Reproductive Sciences
2014, Vol. 21(12) 1472-1482

DOI: 10.1177/1933719114530185
rs.sagepub.com
SAGE

Molecular Cytogenetics of Human Single Pronucleated Zygotes

Ana Raquel Azevedo, MSc[1], Maria João Pinho, PhD[2], Joaquina Silva, MD[3], Rosália Sá, PhD[1], Sólveig Thorsteinsdóttir, PhD[4,5], Alberto Barros, MD, PhD[2,3], and Mário Sousa, MD, PhD[1]

Abstract

The aim of the present study was to use fluorescence in situ hybridization to analyze the chromosome status of zygotes with a single pronucleus from in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatment cycles. In addition, we performed immunocytochemical detection of nuclear lamins and histone H3 trimethylated at lysine-9, Me(3)H3K9. Zygotes were processed 24 hours after insemination or injection to assure the absence of asynchrony. In opposition to previous results, we observed 2 pronuclei in 16 of 18 IVF zygotes and 40 of 64 ICSI zygotes, suggesting premature pronuclear breakdown. In IVF and ICSI zygotes, the rate of normal diploidy was only 6 of 16 and 27 of 56, respectively, suggesting that monopronucleated zygotes should not be used in assisted reproductive treatments. The possible mechanisms are discussed and compared to previous studies of monopronucleated zygotes.

Keywords

FISH, histone H3, human monopronucleated zygotes, immunocytochemistry, lamins

Introduction

The entry of a spermatozoon into the oocyte, which is arrested at metaphase II of meiosis and presents the first polar body (PB1), triggers oocyte activation with female meiosis resumption, extrusion of the second polar body (PB2), and formation of two haploid pronuclei (2 PNs): male (m-PN) and female (f-PN). After a tight connection forms between the 2 PNs, the nuclear envelopes disassemble into small fragments, the chromosomes intermingle, and the zygote undergoes the first mitotic division. After fertilization, the PB2 appears 1 to 8 hours postinsemination in in vitro fertilization (IVF) or postmicroinjection in intracytoplasmic sperm injection (ICSI) cycles. Appearance of the 2 PNs occurs 3 to 20 hours after fertilization, with the f-PN first observed near the site where telophase II occurred. Formation of 2 PNs is synchronous in the large majority of the cases (63%-74%), but when asynchrony occurs, the m-PN appears first and both PNs are observed after 10 to 120 minutes. Pronuclear envelope breakdown (PNBD) is observed 23 to 46 hours after fertilization.[1-5]

In some cases (2.7%-17%), the inseminated or injected oocytes produce zygotes with only 1 PN, either with 1 or 2 polar bodies (PBs), although the majority of zygotes have two PBs. Comparisons between cycles with and without 1 PN zygotes have not revealed any relationship with demographic characteristics, or embryological or clinical outcomes,[1,6-12] with the exception of one study that described a higher number of cumulus–oocyte complexes and clinical pregnancy in cycles with 1 PN zygotes.[13]

After assessments of 1 PN zygotes and respective embryos with cytogenetics, fluorescence in situ hybridization (FISH), antibodies specific for female chromatin, and polymerase chain reaction, several mechanisms have been proposed to explain the appearance of 1 PN zygotes, including parthenogenesis, endoduplication, and karyogamy (PN fusion).[5,8,12]

A recent study reported that the number of diploid single pronucleated zygotes with two PBs, probably originated from karyogamy, is about 86.7% in IVF cycles, and the authors suggested that these zygotes could be used for assisted reproduction. They also found that in ICSI cycles, 69.7% of these zygotes were haploid, indicating that they should not be used in treatments.[14] These observations are consistent with previous findings from embryo

[1] Department of Microscopy, Laboratory of Cell Biology, Multidisciplinary Unit for Biomedical Research-UMIB, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Rua Jorge Viterbo Ferreira, Porto, Portugal
[2] Department of Genetics, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal
[3] Centre for Reproductive Genetics, Porto, Portugal
[4] Department of Animal Biology, Faculty of Sciences, Centre for Environmental Biology, University of Lisbon, Lisbon, Portugal
[5] Instituto Gulbenkian de Ciência, Oeiras, Portugal

Corresponding Author:
Mário Sousa, Department of Microscopy, Laboratory of Cell Biology, Building 1, Floor 2, Room 07, Institute of Biomedical Sciences Abel Salazar, University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050 Porto, Portugal.
Email: msousa@icbas.up.pt

analysis: (IVF: 80.5% diploid, cytogenetics),[1] (IVF: 71.4% diploid; ICSI: 28.6% diploid; FISH),[9] (ICSI: none diploid; cytogenetics),[10] (ICSI: none diploid; FISH)[15]; but not consistent with other previous results from zygotes: (IVF: 58% diploid; cytogenetics, FISH),[13] (IVF: 37.5% diploid; FISH)[16] and embryos: (IVF: 47.6% diploid; PCR and FISH),[7] (IVF: 48.7% diploid; ICSI: 27.9% diploid; FISH).[11] To date, three published reports have described the birth of 5 healthy children from IVF treatment cycles, with embryos derived from 1 PN/2 PB zygotes as no other embryos were available for transfer.[1,17,18]

The aim of the present study was to quantitatively analyze the chromosome status of 1 PN zygotes with 1 and 2 PBs from cycles with IVF and ICSI using FISH analysis. In addition, we performed immunocytochemical detection of nuclear lamins (PNs with intact nuclear envelope) and histone H3, trimethylated at lysine 9, Me(3)H3K9 (specific to female chromatin). We observed the presence of two PNs in all cases with fertilization, suggesting that premature PNBD (pPNBD) occurred. The mechanisms underlying this novel finding are discussed and compared to previous studies that analyzed 1 PN zygotes.

Materials and Methods

Ethics

According to the National Law on Medically Assisted Procreation (Law 32/2006), the National Council on Medically Assisted Procreation guidelines (CNPMA, 2008), and clinical and embryological databases, the zygotes donated for experiments (with 1 PN) were used after patients, who underwent assisted reproductive technology (ART) treatment cycles, were informed about the studies and provided written consent.

Stimulation Protocol

Women underwent controlled ovarian hyperstimulation[19] using a gonadotropin-releasing hormone (GnRH) short antagonist protocol with cetrorelix (Merck Serono, Geneva, Switzerland) or ganirelix (Organon, Oss, The Netherlands) in the large majority of the cases. In a few cycles, patients underwent a long agonist protocol with busereline (Sanofi Aventis, Frankfurt, Germany) or an ultralong GnRH agonist protocol with triptoreline (Ipsen, Paris, France) combined with cetrorelix at final stimulation. Recombinant follicle-stimulating hormone (rFSH) was mainly used (Puregon, Organon, or Gonal-F; Merck Serono) for stimulation, but in several other cases rFSH was combined with human menopausal gonadotropin (Ferring, Kiel, Germany). Human chorionic gonadotropin (HCG) was administered (10 000-15 000 IU; Pregnyl, Organon) to trigger final oocyte maturation. In some cases, the agonist busereline or recombinant HCG (Merck Serono) was administered. Serum levels of estradiol (Elecsys 2010, Roche, France: up to 2009; VIDAS Estradiol II, Biomerieux, Marcy-LÈtolie, France: since 2009) were assayed by electrochemiluminescence (up to 2009) and enzyme-linked fluorescent assay (since 2009) at the day of HCG administration. Basal FSH levels were quantified by microparticle enzyme immunoassay (Abbott Lab, Abbott Park, Illinois).

Intracytoplasmic Sperm Injection

Cumulus–oocyte complexes were denuded under paraffin oil (Medicult Origio, Jyllinge, Denmark or Vitrolife, Kungsbacka, Sweden) with cumulase (Medicult) and incubated in 4-well plastic tissue culture dishes (NUNC, Roskilde, Denmark) in IVF medium (Medicult) or G-IVF-Plus (Vitrolife) until microinjection. The microinjections were performed as previously described[20] using an inverted microscope (Nikon DIAPHOT 200, Nikon ECLIPSE 300; Nikon, Tokyo, Japan) equipped with a thermal stage (37°C), Hoffman optics (Nikon) and Narishige micromanipulators (MO-188; Narishige, Tokyo, Japan).

Embryo Culture and Score

Embryos were cultured in sequential media (Medicult and Vitrolife) under oil in 4-well dishes (NUNC) or multi small-well EGPS dishes (Sun IVF, Guilford, Connecticut) inside a multigas incubator (6% CO_2, 5% O_2, 89% N_2, filtered humidified air; Sanyo, Tokyo, Japan). For embryo transfer, two different media were used (UTM; Medicult or Embryo-Glue; Vitrolife). Normal fertilization was assessed 16 to 20 hours after insemination or microinjection. Evaluation of embryo was performed using established score guidelines.[21,22]

Embryo Transfer, Luteal Support, and Pregnancy Confirmation

Only embryos from 2 PN/2 PB zygotes were transferred. Embryo transfer was at day 3 or 5 (when there were at least 3 to 4 high-quality embryos at day 3). Ultrasound-guided embryo transfer was performed with a Sure View Wallace Embryo Replacement Catheter (23 cm, CE123; Smiths Medical International Ltd, Kent, United Kingdom). Endovaginal tablets of natural micronized progesterone were administered for luteal support (Jaba; Besins International, Montrouge, France) from the day of oocyte aspiration up to 10 weeks of gestation. A clinical pregnancy was established by ultrasound visualization of a gestational sac at 6 weeks of gestation.

Zygote Evaluation

Approximately 16 to 20 hours after IVF or ICSI, zygotes with 1 PN and 1 or 2 PBs were selected for the present study. Before processing, these were inspected at 24 hours to confirm the absence of a delayed appearance of a second PN (Figure 1).

Fluorescence In Situ Hybridization

Each zygote was transferred to a 400-μL drop of 1% sodium citrate (Sigma, St Louis, Missouri) in a Petri dish. After 10

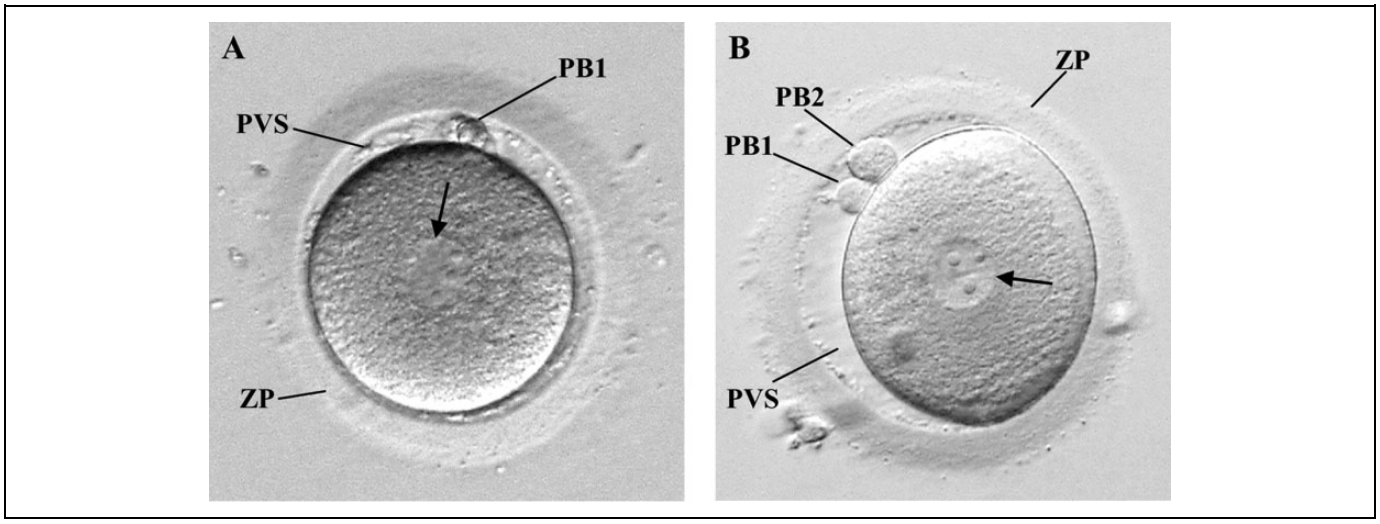

Figure 1. Images obtained by inverted Hoffman optics microscopy of human oocytes with 1 pronucleus (arrows). A, Presence of the first polar body (PB1). B, Presence of PB1 and second polar body (PB2). ZP indicates zona pellucida; PVS, perivitelline space.

minutes, the swollen cell was allocated to a poly-L-lysine-coated glass slide (Menzel-Glazer, Braunschweig, Germany). Fixation was performed with Carnoy (3:1 v/v methanol/acetic acid; Merck, Darmstadt, Germany) by dropping five 10-μL drops starting 5 cm above the dish with sequential decreases of 1 cm. In this way, the PN and PB appear geographically separated.[23] The slide was then washed for 4 minutes in phosphate-buffered saline (PBS; Sigma) and 4 minutes in distilled water, dehydrated in 70%, 96%, and 100% ethanol (Panreac, Barcelona, Spain; 1 minute each), and left to air dry. It was then incubated at 37°C in a water bath for 20 minutes with pepsin (100 μg/mL in water; Sigma) to remove cytoplasmic remnants and enable probe penetration. After a wash in distilled water, the slide was fixed at 4°C for 10 minutes in 1% paraformaldehyde (37% formalin; Sigma) in PBS. The sample was again washed and dehydrated as mentioned earlier and left to air dry.[24-26]

A fluorescent probe mixture (7 μL hybridization buffer-LSI + 1 μL CEP18-blue spectra + 1 μL LSI21-red spectra + 1 μL CEPX-green spectra + 0.5 μL YSATIII-red spectra + 0.5 μL YSATIII-green spectra) was prepared according to the manufacturer's instructions (Vysis, Downers Grove, Illinois) and 3 μL were applied to each zygote. DNA denaturation was performed at 75°C for 4 minutes by plating samples in a thermal plate (HYBrite; Vysis), followed by hybridization in a dark, humid chamber at 37°C for 4 hours or overnight. Thereafter, samples were washed inside vertical jars for 3 minutes at 42°C with 60% formamide (Fluka, Buchs, Switzerland) and then with 2× saline sodium citrate (SSC; Invitrogen, Carlsbad, California), followed by 4× SSC in 0.05% Tween 20 (Sigma) at room temperature (RT) with shaking. After washing in PBS and distilled water and dehydrated as mentioned earlier, slides were mounted and the DNA was counterstained with 10 μL 4′,6-diamidino-2-phenylindole (DAPI; Vectashield; Vector Laboratories, Burlingame, California) and observed in an epifluorescence microscope (AxioImager Z1, Carl Zeiss Inc, Oberkochen, Germany).[24-26]

Immunocytochemistry

Each zygote was washed in a Petri dish at 37°C with up and down micropipette movements in a 400-μL drop of PBS.[27] It was then transferred to a poly-L-lysine-coated glass slide, and 2 μL lysis buffer (2.5 μL Tween 20 + 2.5 mL embryo tested water + 25 μL HCl-1N) was added. More buffer was carefully added until complete cell lysis was observed and then the sample was left to air dry.[26]

Samples were then treated with pepsin as mentioned earlier, washed for 15 seconds with distilled water, and 15 seconds with PBS, with care to not let air dry. They were then fixed by incubation at −20°C for 10 minutes in a 200-μL drop of methanol (Merck) and washed 2 times for 2 minutes each at 4°C with PBS. Samples were then permeabilized for 15 minutes at RT with 0.5% Triton X 100 in PBS (Sigma), and washed 3 times for 5 minutes each with PBS at RT. To block nonspecific antibody binding, samples were treated at RT for 30 minutes with 200 μL 1% bovine serum albumin (BSA; Sigma) in PBS.

Primary antibody labeling was performed by incubating samples in a dark, humid chamber at 4°C overnight with 200 μL rabbit polyclonal antibody to Me(3)H3K9 (ab8898; Abcam, Cambridge, United Kingdom) diluted to 1:500 with 1% BSA in PBS and Tween 20 (PBST; 50 μL Tween 20 + 9.95 mL PBS) and 200 μL mouse monoclonal antibody to pan Lamin (ab20740, Abcam) diluted to 1:50 with 1% BSA in PBST. Samples were then decanted and washed 3 times for 5 minutes each with PBS and incubated in a dark, humid chamber at RT for 1 hour with secondary antibodies: 200 μL goat polyclonal antibody to rabbit immunoglobulin G (IgG)—H&L conjugated with Cy5 (ab6564, Abcam) diluted to 1:600 with 1% BSA in PBST and 200 μL of goat anti-mouse IgG1 conjugated with fluorescein isothiocyanate (sc-2078, Santa Cruz Biotechnology, Santa Cruz, California) diluted to 1:100 with 1% BSA in PBST.[28] After washing as described earlier, the samples were mounted with DAPI and observed

Table 1. Embryological and Clinical Data From IVF cycles.

Parameters	IVF-1 PN	IVF-Control	*P*
Patients	15		
Cycles	15	898	
Female age (mean, range)	35.6 ± 2.6 (30-40)	34 ± 4.1 (21-52)	.133
Male age (mean, range)	37.8 ± 2.8 (32-42)	35.5 ± 4.9 (24-60)	.075
TI (mean, range)	4.7 ± 4.9 (1-17)	3.6 ± 2.9 (1-22)	.175
COC/ETC (mean)	169 (11.3 ± 4.8)	7752 (8.6 ± 4.6)	.028
MII (n)	165	7234	
1 PN (n; %)	21	238 (4.9)	
2 PNs/MII (FR)	117 (71)	5011 (69.3)	.652
No. ET/ETC (mean)	28/14 (2.0 ± 0.0)	1639/858 (1.9 ± 04)	.470
CP/ETC (rate)	7 (50)	432 (50.3)	.979
IR	12/28 (42.9)	582/858 (35.5)	.421
Ab/CP (rate)	1 (14.3)	59 (13.7)	.962
OP/ETC (rate)	6 (42.9)	373 (43.5)	.963
Delivery/ETC (rate)	6 (42.9)	373 (43.5)	.963
LBDLR/ETC (rate)	6 (42.9)	373 (43.5)	.963
NB/ETC (rate)	10 (71.4)	485 (56.5)	.264
Stimulation characteristics of IVF cycles			
bFSH	5.9 ± 2.3	6.6 ± 3.6	.476
Total gonadotrophin dose	1713.5 ± 560.1	1756.9 ± 802.6	.846
Total time of stimulation	8.5 ± 1.76	8.3 ± 1.6	.642
Estradiol	1615.5 ± 1238.5	1310.1 ± 869.1	.213
HCG	9444.4 ± 1666.7	9398.4 ± 1763.6	.911

Abbreviations: IVF, in vitro fertilization; 1 PN, zygotes with 1 pronucleus; TI, time of infertility; COC, cumulus–oocyte complexes; MII, mature metaphase-II oocytes; 2 PNs, normal fertilized oocytes (with 2 pronuclei and 2 polar bodies); FR, fertilization rate; ET, embryo transfer; ETC, embryo transfer cycles; No. ET, number of transferred embryos; CP, clinical pregnancy; IR, implantation rate; Ab, abortion; OP, ongoing pregnancy; LBDLR, live birth delivery rate; NB, newborn; bFSH, basal follicle-stimulating hormone ; HCG, human chorionic gonadotropin.

under an epifluorescence microscope. After observation, samples were washed as mentioned earlier with 2× SSC and then with 4× SSC in 0.05% Tween 20, followed by the primary and secondary antibodies for FISH.

Statistical Analysis

Two types of controls were used (IVF and ICSI) from all consecutive cycles between 2005 and 2011 (date from which all data are computerized). Statistical analysis was carried out using the IBM SPSS Statistics 20 program for Windows (Armonk, New York). We used chi-square tests (Fisher exact test, 2 sided) and independent samples *t*-tests for assessing equality of means (2 sided). A value of $P < .05$ was considered statistically significant.

Results

Patients

Between January 2005 and September 2011, there were 898 IVF cycles and 3998 ICSI cycles. We analyzed 18 zygotes with 1 PN from 15 patients who underwent IVF (15 cycles) and 64 zygotes with 1 PN from 47 patients who underwent ICSI (49 cycles). Zygotes with 1 PN were collected for experiments as they were available during ART treatments. The study group (cases) was compared with all other IVF and ICSI cycles (controls) to ensure that cases did not present clinical and embryological differences in relation to controls.

Embryological and Clinical Data From IVF and ICSI Cycles

There were no significant differences between IVF and ICSI cycles with regard to the means of female and male ages; time of infertility; number of embryos transferred; or the rates of fertilization, clinical pregnancy, implantation, abortion, ongoing pregnancy, live birth delivery, and newborns. However, a significantly higher mean number of cumulus–oocyte complexes were retrieved in cases. There were no significant differences with respect to basal FSH and stimulation data. The rates of 1 PN zygotes were 4.9% and 5.9% for IVF and ICSI cycles, respectively (Tables 1 and 2).

Molecular Cytogenetics and Immunocytochemistry From IVF Cycles

Of the 1 PN zygotes, 16 of 18 exhibited a FISH labeling pattern corresponding to the presence of 2 PNs. One had no sperm in the ooplasm; in the other case, the sperm was present in the ooplasm but its head remained condensed. Of the 1 PN zygotes, 16 of 18 presented 2 PBs and 2 of 18 had 1 PB. The PBs were found after FISH in all cases. The PB1 was identified as the

Table 2. Embryological and Clinical Data From ICSI Cycles.

Parameters	ICSI-1 PN	ICSI-Control	*P*
Patients	47		
Cycles	49	3998	
Female age (mean, range)	33.8 ± 8 (20-43)	35.0 ± 4.5 (18-48)	.067
Male age (mean, range)	35.3 ± 3.9 (28-48)	36.8 ± 5.8 (20-66)	.086
TI (mean, range)	3.2 ± 2.7 (1-14)	4 ± 3.1 (1-23)	
COC/ETC (mean)	495 (10.1 ± 4.2)	30 189 (7.6 ± 4.8)	< .001
MII	425	24 629	
1 PN	64	1448 (5.9)	
2 PNs/MII (FR)	302 (71.1)	17 111 (69.5)	.482
N° ET/ETC (mean)	91 (1.9 ± 0.4)	7025 (1.9 ± 0.5)	.742
CP/ETC (rate)	18/48 (37.5)	1318/3655 (36.1)	.837
IR	23/91 (25.3)	1667/7025 (23.7)	.173
Ab/CP (rate)	4 (22.2)	213 (16.2)	.489
OP/ETC (rate)	14 (29.2)	1104 (30.2)	.876
Delivery/ETC (rate)	14 (29.2)	1098 (30.0)	.896
LBDLR/ETC (rate)	14 (29.2)	1098 (30.0)	.896
NB/ETC (rate)	16 (33.3)	1355 (37.1)	.594
Stimulation characteristics of ICSI cycles			
bFSH	7.6 ± 3.9	7.3 ± 4.0	.657
Total gonadotrophin dose	2119.6 ± 1366.9	2048.2 ± 1012.9	.651
Total time of stimulation	8.5 ± 1.9	8.5 ± 1.8	.940
Estradiol	1334.9 ± 624.7	1258.4 ± 891.9	.589
HCG	9833.3 ± 1962.3	9614.2 ± 1869.7	.523

Abbreviations: ICSI, intracytoplasmic sperm injection; 1 PN, zygotes with one pronucleus; TI, time of infertility; COC, cumulus-oocyte-complexes; MII, mature metaphase-II oocytes; 2 PNs, normal fertilized oocytes (with 2 pronuclei and 2 polar bodies); FR, fertilization rate; ET, embryo transfer; ETC, embryo transfer cycles; No. ET, number of transferred embryos; CP, clinical pregnancy; IR, implantation rate; Ab, abortion; OP, ongoing pregnancy; LBDLR, live birth delivery rate; NB, newborn; bFSH, basal follicle-stimulating hormone; HCG, human chorionic gonadotropin.

one presenting a diploid constitution (each chromosome with 2 chromatids), whereas PB2 exhibited a haploid constitution. While the chromosome number was equal in both PBs, we made no distinction. All zygotes were analyzed with FISH and immunocytochemistry (IF) (Table 3). In total, we studied 18 zygotes (15 patients), including 16 zygotes with 2 PBs (FISH: 11 zygotes, 10 patients; IF: 5 zygotes, 3 patients) and 2 zygotes with 1 PB (FISH: 1 zygote, 1 patient; IF: 1 zygote, 1 patient).

Among the 16 zygotes with 2 PBs, 1 had no sperm in the ooplasm and a single haploid PN, and another exhibited a condensed sperm head and a female metaphase plate. A second PN was observed in the remaining 14 zygotes. When both PNs exhibited an X chromosome, we could not distinguish which was the f-PN or m-PN, except in those cases where identification of the m-PN was based on its higher level of condensation. Of these 14 zygotes, 6 were haploid on both PNs (HH), 2 were diploid (DD), 4 had a diploid f-PN and a haploid m-PN (DH), 1 presented a haploid f-PN and a diploid m-PN (HD), and 1 had a diploid f-PN with no labeling found on the m-PN (D-). Regarding the 2 zygotes with 1 PB, both exhibited 2 PNs on FISH analysis. One was diploid on both PNs (DD), whereas the second zygote presented 2 haploid f-PNs and 1 haploid m-PN (HH-H; Table 3; Figure 2 A-F).

The finding of the 2 PNs in 1 PN zygotes suggested the occurrence of pPNBD. To confirm this mechanism, zygotes were evaluated by immunocytochemistry using a lamin antibody that labels an intact nuclear envelope and an antibody against Me(3)H3K9, which is specific for female chromatin. In 1 case, the labeling failed. In others, pPNBD was confirmed with 2 cases of female pPNBD and 3 cases of male pPNBD (Table 3, Figure 2G-I). There was a case that presented 2 haploid f-PNs and 1 haploid m-PN with male pPNBD. Regarding the 2 haploid f-PNs, immunocytochemistry confirmed the presence of female pPNBD in the second f-PN, which indicates that karyokinesis (PN division) occurred (Table 3, Figure 2I).

Molecular Cytogenetics and Immunocytochemistry From ICSI cycles

Of the 64 zygotes with 1 PN, FISH analysis revealed that 40 exhibited 2 PNs, 1 had no sperm in the ooplasm, and the sperm head remained condensed in 23. Moreover, 56 and 8 zygotes with 1 PN had 2 PBs and 1 PB, respectively. All zygotes were included in FISH and immunocytochemistry analyses (Table 4). Thus, we studied a total of 64 zygotes (47 patients), including 56 zygotes with 2 PBs (FISH: 42 zygotes, 31 patients; IF: 14 zygotes, 12 patients) and 8 zygotes with 1 PB (FISH: 6 zygotes, 2 patients; IF: 2 zygotes, 2 patients).

Among the 56 zygotes with 2 PBs, 1 had no sperm in the ooplasm and a single PN with no labeling, 20 presented a condensed sperm head and a haploid f-PN (with the exception of 3 cases where the f-PN had no labeling and 1 case where the

Table 3. FISH Analysis of IVF Zygotes With 1 PN/2 PBs (11) and 1 PN/1 PB (1).

		FISH		
1 PN/2 PBs	Zygote (n)	f-PN	m-PN	Result
	1	H	Sperm absent	AS
	1	MII	Sperm head	AD
	3	H	H	pPNBD
	1	D	D	f-ED/m-ED; pPNBD
	4	D	H (2 Y)	f-ED; pPNBD
	1	H	D (Y)	m-ED; pPNBD
1 PN/1 PB				
	1	D	D	NE/f-ED; m-ED; pPNBD

FISH and immunocytochemistry analysis of IVF zygotes with 1 PN/2 PBs (5) and 1 PN/1 PB (1)

		f-PN			m-PN			
1 PN/2 PBs	Zygote (n)	FISH	Lamin	H3	FISH	Lamin	H3	Result
	2	H	+	+	H (1Y)	−	−	(m) pPNBD
	1	H	−	+	H (Y)	+	−	(f) pPNBD
	1	D	−	+	No labeling	+	−	f-ED; (f) pPNBD
	1	D	−	−	D (Y)	−	−	f-ED/m-ED; pPNBD
1 PN/1 PB								
	1	H	+	+	H	−	−	NE/f-ED; (m) pPNBD
		H	−	+	−			Female karyokinesis

Abbreviations: FISH, fluorescence in situ hybridization; IVF, in vitro fertilization; 1 PN, zygotes with one pronucleus; 2 PBs, 2 polar bodies; 1 PB, one polar body; f-PN, female pronucleus; m-PN, male pronucleus; MII, metaphase II plate of the oocyte; H, haploid; D, diploid; AS, sperm head not found in the ooplasm; AD, presence of the sperm head without decondensation; pPNBD, premature pronucleus break down (male and female); f-ED, DNA endoduplication in the female pronucleus; m-ED, DNA endoduplication in the male pronucleus or diploid sperm; NE, nonextrusion of the second polar body; No labeling, presence of the male pronucleus with no FISH signals; Zygote 17, failure of the immunocytochemistry technique.

f-PN was diploid), and a second PN was observed in 35. Of these, 27 were haploid on both PNs (HH), 5 had a diploid f-PN and a haploid m-PN (DH), 1 presented a haploid f-PN and a diploid m-PN (HD), 1 had a haploid f-PN and an aneuploid m-PN (HA), and 1 had a haploid f-PN with no labeling found on the m-PN (H-). Regarding the 8 zygotes with 1 PB, there were 3 cases with a condensed sperm head and a f-PN (2 diploid and 1 chaotic) and 5 cases where 2 PNs were observed. Of these, 1 was haploid on both PNs (HH) and 4 had a diploid f-PN and a haploid m-PN (DH; Table 4). Immunocytochemistry data allowed us to confirm the presence of pPNBD in all cases, with 5 cases of female pPNBD and 5 cases of male pPNBD. In 1 case, the f-PN was labeled for H3 but lamin labeling failed (Table 4).

Discussion

The appearance of 2 PNs during normal fertilization occurs at a maximum of 20 hours after insemination or sperm injection. When asynchrony occurs, the maximal delay is 2 additional hours, which gives 22 hours in total for the appearance of both PNs. However, after this time, some zygotes exhibit only 1 PN. In the present work, the incidence of 1 PN zygotes was 4.9% in IVF and 5.9% in ICSI cycles, which is similar to previous reports (2.2%-17%). Also, as reported by other studies, comparisons to controls regarding demographic, stimulation, clinical, and embryological outcomes showed no significant differences.[1,6-12] However, we found a significantly higher mean number of cumulus–oocyte complexes in cycles with 1 PN zygotes, which is in accordance with a previous study.[13]

To explain the occurrence of a normal diploid content in the single PN observed with FISH, others have suggested that this could be due to PN asynchrony[1,7,9] or PN karyogamy.[14] We also processed single pronucleated zygotes after 24 hours and found FISH signals corresponding to 2 PNs in 16 of 18 IVF zygotes and 40 of 64 ICSI zygotes, and karyogamy cannot explain our findings. Regarding PN asynchrony, 1 PN zygotes were processed after 24 hours of insemination or sperm injection, which is the longest observation time in comparison to previous reports so this cannot explain our observations either. Thus, the present results suggest the occurrence of pPNBD, which was further confirmed by immunocytochemistry. We have no plausible explanation for this difference regarding all other previous studies, where authors used FISH and only found a single labeled area corresponding to the single PN previously observed. Our FISH analysis results revealed 2 distinct PNs in all cases of 1 PN zygotes (except those with absent sperm or a condensed sperm head in the ooplasm); therefore, our data suggest that 1 PN zygotes do in fact have pPNBD.

The nuclear envelope is supported by the nuclear lamina, which is a net of a bidimensional leaves of intermediate

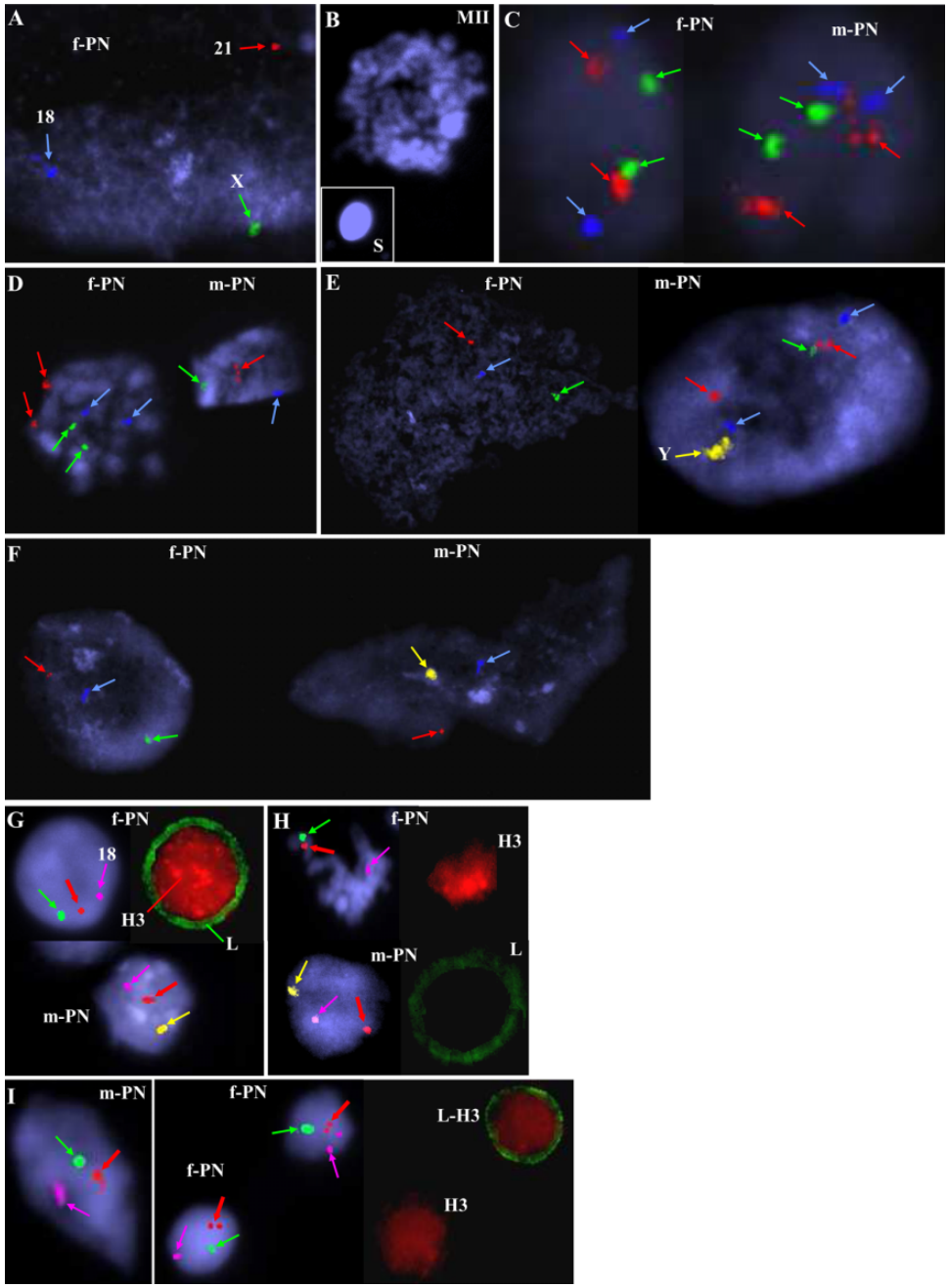

Figure 2. Images obtained by epifluorescence microscopy of human mature metaphase II oocytes with 1 pronucleus. A-I, Chromosomes 18 (blue; pink), 21 (red), X (green), and Y (yellow) were detected with fluorescence in situ hybridization (FISH). A, Patient with presence of a haploid female pronucleus (f-PN) with the absence of sperm in the ooplasm. B, Patient with presence of a metaphase II plate (MII) and a condensed sperm head (S). C, Patient with diploid pronuclei, f-PN and male pronucleus (m-PN). D, Patient with a diploid f-PN and a haploid m-PN. E, Patient with a haploid f-PN and a diploid m-PN. F, Patient with haploid pronuclei. G-I, FISH coupled with immunocytochemistry to detect the presence of the nuclear envelope using a pan-Lamin (L: green) antibody and the female chromatin using an antibody specific for histone trimethylated at lysine 9 (H3: red) to evaluate premature pronucleus breakdown (pPNBD). G, Patient with haploid pronuclei and m-PN pPNBD (presence of lamin staining in the f-PN with absence of lamin staining in the m-PN). H, Patient with haploid pronuclei and f-PN pPNBD (presence of lamin staining in the m-PN with absence of lamin staining in the f-PN). I, Patient with 1 haploid m-PN and 2 haploid f-PNs. In this patient, there is m-PN pPNBD (absence of lamin staining), whereas of the 2 f-PNs, one presented lamin and H3 staining and the other showed pPNBD (absence of lamin staining and presence of H3 staining). (The color version of this figure is available at rs.sagepub.com.)

Table 4. FISH Analysis of ICSI Zygotes With 1 PN/2 PBs (42) and 1 PN/1 PB (6).

		FISH		
1 PN/2 PBs	Zygote (n)	f-PN	m-PN	Result
	2	No labeling	Sperm head	AD
	14	H	Sperm head	AD
	22	H	H (8 Y)	pPNBD
	2	D	H (1 Y)	f-ED; pPNBD
	1	H	D	m-ED; pPNBD
	1	H	Aneuploid	pPNBD
1 PN/1 PB				
	1	D	Sperm head	NE/f-ED; AD
	1	Chaotic	Sperm head	NE/f-ED; AD
	4	D	H (1 Y)	NE/f-ED; pPNBD

FISH and immunocytochemistry analysis of ICSI zygotes with 1 PN/2 PBs (14) and 1 PN/1 PB (2)

		f-PN			m-PN			
1 PN/2 PBs	Zygote (n)	FISH	Lamin	H3	FISH	Lamin	H3	Result
	1	No labeling	−	+	Sperm absent	−	−	AS
	1	No labeling	−	−	Sperm head	−	−	AD
	1	H	+	+	Sperm head	−	−	AD
	1	H	−	−	Sperm head	−	−	AD
	1	D	+	−	Sperm head	−	−	f-ED; AD
	1	H	+	+	H (Y)	−	−	(m) pPNBD
	3	H	−	+	H (2 Y)	+	−	(f) pPNBD
	1	H	−	−	H	+	−	(f) pPNBD
	1	H	+	+	No labeling	−	−	(m) pPNBD
	2	D	+	+	H	−	−	f-ED; (m) pPNBD
	1	D	−	+	H	−	−	f-ED; pPNBD
1 PN/1 PBs								
	1	H	−	+	H (Y)	+	−	(f) pPNBD
	1	D	−	+	Sperm head	−	−	NE/f-ED; AD

Abbreviations: FISH, fluorescence in situ hybridization; ICSI, intracytoplasmic sperm injection; 1 PN, zygotes with one pronucleus; 2 PBs, 2 polar bodies; 1 PB, 1 polar body; f-PN, female pronucleus; m-PN, male pronucleus; No labeling, presence of the female or male pronucleus with no FISH signals; H, haploid; D, diploid; AD, presence of the sperm head without decondensation; pPNBD, premature pronuclear break down (male and female); f-ED, DNA endoduplication in the female pronucleus; m-ED, DNA endoduplication in the male pronucleus or diploid sperm; NE, nonextrusion of the PB2; AS, sperm head not found in the ooplasm; Zygotes 50, 52, failure of the immunocytochemistry technique (cases with an uncondensed sperm head). Zygote 62, failure of lamin labeling (diploid f-PN with a haploid m-PN).

filaments. This is a dynamic structure, as lamin phosphorylation causes pronuclear breakdown at mitosis and lamin dephosphorylation and enables nuclear envelope reformation at telophase. Seven lamin isoforms are encoded by 3 genes, *LMNA* (4 genes), *LMNB1*, and *LMNB2* (2 genes). Lamins C2 (encoded by *LMNA*) and B3 (encoded by *LNMB2*) are specifically expressed in the germ line.[29] Histones represent the main structural proteins of chromosomes and enable DNA compaction. Together with DNA, they form nucleosomes that are comprised of 4 nucleosome proteins, histones H2A, H2B, H3, and H4, as well as the linkage histone H1. In zygotes, only the female chromatin possesses Me(3)H3K9.[30] The presence of lamin and Me(3)H3K9 labeling identifies an intact f-PN and a male pPNBD, whereas the absence of lamin and presence of Me(3)H3K9 labeling in the f-PN associated with lamin-positive labeling in the m-PN identify a female pPNBD.

A previous study using FISH and an antibody against Me(3)H3K9 showed that, of the 2 PB IVF zygotes, about 86.7% presented a normal diploid PN (suggested to be due to karyogamy), whereas the PN only had a normal diploid content in 30.3% of ICSI cycles.[14] Our present results using combined FISH with antibodies against lamins and Me(3)H3K9 confirmed that pPNBD occurs with a similar rate between f-PN and m-PN in both IVF (2 female, 3 male) and ICSI cycles (5 female, 4 male). This premature disassembly of the nuclear envelope justifies the absence of observation of 2 PNs during the regular observation of single pronucleated zygotes. However, immunofluorescence also revealed that in 2 PB zygotes, only 3 of 5 (IVF) and 5 of 14 (ICSI) were normally diploid.

There was a unique case of a 1 PB zygote (IVF) with 1 male haploid PN and 2 haploid f-PNs, indicating female karyokinesis (PN division) after probable inclusion of the genetic material of the second PB. This was confirmed by immunocytochemistry.

The occurrence of zygotes with a single PN has been attributed to several mechanisms. In the case of female haploid 1 PB zygotes (without extrusion of the PB2), oocyte activation

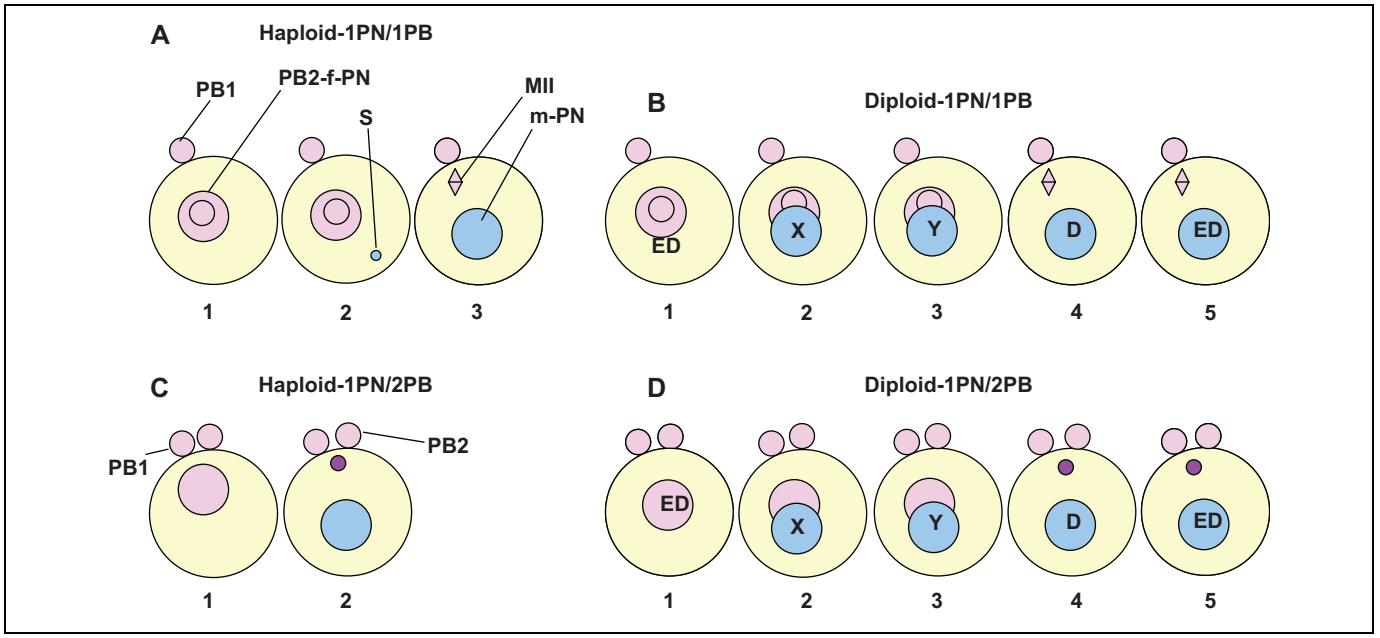

Figure 3. Scheme of the mechanisms exclusively related to cases where only one pronucleus (1 PN) was observed after fluorescence in situ hybridization (FISH). PB1 indicates first polar body; PB2, second polar body; PB2-f-PN, inclusion of the second polar body to originate one female haploid pronucleus (f-PN); S, condensed sperm head; MII, metaphase plate of the second female meiosis; m-PN, male pronucleus; ED, endoduplication; D, diploid sperm; X and Y chromosomes.

would be due to parthenogenic activation with formation of a f-PN due to inclusion of the genetic material of the PB2 (Figure 3A1 and A2). Parthenogenesis can be primary when there is absence of sperm in the ooplasm (IVF: absence of gamete membrane fusion or sperm extrusion from the ooplasm, ICSI: sperm extrusion from the ooplasm with activation as a consequence of cytoplasmic aspiration; Figure 3A1) or secondary with presence of sperm in the ooplasm and absence of sperm head decondensation (IVF: gamete membrane fusion triggers oocyte activation, ICSI: the sperm could have released the oocyte activation factor or activation was a consequence of cytoplasmic aspiration; Figure 3A2). If the PN is male, then the cell must have maintained the second meiotic metaphase plate, and failure of meiosis resumption could be due to an abnormal meiotic spindle (Figure 3A3).

In the case of female diploid 1 PB zygotes (without extrusion of the PB2; Figure 3B), our data suggest f-PN formation due to the inclusion of the genetic material of the PB2 followed by DNA endoduplication (Figure 3B1). In this case, parthenogenesis has occurred under the mechanisms described earlier. Alternatively, there has been a fusion with a m-PN (with an X or Y chromosome), thus indicating karyogamy with asynchronous PN development (Figure 3B2 and B3). The other possibility is that the oocyte has maintained the second meiotic metaphase plate, and the m-PN was due to fertilization by a diploid sperm (Figure 3B4) or the m-PN has had endoduplication (Figure 3B5).

In the case of female haploid 2 PB zygotes (with extrusion of the PB2; Figure 3C), the explanation is that parthenogenesis has occurred (Figure 3C1), whereas in the case of a m-PN, there was a female incompetence to develop a PN (Figure 3C2). In the case of female diploid 2 PB zygotes (with extrusion of the PB2; Figure 3D), it is likely that there was parthenogenic activation with endoduplication (Figure 3D1). Cases with a m-PN are suggestive of karyogamy with a sperm carrying a female (Figure 3D2) or male (Figure 3D3) chromosome. Alternatively, fertilization occurred with a diploid sperm (Figure 3D4) or the m-PN had endoduplication (Figure 3D5), both of which are associated with female incompetence to develop a PN.

The absence of sperm in the ooplasm was only observed in 2 cases of 2 PB zygotes. In IVF cycles, we considered absent fertilization, whereas we considered sperm extrusion in ICSI cases, both with parthenogenic activation, taking into account that this could be due to the aspiration procedure during microinjection in ICSI. These mechanisms have been described previously.[1,6-10,13,16] The presence of a condensed sperm head in the ooplasm was observed in 1 case of a 2 PB zygote from IVF cycles with presence of the metaphase II plate, indicating absence of f-PN formation, probably due to a defective meiotic spindle. In ICSI cycles, 20 of 56 zygotes with 2 PBs and 3 of 8 zygotes with 1 PB presented a condensed sperm in the ooplasm, which suggests that oocyte activation could either be due to a consequence of the ICSI procedure or release of the oocyte-activating factor from the sperm. These mechanisms have been previously described.[9,11,13,31] Regarding zygote diploidy, in 2 PB zygotes from IVF cycles, we found higher diplody in the f-PN (7 of 16) than the m-PN (3 of 16), with the same observed in ICSI cycles although with lower diploidy rates (f-PN: 5 of 56, m-PN: 1 of 56). In these cases, female diploidy can be explained by endoduplication, whereas male diploidy could result from endoduplication or fertilization by a diploid sperm. Regarding diploidy in 1 PB zygotes, our observations revealed diploidy in half of the IVF

(in both female and male PNs) and ICSI (only in the female PN) cycles. In these cases, female diploidy can be explained by nonextrusion of the PB2 with inclusion of the haploid genetic material followed by endoduplication. These mechanisms have also been described in other reports.[1,7,11]

Previous studies with 2 PB zygotes, where only 1 PN was found after FISH, suggested the potential use of 1 PN zygotes in IVF cycles due to the finding that the PN was normally diploid in 86.7% of the cases, possibly because of asynchronous PN development or karyogamy.[1,14] We observed different values in the present study. From the 18 zygotes of IVF cases, 16 had 2 PBs and 14 of these presented pPNBD; however, only 6 of 16 were haploid on both PNs. Similarly, in ICSI cycles, of the 64 zygotes evaluated, 56 had 2 PBs, and 35 of these presented pPNBD but only 27 of 56 were haploid on both PNs. Our results are nevertheless in line with other previous reports,[11,13,16] which suggested that single pronucleated zygotes should not be used in IVF or ICSI treatments.

In conclusion, we described two new mechanisms in single pronucleated zygotes: pPNBD and karyokinesis. Our findings indicate that these zygotes should not be used in IVF or ICSI treatments, as the rates of normal diploidy (2 haploid PNs) were 37.5% (6 of 16) in IVF and 48.2% (27 of 56) in ICSI cycles. Nevertheless, we advise the use of preimplantation genetic diagnosis in special clinical cases where only embryos derived from single pronucleated zygotes with 2 PBs are available for transfer.

Acknowledgments

We would like to acknowledge Jorge Beires, MD, PhD, Gynecologist, from the Department of Gynecology and Obstetrics, Director of the Unit of Gynecology and Reproductive Medicine, Hospital of S. João, E.P.E, Porto, Portugal, and José Manuel Teixeira da Silva, MD, Gynecologist for oocyte retrieval; José Correia, MD, Anesthesist (Department of Anesthesiology, Hospital of S. João, E.P.E, Porto, Portugal) for anesthesiology; José Teixeira da Silva, MD, Gynecologist, and Cristiano Oliveira, MD, Gynecologist, from the Centre for Reproductive Genetics Prof Alberto Barros, Porto (CGR-ABarros) for patient evaluation and treatment; Mariana Cunha, BSc, Biologist, and Paulo Viana, BSc, Biologist, for embryology laboratorial work; Cláudia Osório, BSc, Biologist, Ana Gonçalves, BSc, Biochemist, and Nuno Barros BSc, Microbiologist, from CGR-ABarros for spermiology laboratorial work; Sofia Dória, PhD, and Carolina Almeida, PhD, from the Departmeny of Genetics, FMUP for molecular cytogenetics support; and Elsa Oliveira and Ângela Alves from ICBAS for immunocytochemical technical support.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The UMIB is funded by National Funds through FCT - Foundation for Science and Technology under the «Fcomp-01-0124-FEDER-015893»project.

Funding

The author(s) received the following financial support for the research, authorship, and/or publication of this article. The UMIB is funded by National Funds through FCT - Foundation for Science and Technology under the «Fcomp-01-0124-FEDER-015893» project.

References

1. Staessen C, Janssenswillen C, Devroey P, Van Steirteghem AC. Cytogenetic and morphological observations of single pronucleated human oocytes after in-vitro fertilization. *Hum Reprod*. 1993;8(2):221-223.
2. Nagy ZP, Liu J, Joris H, Devroey P, Van Steirthghem A. Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum Reprod*. 1994;9(9):1743-1748.
3. Capmany G, Taylor A, Braude PR, Bolton VN. The timing of pronuclear formation, DNA synthesis and cleavage in the human 1-cell embryo. *Mol Hum Reprod*. 1996;2(5):299-306.
4. Payne D, Flaherty SP, Barry MF, Matthews CD. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Hum Reprod*. 1997;12(3):532-541.
5. Feenan K, Herbert M. Can 'abnormally' fertilized zygotes give rise to viable embryos? *Hum Fertil*. 2006;9(3):157-169.
6. Plachot M, Mandelbaum J, Junca AM, de Grouchy J, Salat-Baroux J, Cohen J. Cytogenetic analysis and developmental capacity of normal and abnormal embryos after IVF. *Hum Reprod*. 1989;4(suppl):99-103.
7. Munné S, Tang YX, Grifo J, Cohen J. Origin of single pronucleated human zygotes. *J Assist Reprod Genet*. 1993;10(4):276-279.
8. Flaherty SP, Payne D, Swann N, Matthews CD. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod*. 1995;10(10):2623-2629.
9. Sultan KM, Munné S, Palermo GD, Alikani M, Cohen J. Chromosomal status of uni-pronuclear human zygotes following in-vitro fertilization and intracytoplasmic sperm injection. *Hum Reprod*. 1995;10(1):132-136.
10. Macas E, Imthurn B, Roselli M, Keller PJ. Chromosome analysis of single- and multipronucleated human zygotes proceeded after the intracytoplasmic sperm injection procedure. *J Assist Reprod Genet*. 1996;13(4)345-350.
11. Staessen C, Van Steirteghem AC. The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in-vitro fertilization. *Hum Reprod*. 1997;12(2):321-327.
12. Munné S, Cohen J. Chromosome abnormalities in human embryos. *Hum Reprod Update*. 1998;4(6):842-855.
13. Balakier H, Squire J, Casper RF. Characterization of abnormal one pronuclear human oocytes by morphology, cytogenetics and in-situ hybridization. *Hum Reprod*. 1993;8(3):402-408.
14. Van Der Heijden GW, Van Den Berg IM, Baart EB, Derijck AAHA, Martinini E, De Boer P. Parental origin of chromatin in human monopronuclear zygotes revealed by asymmetric histone methylation patterns, differs between IVF and ICSI. *Mol Reprod Dev*. 2009;76(1):101-108.
15. Mateo S, Parriego M, Boada M, Vidal F, Coroleu B, Veiga A. In vitro development and chromosome constitution of embryos derived from monopronucleated zygotes after intracytoplasmic sperm injection. *Fertil Steril*. 2013;99(3):897-902.
16. Levron J, MunnéWilladsen S, Rosenwaks Z, Cohen J. Male and female genomes associated in a single pronucleus in human zygotes. *Biol Reprod*. 1995;52(3):653-657.

17. Gras L, Trouson A. Pregnancy and birth resulting from transfer of a blastocyst observed to have one pronucleus at the time of examination for fertilization. *Hum Reprod*. 1999;14(7):1869-1871.
18. Dasig D, Lyon J, Behr B, Milki AA. Monozygotic twin birth after the transfer of a cleavage stage embryo resulting from a single pronucleated oocyte. *J Assist Reprod Genet*. 2004;21(12): 427-429.
19. Pinto F, Oliveira C, Cardoso MF, Teixeira-da-Silva J, Silva J, Sousa M, Barros A. Impact of GnRH ovarian stimulation protocols on intracytoplasmic sperm injection outcomes. *Reprod Biol Endocrinol*. 2009;7(5):1-10.
20. Tesarik J, Sousa M. Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dislocation. *Fertil Steril*. 1995;64(4):770-776.
21. Vandervorst M, Liebaers I, Sermon K, et al. Successful preimplantation genetic diagnosis is related to the number of available cumulus-oocyte complexes. *Hum Reprod*. 1998;13(11):3169-3176.
22. Gardner DK, Lane M, Stevens J, Schlenker T, Schollcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril*. 2000;73(6): 1155-1158.
23. Tarkowski AK. An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics*. 1966;5(6):394-400.
24. Coonen E, Dumoulin JCM, Ramaekers FCS, Hopman AHN. Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence in-situ hybridization. *Hum Reprod*. 1994;9(3):533-537.
25. Harper JC, Coonen E, Ramaekers FCS, et al. Identification of the sex of human preimplantation embryos in two hours using an improved spreading method and fluorescent in-situ hybridization (FISH) using directly labelled probes. *Hum Reprod*. 1994;9(4):721-724.
26. Alves C, Sousa M, Silva J, Barros A. Preimplantation genetic diagnosis using FISH for carriers of Robertsonian translocations: the Portuguese experience. *Prenat Diagn*. 2002;22(12):1153-1162.
27. Santos F, Peters AH, Otte AP, Reik W, Dean W. Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. *Dev Biol*. 2005;280(1):225-236.
28. Baart EB, de Rooij DG, Keegan KS, de Boer O. Distribution of Atr protein in primary spermatocytes of a mouse chromosomal mutant: a comparison of preparation techniques. *Chromosoma*. 2000;109(1):139-147.
29. Goldman RD, Gruenbaum Y, Moir RD, Shumaker DK, Spann TP. Nuclear lamins: building blocks of nuclear architecture. *Genes Dev*. 2002;16(5):533-547.
30. Cowell IG, Aucott R, Mahadevaiah SK, et al. Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. *Chromosoma*. 2002;111(1):22-36.
31. Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. *Hum Reprod*. 1994;9(12): 2374-2380.