LifeGlobal Total® w/HEPES. A laser (NaviLase, OCTAX Microscience GmbH) attached to a microscope (U-LH100L-3, Olympus®) was used. Three laser pulses of 1.3ms were applied in the zona pellucida of the blastocyst, opposite to the inner cell mass. After zona drilling, the blastocysts were placed in LifeGlobal total® media (LifeGlobal®) for at least 8 hours, to facilitate the trophectoderm (TE) herniation for later biopsy. Biopsy of two different TE fragments of each blastocyst were collected by aspiration (between 4 and 16 cells each, depending on the characteristics of the TE).

Chromosomal analysis

FISH was performed in one of the TE fragments and aCGH analysis in the other.

FISH analysis

TE cells were washed in phosphate-buffered saline solution (PBS) (6% human serum albumin) and were fixed on a slide by adding Carnoy's solution (methanol-acetic acid; 3:1). The fixation procedure was performed under an inverted microscope and was adapted to the fragment's characteristics for accurate cell spreading. Biopsies were individually fixed on different slides to avoid cell contamination from other blastocysts. After fixation, the slides were left to dry and they were stored at -4°C until they were processed for FISH analysis.

Since the objective of the FISH analysis was ploidy assessment, the slides were processed for FISH using a commercial probe panel, specific for chromosomes X, Y and 18 (AneuVysion Multicolor DNA Probe Kit, Vysis CEP 18/X/Y, Abbott Molecular) according to the manufacturer's protocols. Interphase nuclei were evaluated using an Olympus BX-61 fluorescent microscope (Olympus®) equipped with specific filters for FITC, Cy3, and Aqua and a multiband pass filter (DAPI/FITC/Texas Red). Overlapped nuclei, metaphase figures and chromatin fragments were discarded from the analysis. Embryos were further classified according to their chromosomal constitution. According to FISH results, the embryos were classified into haploid (H), diploid (D) or mosaic (M), and further gender identified as f (female) or m (male) depending on the results of the cells analyzed. According to this, Df and Dm were those embryos with all the cells analyzed containing two chromosomes 18 and XX or XY, respectively. Mf and Mm corresponded to those mosaic embryos with more than one cell line but at least one cell with two copies of chromosome 18 and XX or XY respectively. H were those embryos with all the cells analyzed with one chromosome 18 and one sex chromosome.

aCGH analysis

TE cells were washed separately in four drops of 10µl of PBS/PVA solution and were transferred to a PCR tube with a drop of 0.1µl of PBS solution. The PCR tubes with the samples were stored at -80°C until they were processed. The trophectoderm cells were processed for DNA amplification and aCGH analysis using 24 sure kit and Fluorescent labelling system (Illumina®), according to the manufacturer's protocols. Hybridization results were processed and analyzed with the BlueFuse software (Illumina®). The embryos were further classified according to their chromosomal constitution. According to aCGH results, the embryos were classified into diploid (D) or mosaic (M), and further gender identified as f (female) or m (male). According to this classification, Df and Dm were those embryos showing diploid female or diploid male homogeneous chromosomal complements, respectively. Mf and Mm corresponded to mosaic embryos with diploid female or male cell lines, respectively.

RESULTS

Fifteen 1PN 2PB-derived blastocysts were successfully thawed (100% survival rate) and all had re-expanded their blastocoel cavity after 8h of culture.

Results from FISH and aCGH analysis and embryo classification are detailed in Tables 1 and 2 respectively.

Concerning FISH results, haploid, diploid female, diploid male and tetraploid cells were found. Diploid cells were found in all the embryos studied. Haploid cell lines were observed in 46.7% of the embryos (7/15) and tetraploid cells were found in 20% of the embryos (3/15). No aneuploidy for any of the three chromosomes analyzed was observed (Table 1).

According to FISH results, 20% (3/15) of the embryos were classified as diploid female, 26.7% (4/15) as diploid male and 46.7% (7/15) as mosaic female. One embryo (E12) was classified as mosaic (6.7%; 1/15) with coexisting diploid male, diploid female and haploid cell lines. No haploid embryos and no mosaic diploid male embryos were found (Table 1).

Concerning aCGH, the embryos were classified as diploid female in 60% (9/15), 26.7% (4/15) as diploid male, one mosaic female (6.7%) and one mosaic for the sex chromosomes (6.7%). Aneuploidy was found in 26.7% of the embryos (4/15). In two diploid male embryos (E6 and E8), the results were consistent with homogeneous aneuploidy for all the cells analyzed. The other two aneuploidy embryos (E4 and E12) were mosaic (Table 2).

Total agreement between FISH and aCGH results was found in 60% (9/15) of the embryos, corresponding to embryos diagnosed by FISH as diploid (E1, E6, E8, E10, E11, E14, E15) and mosaic embryos E4 and E12, the latter with mosaicism for the sex chromosomes. Similar percentages between male and female embryos were observed in embryos diagnosed as diploid, representing 46.7% of the total embryos (Table 3).

There was no agreement in 40% of the embryos (6/15). Seven mosaic female embryos were detected by FISH and, six of them, were classified as diploid females by aCGH.

Table 1. Results from FISH analysis, embryo classification and percentage of diploid and haploid cells in 1PN 2PB-derived blastocyst. Df: diploid female; Dm: diploid male; Mf: mosaic female; Mm: mosaic male; M: mosaic for the sex chromosomes

EMBRYO CODE	FISH RESULTS [number of cells]	% DIPLOID CELLS	% HAPLOID CELLS	FISH CLASSIFCATION
E1	XX1818 [12]	100% (12/12)	0%	Df
E2	XX1818[6]; X18 [1]	85.7% (6/7)	14.3% (1/7)	Mf
E3	XX1818 [3]; X18 [2]	60% (3/5)	40% (2/5)	Mf
E4	XX1818 [7]; XXXX18181818[1]	87.5% (7/8)	0%	Mf
E5	X18 [4]; XXXX18181818[4]; XX1818[2]	20% (2/10)	40% (4/10)	Mf
E6	XY1818 [5]	100% (5/5)	0%	Dm
E7	X18 [5]; XX1818 [1]; XXXX18181818 [1]	14.3% (1/7)	71.4% (5/7)	Mf
E8	XY1818 [6]	100% (6/6)	0%	Dm
E9	X18 [15]; XX1818 [1]	6.3% (1/16)	93.8% (15/16)	Mf
E10	XY1818 [4]	100% (4/4)	0%	Dm
E11	XY1818 [4]	100% (4/4)	0%	Dm
E12	X18 [7]; XY1818 [5]; XX1818 [2]	50% (7/14)	50% (7/14)	M
E13	XX1818 [4]; X18 [1]	80% (4/5)	20% (1/5)	Mf
E14	XX1818 [5]	100% (5/5)	0%	Df
E15	XX1818 [13]	100% (13/13)	0%	Df

Table 2. Results from aCGH analysis and embryo classification of 1PN 2PB-derived blastocysts. Df: diploid female; Dm: diploid male; Mf: mosaic female; Mm: mosaic male; M: mosaic for the sex chromosomes. (*) Non-mosaic aneuploid embryo

EMBRYO CODE	aCGH RESULTS	aCGH CLASSIFICATION
E1	46, XX	Df
E2	46, XX	Df
E3	46, XX	Df
E4	46, XX/45, XX (-8)	Mf
E5	46, XX	Df
E6	47, XY (+22)	Dm*
E7	46, XX	Df
E8	46, XY (-16, +21)	Dm*
E9	46, XX	Df
E10	46, XY	Dm
E11	46, XY	Dm
E12	46, XY/46, XXY (-6)	M
E13	46, XX	Df
E14	46, XX	Df
E15	46, XX	Df

Classification disagreements between the two techniques were due to a higher detection of mosaicism by FISH when compared to aCGH (53.3%; 8/15 vs. 13.3%; 2/15). The remaining embryo was classified as mosaic female, an agreement between both techniques (Table 3).

In all the embryos analyzed, gender assignment was concordant between the two techniques. Special attention should be paid to embryo E12, which showed mosaicism for the sex chromosomes (Table 3).

DISCUSSION

There are few papers where monopronucleated ICSI zygotes were cultured until blastocyst stage (Otsu *et al.*, 2004; Mateo *et al.*, 2013; 2017; Yao *et al.*, 2016) and all came from a small study cohort. The reason for the low number of blastocysts included in the present study was the low incidence of monopronucleated ICSI zygotes in the IVF program (3.1%, unpublished data, Dexeus Women's Health), the restrictive 1PN 2PB assessment using time-lapse and the low development potential of 1PN 2PB ICSI zygotes with regard to reaching the blastocyst stage (14.8%, Mateo et al., 2013; 3.6%, Yao et al., 2016; 3.4%, Mateo et al., 2017).

The disagreements found between the techniques used can be explained because a high number of mosaic female embryos detected by FISH were misclassified by aCGH, due to their failure in detecting haploid and tetraploid cells. Even so, it cannot be ruled out that the disagreements were the result of analyzing two different TE fragments; it seems unlikely because the most commonly observed mosaicism involved ploidy alterations. The coexistence

Table 3. Agreement between classifications obtained after FISH and aCGH analysis of 1PN 2PB-derived blastocysts. H: Haploid; Df: diploid female; Dm: diploid male; Mf: mosaic female; Mm: mosaic male; M: mosaic for the sex chromosomes

		aCGH						
		H	Df	Dm	Mf	Mm	M	TOTAL
FISH	H	0	0	0	0	0	0	0
	Df	0	3	0	0	0	0	3
	Dm	0	0	4	0	0	0	4
	Mf	0	6	0	1	0	0	7
	Mm	0	0	0	0	0	0	0
	M	0	0	0	0	0	1	1
	TOTAL	0	9	4	1	0	1	60% (9/15)

of diploid, haploid and tetraploid cells has been already reported in embryos from monopronucleated zygotes as well as in those which were normally fertilized (Staessen & Van Steirteghem, 1997; Daphnis *et al.*, 2005). Female embryos showing a high percentage of diploid cells, and few haploid and tetraploid cells could originate from fertilization involving paternal and maternal genomes. The presence of tetraploid cells is a common phenomenon and attributed to the endoreduplication of diploid cells (Daphnis *et al.*, 2005). The presence of few haploid cells could be explained by the formation of binucleate cells with a subsequent cytokinesis (Delhanty *et al.*, 1997). Mosaic female embryos with a high percentage of haploid cells could have originated from oocyte parthenogenetic activation. The embryo initially would be haploid, and later some cells will turn into diploid cells by endoreduplication.

With regards to these findings, embryos in which all the cells analyzed were diploid, those mosaics with diploid/tetraploid cells and diploid/tetraploid/haploid cells with a low presence of haploid cells (<50%) could be considered fertilized; this accounts for 86.7% (13/15). The percentage of diploidy has been shown to be lower when chromosomal assessment is performed in early development (Sultan *et al.*, 1995; Staessen & Van Steirteghem, 1997; Van Der Heijden *et al.*, 2009; Mateo *et al.*, 2013) but higher, when it is performed in blastocysts (Mateo *et al.*, 2013). The selection against haploid embryos through culturing up to the blastocyst stage could explain these differences (Gras & Trounson, 1999).

Embryo E12 needs special consideration as coexistent haploid, diploid female and diploid male cells have been observed. These cells could come from the rescue of a triploid XXY embryo, resulting in one diploid male cell line and another haploid cell line, with subsequent diploidization of some haploid cells. Although the rescue of a triploid embryo has been reported after dispermic fertilization, it has not been confirmed after ICSI (Golubovsky, 2003). The origin of this triploid XXY embryo could be the result of either the fecundation of the oocyte by a diploid spermatozoa or by a spermatozoa carrying the Y chromosome together with endoreduplication of the oocyte genome (Rosenbusch, 2008; 2014).

The combination of FISH and aCGH techniques has been a suitable approach to expand the knowledge 1PN 2PB ICSI-derived embryos, and to allow the detection of mosaicism, which would not be detected by applying only one technique. It has been shown that the application of aCGH for analysis may lead to an underestimation of embryos with a high proportion of haploid cells and fully haploid embryos that could compromise the correct development of further pre-implantation stages. Furthermore, it has been demonstrated that FISH approach is not a safe option for the analysis of 1PN 2PB-derived embryos for clinical use, as it cannot distinguish between fertilized and non-fertilized diploidized embryos. Even more, FISH analysis where only few chromosomes are analyzed can underestimate the impact of aneuploidy. This is of special importance in 1PN-derived embryos, as high aneuploidy rates have been observed among them (Mateo *et al.*, 2013). Consequently, we recommend an initial analysis with a CCS technique to evaluate the euploidy of 1PN-derived embryos.

In case those embryos would be needed for reproductive purposes, blastocysts diagnosed as diploid males could be considered, as the presence of the Y chromosome ensures the paternal genome contribution. Despite that, the possibility of an androgenetic origin, with the contribution of only the paternal genome, is not ruled out, although it may seem like a very rare phenomenon (Azevedo *et al.*, 2014; Kai *et al.*, 2015). As aCGH was concordant with FISH in gender assignment, the use of FISH for detecting male embryos will not be necessary. Considering the possible use of diploid female embryos or female mosaics with a high percentage of diploid cells, the use of both FISH and aCGH would not solve the dilemma, as the possible origin by parthenogenesis followed by endoreduplication is not ruled out. The low frequency of uniparental disomy observed in human blastocysts, which is equivalent to those observed in live births, indicates that the presence of uniparental disomy is a rare phenomenon, but it could increase among abnormally fertilized embryos (Gueye *et al.*, 2014; Xu *et al.*, 2015). For better assessment, despite an euploid diagnosis after aCGH has been achieved, other diagnostic methods covering the analysis of parental genome origin should be performed in blastocysts derived from 1PN 2PB ICSI zygotes.

CONCLUSIONS

The additional use of FISH in the analysis of blastocysts derived from 1PN 2PB ICSI zygotes enabled the conclusion that aCGH underestimates haploidy. Furthermore, some diploid embryos diagnosed by aCGH are, in fact, mosaic. In cases where 1PN 2PB ICSI-derived embryos would be used for reproductive purposes, despite an euploid diagnosis after aCGH has been achieved, an extra analysis of parental genome origin should be performed.

CONFLICT OF INTERESTS

No conflict of interests has been declared.

Corresponding author:
Silvia Mateo
Reproductive Medicine Service
Department of Obstetrics, Gynaecology and Reproduction,
Women's Health Dexeus, Barcelona, Spain.
E-mail: silmat@dexeus.com

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4.4. Article 4:

Títol: *Morphokinetics and developmental potential of monopronucleated ICSI zygotes*.

Autors: Sílvia Mateo, Francesca Vidal, Beatriz Carrasco, Ignacio Rodríguez, Ventura Coroleu, Anna Veiga, Montserrat Boada.

En preparació.

MORPHOKINETICS AND DEVELOPMENTAL POTENTIAL OF MONOPRONUCLEATED ICSI ZYGOTES

ABSTRACT:

Introduction: Time-lapse (TL) technologies have been used extensively for analysing *in vitro* development of embryos coming from zygotes showing two pronuclei (2PN). However, the developmental capacity of embryos derived from monopronucleated (1PN) ICSI zygotes has not been yet deeply analysed using this technology yet. Using the TL technology with the aim of increasing knowledge on *in vitro* development of the 1PN ICSI zygotes, the morphokinetic parameters from the zygote to the blastocyst stage between embryos derived from the 1PN and 2PN ICSI zygotes have been compared.

Material and methods: The study group included 149 1PN two polar bodies (2PB) ICSI zygotes and the control group comprised 195 2PN 2PB ICSI zygotes. The embryo development, including the blastocyst rate, was evaluated. Morphokinetic parameters, including the pronucleus diameter and kinetics of the *in vitro* development, were analysed as well.

Results: Embryos derived from the 1PN 2PB zygotes showed impaired development compared to the 2PN-derived embryos, showing 28.9% and 67.2% blastocyst rate, respectively. The diameter of the 1PN zygotes was larger than that of the 2PN zygotes. Embryos derived from the 1PN zygotes showed a shorter time of the visible pronucleus, longer time of syngamy and slower kinetic behaviour from 2 to 9 cells when compared to the 2PN-derived embryos. When blastocysts arising from the 1PN and 2PN zygotes were compared, it was observed that the developmental kinetics was similar in both groups.

Conclusions: The monopronucleated ICSI zygotes have a different developmental capacity and morphokinetic behaviour than the 2PN ICSI zygotes do, showing characteristic morphokinetic parameters involving the pronucleus formation. Only blastocysts derived from the 1PN ICSI zygotes have similar morphokinetic development to the 2PN-derived blastocysts.

INTRODUCTION:

Time-lapse (TL) technologies provide an opportunity to perform continuous monitoring of embryo *in vitro* development. Beyond the annotation of the times of division, the constant dynamic monitoring allows accurate morphokinetic evaluation of embryos from initial stages to blastocyst formation (Wong *et al.*, 2010; Meseguer *et al.*, 2011; Cruz *et al.*, 2012; Milewski *et al.*, 2015). It is therefore possible to detect alterations of expected normal development that could not be identified through classical morphological evaluation (Rubio *et al.*, 2012; Athayde Wirka *et al.*, 2014; Liu *et al.*, 2014b).

The fertilization process has been studied using TL systems analysing the morphometry of zygotes and pronuclei, the kinetics of second polar body (2PB) extrusion, pronuclear (PN) formation and fading, as well as the timing of the first cleavage (Payne *et al.*, 1997; Montag *et al.*, 2011; Azzarello *et al.*, 2012; Aguilar *et al.*, 2014; Liu *et al.*, 2014a; Iwata and Mio, 2016). The technology also enabled the detection and evaluation of embryos coming from pronuclear patterns different from the two pronuclei.

Similarly, embryos originating from tripronucleated (3PN) zygotes have also been evaluated using the TL systems (Joergensen *et al.*, 2014; Grau *et al.*, 2015). Those studies have shown different morphokinetic behaviour of the 3PN-derived embryos when compared to the 2PN-derived ones. The monopronucleated zygotes (1PN) have also been also assessed by continuous monitoring in preliminary studies focusing on the fertilization process and early development (Boada *et al.*, 2012; Yamaguchi *et al.*, 2013; Igashira *et al.*, 2016; Tanaka *et al.*, 2016). The technology enabled the detection of a novel mechanism of the 1PN formation which involves the extrusion of the female genetic material in a third polar body-like (Mio *et al.*, 2014; Iwata and Mio, 2016). However, very little is known about the developmental morphokinetics of the 1PN-derived embryos and no studies employing the TL technology to evaluate pronuclear behaviour, cleavage patterns and blastocyst formation have been published so far.

The objective of this study was to compare the morphokinetic behaviour from the zygote to blastocyst stage of embryos derived from the monopronucleated and normally fertilized zygotes, in order to evaluate the *in vitro* development of the monopronucleated zygotes coming from an intracytoplasmic sperm injection (ICSI).

MATERIAL AND METHODS:

Study and control groups

The study group included 149 monopronucleated zygotes from 129 ICSI cycles with own gametes. Only the zygotes displaying one pronucleus and two polar bodies were considered for the study. Zygotes temporarily showing 1PN due to asynchronous formation or fusion of 2PN or zygotes showing one pronucleus and micronuclei, were not considered valid 1PN and were therefore were not included in the study group. Furthermore, zygotes in which 1PN did not disappear or the ones showing only one polar body were also excluded from the study.

The control group included 195 zygotes with two pronuclei and two polar bodies coming from 30 ICSI cycles with own gametes.

Zygotes from both the study and the control groups were obtained at the IVF Dexeu Mujer laboratory in Barcelona between September 2011 and May 2015.

Patients' populations from the 1PN and the control group showed similar characteristics (Table I). The characteristics of the cycles from the study and the control groups have been summarized in Table II.

	STUDY 1PN GROUP	**CONTROL 2PN GROUP**	***p val.***
Maternal age (mean±SD)	36.61±4.01	36.21±3.15	*.462*
Indication (n)			*.191*
Female infertility	22.5% (29)	10% (3)	
Male infertility	29.5% (38)	23.3% (7)	
Male and female infertility	17% (22)	16.7% (5)	
Idiopathic infertility	31% (40)	50% (15)	
Total	100% (129)	100% (30)	

Table I. Patients' characteristics from the study (1PN) and the control (2PN) groups.

Parameter (mean±SD)	STUDY 1PN GROUP	CONTROL 2PN GROUP	*p value*
Gonadotrophin dose	2103.63±919.87	1909.79±566.85	*.403*
Oocytes retrieved	11.65±4.95	10.33±5.04	*.296*
Mature (MII) oocytes	10.02±3.94§	8.57±4.58§	*.045*
Inseminated oocytes	10.02±3.99§	8.57±4.58§	*.048*
Oocytes fertilized (2PN)	7.00±3.48	6.83±4.03	*.713*
Embryos transferred	1.58±0.83§	1.23±0.50§	*.002*
Embryos frozen	2.36±2.40	2.37±2.58	*.889*

Table II. Characteristics of the cycles from the study (1PN) and the control (2PN) groups.

Ovarian stimulation and pick up

The patients underwent an ovarian stimulation with the use of gonadotrophins (FSH or hMG) and GnRH analogues. The monitoring of the response was performed by a transvaginal ultrasound scan and determining the levels of estradiol and progesterone according to our protocols (Martinez *et al.*, 2016). The patients received hCG when the diameter of the leading follicle was >18mm. the ultrasound-guided oocyte retrieval was carried out 36h post the hCGH administration.

In vitro fertilization and embryo culture

Oocytes were fertilized by ICSI 40h after the hCG administration. After insemination, oocytes from both groups were individually placed in an EmbryoSlide (Vitrolife®), preequilibrated at 37ºC and 6% CO_2. Oocytes after ICSI

were cultured either in G1/G2 or G-TL™ culture media (Vitrolife®) up to blastocyst formation or their arrest in an Embryoscope™ (Vitrolife) at 37ºC, 5% O_2 and 6% CO_2.

Embryo development

The time-lapse images of each embryo development were acquired at five equidistant (20μm) focal planes every 15 minutes.

The presence of early cleavage (EC) at 26 hours post insemination (hpi) and the number of cells on day 4 (44 hpi) and day 3 (68 hpi) were studied. The percentage of embryos with at least 4 cells at 44 hpi and 8 cells at 68 hpi were calculated. The maximum state of division achieved for each embryo was recorded and the blastocyst rate at days 5-7 (120-185 hpi) was also calculated.

Morulae were classified as fully compacted when all the cells of the embryo were included in the morulae and partially compacted when at least one cell from the morulae was excluded.

Blastocysts morphology was evaluated when a full blastocyst was observed. Characteristics of the inner cell mass and the trophectoderm cells were evaluated and blastocysts were further classified in A, B, C or D categories, where A was the best and D the worst according to the ASEBIR score categorization (ASEBIR, 2015).

The presence of multinucleation (MN), irregular division (ID) (cellular fusion/reverse cleavage and failed cytokinesis), and direct cleavage (DC) (observation of cleavage from 1 cell to 3 daughter cells at any moment of development) was analysed (Ciray *et al.*, 2014; Liu *et al.*, 2014b).

Zygote morphometric parameters

Different measurements of zygotes were acquired at mid-time of the visible pronucleus (mtvPN): the maximum diameter and the area of PN, the maximum diameter and the area of zygotes with and without the zona pellucida (ZP), and the maximum and minimum thickness of the ZP.

Within the control group, the average diameter and area of the two pronuclei were calculated and used for the comparison with the corresponding measures from the 1PN ICSI zygotes.

Kinetic analysis of zygotes and embryos

Embryo kinetics was evaluated retrospectively using the images acquired periodically every 15 minutes from the ICSI time until a maximum of 7 days of development. The reference starting point (t0) was the mid-time between the first and the last inseminated oocyte.

The times recorded for each morphokinetic variable were adapted from those proposed by Ciray and others (Ciray *et al.*, 2014) (Table III). The time for the PN appearance for the 2PN group was recorded when the first pronuclei was visible for the first time. The time for the PN fading was the first frame when neither of the two pronuclei were visible. The morphokinetic variables for the cleavage, morula, blastocyst and other events observed during the embryo development are shown in Table III.

Recorded morphokinetic events	
tPB2	Second polar body appearance
tPNa	Appearance of PN
tPNf	PN fading
t2 to t9	Cleavage from 2 to 9 cells
tSC	First evidence of compaction
tM	Morulae/End of compaction process (last frame before cavity formation)
tSB	Initiation of blastulation (first frame of cavity formation)
tB	Full blastocyst (last frame before zona starts to thin)
tEB	Initiation of expansion (first frame of zona thinning)
tHB	Herniation; initiation of hatching process
tBcol	Blastocyst collapse
tBre-exp	Blastocyst re-expansion

Table III. The events recorded during the kinetic analysis of the 1PN and the 2PN ICSI zygotes (adapted from Ciray et al., 2014).

The associated calculated variables were adapted from other available publications (Meseguer *et al.*, 2011; Aguilar *et al.*, 2014; Athayde Wirka *et al.*, 2014; Ciray *et al.*, 2014) and are summarized in Table IV. The times at which each event occurred were noted as hours after insemination. If blastocyst collapses were observed, the numbers of collapses was noted as was the time of the first collapse and re-expansion.

Calculated morphokinetic variables	
vPN	PN duration or S-phase length
mtvPN	Mid-time of visible PN ([(PNf-PNa)/2] + PNa)
Syngamy	Duration from PNf and t2 (t2-PNf)
ECC1	Duration of first cell cycle (t2-tPB2)
ECC2	Duration of second embryo cell cycle (t4-t2)
cc2	Duration of second cell cycle (t3-t2)
ECC3	Duration of third embryo cell cycle (t8-t4)
cc3	Duration of the third cell cycle (t5-t3)
s2	Synchronization of second cell cycle (t4-t3)
s3	Synchronization of third cell cycle (t8-t5)
dcom	Duration of compaction (tM-tSC)
dB	Duration of blastulation (tB-tSB)
Ncol	Number of episodes of collapses
dcol	Duration of blastocyst collapse (tBre-exp – tBlcol)

Table IV. The calculated morphokinetic variables during the kinetic analysis of the 1PN and the 2PN ICSI zygotes (adapted from Meseguer *et al.*, 2011; Aguilar *et al.*, 2014; Athayde Wirka *et al.*, 2014; Ciray *et al.*, 2014).

Statistical analysis

Comparisons of the results obtained from the development, morphometric and kinetic analyses were compared between the study and control groups. Furthermore, the 1PN ICSI zygotes that reached the blastocyst stage were differentiated from those 1PN zygotes that arrested their development before the blastocyst stage. The results obtained from both subgroups were compared. The morphokinetic parameters of the 1PN blastocysts were also compared to the 2PN blastocysts results.

Continuous variables have been described using median, ranges (maximum-minimum) and the mean and standard deviation (±SD). The nominal variables have been showed in frequency tables.

In order to compare the distribution of the continuous variables, the non-parametric test of Wilcoxon-Man Whitney was used. The association between the nominal variables has been evaluated by the Pearson Chi-square Test. All the tests were bilateral with a significance level of 5%. All the statistical analyses have been performed using the IBM SPSS Statistics 22 software.

RESULTS:

Embryo development

The monopronucleated zygotes showed impaired development compared with that of the 2PN ones, evidencing higher rates of embryo arrest at the early stages and a lower blastocyst rate (28.9% vs 67.2%) (Table V). The morulae characteristics were different between the two groups, showing a higher percentage of morulae that excluded some cells during compaction in the 1PN group (Table VI). Anomalies during the embryo development were observed more often during the development of the 1PN zygotes rather than that of the 2PN ones, with similar rates of direct cleavage but higher rates of irregular divisions (33.6% vs 10.3%; p<0.001) (Table VII).

	STUDY 1PN GROUP	CONTROL 2PN GROUP	*p value*
EMBRYOS (n)	149	195	*.001*
ARRESTED (n)	71.1% (106)	32.8% (64)	
No cleaved (n)	6.0% (9)	0.0% (0)	
Arrested before compaction (n)	49.0% (73)	17.9% (35)	
Arrested at morula (n)	16.1% (24)	14.9% (29)	
BLASTOCYST (n)	28.9% (43)	67.2% (131)	

Table V. Embryo development from 1PN and 2PN ICSI zygotes.

Embryos from the 1PN subgroup that did not achieve the blastocyst stage showed differences in development from day 2 onwards, with less cells on day 2 (44hpi) and day 3 (68hpi). No differences were observed in the abnormal cleavages in both subgroups of 1PN-derived embryos. A similar percentage of embryos showing early cleavage and blastomeric multinucleation was observed between the 1PN and the 2PN groups (Table VII).

	STUDY 1PN GROUP			CONTROL 2PN GROUP				
	TOTAL §	ARRESTED ‡	BLASTOCYSTS ‡¥	TOTAL§	BLASTOCYSTS ¥	*p val.* §	*p val.* ‡	*p val.* ¥
Morulae (n)	67	24	43	160	131	*.001*	*.008*	*.017*
Fully compacted (n)	22.4% (15)	4.2% (1)	32.6% (14)	46.9% (75)	53.4% (70)			
Partially compacted (n)	77.6% (52)	95.8% (23)	67.4% (29)	53.1% (85)	46.6% (61)			

Table VI. The morphology of the morulae from the 1PN and the 2PN groups. §1PN *vs* 2PN total groups. ‡Arrested 1PN *vs* 1PN blastocyst groups. ¥ 1PN blastocyst *vs* 2PN blastocyst groups.

	STUDY 1PN GROUP			CONTROL 2PN GROUP				
	TOTAL	**ARRESTED**	**BLASTOCYSTS**	**TOTAL**	**BLASTOCYSTS**	***p val.*§**	***p val.*‡**	***p val.*¥**
Number of embryos	140	97	43	195	131			
EC (n)	48.6% (68)	45.4% (44)	55.8% (24)	47.7% (93)	55.0% (131)	*.051*	*.155*	*.067*
Cells at 44hpi mean(±SD)	3.68 ±1.42§	3.39 ±1.36‡	4.33 ±1.34‡	4.27 ±1.19§	4.30 ±1.11	*.000*	*.000*	*.875*
Cells at 68hpi mean(±SD)	7.01 ±2.17§	6.47 ±2.27‡	8.05 ±1.51‡	8.18 ±1.97§	8.58 ±1.72	*.000*	*.000*	*.349*
MN (n)	46.4% (65)	42.3% (41)	55.8% (24)	40.5% (79)	39.7% (52)	*.281*	*.138*	*.064*
Abnormal Cleavage (n)	42.1% (59)§	45.4% (44)	34.9%¥ (15)	23.6%§ (46)	14.5%¥ (19)	*.001*	*.454*	*.003*
ID (n)	33.6% (47)§	38.1% (37)	23.3%¥ (10)	10.3%§ (20)	6.1%¥ (8)	*.000*	*.085*	*.001*
DC (n)	23.6% (33)	24.7% (24)	20.9% (9)	17.9% (35)	9.9% (13)	*.624*	*.474*	*.060*

Table VII. The results from the embryo developmental analysis of the 1PN and the 2PN ICSI zygotes. EC: Early cleavage; MN: Multinucleation; ID: Irregular Division; DC: Direct Cleavage. §1PN *vs* 2PN total groups. ‡Arrested 1PN *vs* 1PN blastocyst groups. ¥1PN blastocyst *vs* 2PN blastocyst groups.

The monopronucleated zygotes that reached the blastocyst stage showed a similar number of cells on day 2 and day 3 as than those coming from 2PN blastocysts (Table VII). The morphology of the blastocysts from the 1PN zygotes was significantly poorer than that of the 2PN-derived blastocyst, even though grade A or B was observed in 32.5% of the 1PN-derived blastocysts (Table VIII).

	STUDY 1PN GROUP	CONTROL 2PN GROUP	*p value*
Blastocyst (n)	43	131	.001
Grade A %(n)	11.6% (5)	40.5% (53)	
Grade B %(n)	20.9% (9)	21.4% (28)	
Grade C %(n)	39.5% (17)	28.2% (37)	
Grade D %(n)	27.9% (12)	9.9% (13)	

Table VIII. The morphology of blastocysts from the 1PN and the 2PN groups.

Zygote morphometric parameters

The analysis of morphometric measures shows differences between the 1PN and the 2PN zygotes across all the studied parameters. The results from the morphometric analysis are represented in Table IX. The monopronucleated zygotes showed bigger sizes of pronucleus, oocyte and zona pellucida.

The parameters of the 1PN zygotes that form the blastocyst were compared with those from the 1PN zygotes whose development was arrested before this stage. Both groups showed similar sizes in the oocyte measures except for the PN diameter and area, which were bigger in the 1PN blastocyst group ($p=0.036$; $p=0.015$) (Table IX).

The morphometries of the 1PN and the 2PN blastocyst groups were compared and no differences were observed in the zygote diameter and area without ZP between both groups. The pronuclear diameter and area, the oocyte diameter including ZP and the ZP thickness were larger in the 1PN blastocyst group compared to the 2PN blastocysts (Table IX).

	STUDY 1PN GROUP			CONTROL 2PN GROUP				
PARAMETER (mean ±SD)	**TOTAL**	**ARRESTED**	**BLASTOCYST**	**TOTAL**	**BLASTOCYST**	***p val.* §**	***p val.* ‡**	***p val.* ¥**
N	149.00	106.00	43.00	195.00	131.00			
PN diameter (μm)	26.74 ±2.91[§]	26.42 ±2.64[‡]	27.51 ±3.42[‡¥]	24.09 ±1.54[§]	24.04 ±1.56[¥]	.000	.036	.000
PN area (μm^2)	571.84 ±106.38[§]	556.74 ±93.02[‡]	609.07 ±127.48[‡¥]	478.43 ±54.34[§]	476.56 ±54.17[¥]	.000	.015	.000
Zygote diameter without ZP (μm)	113.01 ±5.14[§]	113.02 ±5.15	112.98 ±5.16	111.65 ±5.59[§]	111.90 ±6.16	.004	.926	.108
Zygote area without ZP (μm^2)	9567.23 ±821.59[§]	9552.37 ±831.66	9603.86 ±804.70	9360.99 ±560.05[§]	9372.01 ±579.12	.022	.757	.108
Zygote diameter with ZP (μm)	163.59 ±8.84[§]	163.89 ±9.45	162.86 ±7.16[¥]	159.11 ±9.18[§]	159.00 ±9.77[¥]	.000	.664	.005
Zygote area with ZP (μm^2)	20754.17 ±2021.42[§]	20866.97 ±2007.16	20476.09 ±2053.24[¥]	19772.08 ±1555.36[§]	19813.09 ±1513.07[¥]	.000	.390	.022
max. ZP (μm)	21.46 ±3.02[§]	21.57 ±3.09	21.21 ±2.87[¥]	19.17 ±3.04[§]	19.31 ±3.06[¥]	.000	.600	.001
min. ZP (μm)	17.54 ±3.06[§]	17.42 ±3.10	17.84 ±2.98[¥]	15.03 ±3.20[§]	14.97 ±3.14[¥]	.000	.430	.000

Table IX. The morphometric analysis of the 1PN and the 2PN ICSI zygotes. [§]1PN *vs* 2PN total groups. [‡]Arrested 1PN *vs* 1PN blastocyst groups. [¥]1PN blastocyst *vs* 2PN blastocyst groups.

Zygote and embryo kinetics

The analysis of zygote kinetics showed differences in PN appearance and fading between the 1PN and the 2PN groups (Table X). The pronuclei from the 1PN zygotes appeared latter and faded earlier than pronuclei from the 2PN zygotes, which resulted in a shorter time of visible pronuclei in the 1PN group (13.76 h vs 16.20 h; $p<0.001$). The time of syngamy was longer in the 1PN group as was the embryo second cell cycle. The cleavages in the second and third cell cycles were less synchronic in the 1PN-derived embryos.

The comparison of kinetics from the subgroups of the arrested 1PN-derived embryos and the 1PN-derived blastocysts revealed similar behaviour in the PN appearance in both groups but earlier fading was recorded in the 1PN-blastocyst group (Table X). Different kinetics was observed in t2, t4, t5, t7, tSC and tM between both subgroups, showing delayed cleavages in the arrested 1PN group. The length for syngamy showed similar values in both subgroups from the 1PN–derived embryos ($p=0.148$).

The kinetics from the 1PN-derived blastocyst and the 2PN-derived blastocyst were compared and it was observed that the time for visible PN was shorter in the 1PN blastocysts due to their later appearance and earlier disappearance of the pronucleus in the 1PN blastocyst group (Table X). The embryo kinetics was similar in both groups from t2 to t9. Afterwards, the kinetics of the 1PN blastocyst group showed delayed times from tSC to tHB compared to the 2PN blastocysts. Blastocyst collapses were more frequent in the 1PN blastocysts than in the 2PN blastocysts (2 ± 1.05 vs 1.17 ± 0.51; $p=0.001$). The time of the first collapse and re-expansion occurred latter in the 1PN than the 2PN blastocyst but the duration of the collapse was similar (Table X).

Detailed data resulting from all the kinetic analysis and the calculated parameters are shown as supplementary material (Supplemental Table I).

	STUDY 1PN GROUP			CONTROL 2PN GROUP				
median (range)	**TOTAL**	**ARRESTED**	**BLASTOCYST**	**TOTAL**	**BLASTOCYST**	***p val.*[§]**	***p val.*[‡]**	***p val.*[¥]**
t2PB (hpi)	3.95 (1.46-12.70)	3.86 (1.46-10.60)	4.25 (1.76-12.70)	3.70 (1.93-8.60)	3.75 (1.93-8.60)	.091	.517	.091
tPNa (hpi)	8.79[§] (3.45-26.06)	8.87 (3.45-26.06)	8.65[¥] (4.92-20.21)	7.80[§] (3.90-14.00)	7.70[¥] (5.40-12.90)	.000	.338	.011
tPNf (hpi)	22.90[§] (16.65-65.74)	23.27[‡] (16.65-65.74)	22.00[‡¥] (17.29-29.47)	24.10[§] (18.10-61.30)	23.40[¥] (18.10-31.20)	.008	.016	.003
vPN (h)	13.76[§] (5.25-50.74)	14.335 (5.25-50.74)	13.10[¥] (7.00-20.40)	16.20[§] (10.00-52.10)	15.80[¥] (10.00-24.00)	.000	.081	.000
Syngamy (h)	3.53[§] (0.34-34.05)	3.75 (0.34-34.05)	3.07[¥] (2.23-16.50)	2.72[§] (0.54-34.00)	2.62[¥] (0.54-13.60)	.000	.148	.000
t2 (hpi)	27.36 (19.20-66.29)	27.61[‡] (19.20-66.29)	26.50[‡] (19.80-45.97)	26.92 (20.15-66.40)	26.10 (20.15-44.80)	.123	.008	.928
t8 (hpi)	61.92 (42.70-95.80)	63.35 (43.75-95.80)	61.25 (42.70-84.43)	57.95 (43.53-137.59)	57.05 (43.53-110.68)	.193	.057	.566
tM (hpi)	118.40[§] (88.83-150.40)	125.05[‡] (103.6-150.4)	114.30[‡¥] (88.83-138.29)	100.35[§] (81.70-139.40)	99.60[¥] (81.70-139.40)	.000	.003	.000
dcom (h)	21.30[§] (3.50-51.80)	21.19 (3.50-49.70)	21.30[¥] (7.70-51.80)	15.60[§] (4.00-39.50)	15.20[¥] (4.00-39.50)	.000	.628	.001
tB (hpi)	128.10[§] (96.59-145.03)		128.10[¥] (96.59-145.03)	110.80[§] (85.40-155.60)	110.80[¥] (85.40-155.60)	.000		.000
dB (h)	10.56 (3.25-23.70)		10.56 (3.25-23.70)	10.20 (1.30-28.30)	10.20 (1.30-28.30)	.615		.615

Table X. The results of the kinetic analysis of the selected parameters of the 1PN (study group) and 2PN (control group) ICSI zygotes. [§]1PN *vs* 2PN total groups. [‡]Arrested 1PN *vs* 1PN blastocyst groups. [¥]1PN blastocyst *vs* 2PN blastocyst groups.