

Pronuclear Number does Not Fully Reflect Ploidy Number in Human Embryos

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ABSTRACT

Abnormal fertilization is defined by more or less than 2 pronuclei (2PN) in human zygote, especially presence of 3 pronuclei. In this study, the incidence of abnormal fertilization was about 5% and related with maternal age and insemination method. The pronuclear number usually assumed corresponding to the ploidy number of zygotes. This study intended to examine the validity of the assumption. All preimplantation diagnosis cases with Fluorescent In Situ Hybridization (FISH) during 1/1/2004 to 7/31/2009 were included. A total of 497 ICSI cases with 3735 2PN embryos and 189 embryos with either more or less than 2PN were included. The ploidy of embryos was reflected from biopsied blastomeres with probes for chromosomes 13, 15, 16, 18, 21, 22, X, and Y. For embryos from 2PN zygotes, 67.3% were diploidy. For non-2PN group, 40% were diploidy. Except for the 2PN group, the chance of the number of pronucleus matching with the number of ploidy was less than 50%. The results show that the number of pronucleus is not full correspondent to the number of ploidy of embryos. One set of chromosomes can form more than 1 pronucleus. And 1 pronucleus can contain more than 1 set of chromosomes.

Keywords

Abnormal fertilization, Embryos, *In vitro* fertilization, Pronucleus.

Introduction

In the *in vitro* fertilization field, normal fertilization is defined by presence of 2 pronuclei (2PN). The number of pronucleus different from 2 is considered abnormal fertilization. Traditional understanding of abnormal fertilization is oocyte self-activation, fertilization by more than 1 spermatozoon, and retaining second polar body. The observation of abnormal fertilization can be 0 pronucleus, 1 pronucleus, 3 or more pronuclei. Terms of diandry (fertilization by 2 spermatozoa) or digyny (fertilized with binucleate oocyte or retention of second polar body) have been used to describe the 3 pronuclei (3PN) fertilization. It is general assumed that 3PN corresponds to 3 sets of human chromosomes (triploidy) [1-4].

These terms and concepts seem to suggest that the number of pronucleus reflects the number of ploidy of zygote. With this concept in mind, aspirating out the extra pronucleus from 3PN

zygotes has been suggested as a way to correct the presumed triploidy status [1-6].

The counting of pronucleus and polar body should not be used to ascertain the origin of trippronuclear oocytes (digynic or diandric) [2,7]. Grossmann et al. [8] reported 36% of 3PN zygotes were diploid. Chen et al. [9] reported 25% of 3PN zygotes were diploid. These discrepancies are even further complicated by consideration of all abnormal number of pronuclei (less/more than 2PN). Apparently, digyny or diandry cannot explain all these phenomena. Along with maternal age with incidence of abnormal fertilization and insemination method, this study examines whether the number of pronucleus matches the number of ploidy in a large cohort of embryos.

Materials and Methods

This is a retrospective study. The data obtained from in house database without specific patients' information. The study was IRB exempted.

To examine the incidence of abnormal fertilization, all assisted reproductive technology (ART) cases during 1/1/1989 - 3/31/2010 is included. Totally 2316 conventional IVF cycles with 28921 eggs and 6433 ICSI cycles with 73273 eggs in this cohort study. For statistical analysis, Cochran-Mantel-Haenszel analysis is used to examine the trend of incidence of polypronuclei (PPN) along with maternal age. Fisher's Exact test (2-sided) used to compare the incidence of PPN by different insemination method at each age category.

For insemination, raw Semen was processed by swim up or density gradient centrifugation after egg retrieval. The initiation of insemination process was 3-5 hours after egg retrieval. For IVF, 4 well culture dish was used. Each well contained 10 cumulus masses or less in 600 ul insemination medium (HTF-10% human serum albumin). Each well inseminated with 30,000 motile sperm. The insemination volume was 10 ul or less and final concentration of spermatozoa was 50,000/ml. The spermatozoa and eggs remained together until denuding (about 18 hours after insemination) and checking for presence of pronucleus. For ICSI, the oocyte denuding process started 3 hours after egg retrieval. The denuding medium was with 200 iu hyaluronidase/ml and denude pipet was about 120micron inner diameter. Oocytes got ICSId about 30 minutes after denuding.

All ART cases with preimplantation genetic screening (PGS) by Fluorescent in situ hybridization (FISH) are included during 1/1/2004- 7/31/2009. A total of 497 ICSI cases were included in the study. The maternal age ranged from 22 to 52, with median age at 39 and mean age at 38. Only those embryos with more than 5 blastomeres had biopsy for FISH procedure. FISH procedure has reported previously [10]. In brief, blastomeres were exposed to 1 ul hypotonic solution (1% fetal bovine serum in 1% sodium citrate). As the blastomeres began drying, 15 ul of Carnoy's fixative (methanol: acetic acid 3:1) was placed on the slide. The fixative was allowed to slowly wash over the blastomeres without washing away the nuclei. DNA probes from Vysis (for chromosomes 13, 16, 18, 21, 22, X, and Y) and Cytocell (for chromosome 15) were used to examine the presence of these chromosomes.

A total of 3735 embryos with 2PN and 189 embryos (from 112 FISH cases) with an abnormal number of pronucleus were

included the study. For a specific chromosome, the number of FISH signals is defined as the number of that specific chromosome (NSC). The ploidy of an embryo is defined by the majority of NSC (more than 50% or at least 4 specific chromosomes have the same NSC), i.e. triploid is defined by at least 4 specific chromosomes with 3 chromosomes for each specific chromosome. For ploidy determination purpose, the number of chromosomes X and Y are summed as NSC. If no majority NSC found, the ploidy of the embryo is defined as chaotic. Euploid is defined as all examined chromosomes having 2 chromosomes. Aneuploid is defined by at least 4 specific chromosomes having 2 chromosomes but not all chromosomes. Either euploidy or aneuploidy is defined to be diploidy. The number of diploid embryos is the sum of the number of euploidy and aneuploidy.

Results

Table 1 shows the incidence of PPN significantly correlates with maternal age by both insemination method. The IVF group has significant level at $p < 0.05$ and $p < 0.0001$ for ICSI group. By the comparison of each age group, IVF group consistently shows significantly higher incidence of PPN than the ICSI group does except in the age group 40 and beyond ($p = 0.52$). The incidence of PPN by donor egg group also shows significant difference with IVF group gives.

Age group	<30	30-34	35-39	=>40	Donor egg	Total
IVF $P < 0.05$ along with maternal age*						
# cycle	213	717	718	430	238	2316
# egg	3230	9847	8189	4021	3634	28921
# fert.	2135	6567	5487	2588	2386	19163
# poly	167 (5.2%)*	472 (4.8%)	402 (4.9%)	222 (5.5%)	155 (4.2%)	1419 (4.9%)
ICSI $p < 0.0001$ along with maternal age						
# cycle	623	1829	2132	1230	619	6433
# mature egg	8601	22191	21827	9979	10675	73273
# fert.	6922	17886	17436	8040	8978	59262
# poly	318 (3.7%)	852 (3.8%)	955 (4.4%)	581 (5.8%)	373 (3.5%)	3079 (4.2%)
Analysis	$P < 0.0005^{**}$	$P < 0.0001$	$P < 0.05$	$P = 0.52$	$P < 0.05$	$P < 0.000001$

Ploidy status # pronucleus	No result	Hap ^a -	Eu-	Aneu-	Tri-	Tetra-	Penta-	Sextu-	Chaotic	Total #	Diploid rate
	2 (normal)	378	187	1190	1328	96	99	4	4	449	3735
0	8	5	13	7	1	2	0	0	5	41	49%
1	11	12	8	12	1	1	0	0	8	53	38%
3	13	4	11	22	21	2	0	0	7	80	41%
4	0	1	0	0	2	0	1	0	3	7	0%
5	0	0	0	0	1	0	0	0	0	1	0%
6	2	0	0	1	0	0	0	0	0	3	33%
9	0	0	0	2	0	0	0	0	2	4	50%
Total abnormal	34	22	32	44	26	5	1	0	25	189	40%

Table 2: The ploidy distribution of normal and abnormal fertilized populations.

Table 1: Comparison of polypronuclei between 2 insemination methods along with maternal age.

&: # poly/# egg x 100%; @: By Fisher Exact Test; *: By Cochran-Mantel-Haenszel analysis higher incidence ($p < 0.05$). The totally incidence of PPN is highly significant difference ($p < 0.000001$) by insemination method. Hap^a-.: Haploid; Eu-: Euploid; Aneu-: Aneuploid; Tri-: Triploid; Tetra-: Tetraploid; Penta-: Pentaploid; Sextu-: Sextuploid; nona-: Nonaploid.

Ploidy status	No result	Hap ^a -	Eu-	Aneu-	Tri-	Tetra-	Penta-	Sextu-	Nona-	Chaotic	Total
# blastomere	378	187	1190	1328	96	99	4	4	2	447	3735
% of total	10.1	5.0	31.8	35.5	2.6	2.6	0.1	0.1	0.05	12.0	100

Table 3: The distribution of ploidy among normal fertilization (2 pronuclei).
*: The abbreviation is the same as in Table 2.

no tetraploid. The euploidy rate and diploidy rate are 0% (0/7). For 5PN group, no pentaploid embryo is observed. The only 5PN embryo is triploid. For 6PN group, no sextuplet ploidy embryo is observed. The only embryo with any result is an aneuploid. For 9PN group, no nona-ploid embryo is observed. There are only 2 aneuploid and 2 chaotic embryos observed within the group.

	Euploid	Diploid	<> Diploid	No FISH results
2PN	1190/3735 (32%)	2518/3735 (67%)	879/3735 (24%)	378/3735 (10%)
<> 2PN	32/189 (17%)	76/189 (40%)	79/189 (42%)	34/189 (20%)
Analysis	P < 0.0001@	P < 0.0001	P < 0.0001	P < 0.001

Table 4: Comparison ploidy status of normal vs. abnormal fertilization.
@: By Fisher Exact Test.

Comparison of ploid status between 2PN and non-2PN groups is summarized in Table 4. The 2PN embryos have significantly more euploid and diploid embryos than the non-2PN embryos (Table 3). If considering only those embryos with FISH results, about only 50% of non-2PN group matches with non-diploid while the 2PN group has about 75% of embryos matches with diploid. At most scenario, about half of time the number of pronucleus corresponds to the number of ploid for non-2PN situation. For the 2PN group, the chance is about 75%. No one group shows a complete match of pronuclear number with ploid number.

Discussion

For Table 1, it must emphasize the difference of insemination method to interpret the data. For conventional IVF, it is all retrieved cumulus masses with oocytes inseminated with spermatozoa. It includes both mature and immature oocytes. The calculation of PPN includes those immature eggs in the denominator. It makes the PPN rate smaller than real incidence (due to same numerator with bigger denominator). For ICSI method, it is only the mature oocytes have ICSI. It is real incidence. Logically the significant difference between the 2 insemination methods will even more significant. The same concept can lead to more significant of trend of PPN along with maternal age in IVF group. Nerveless the data strongly indicates maternal age is a strong indicator for the incidence of PPN. It is known that aneuploidy rate increases along with maternal age [11]. The meiotic spindle apparatus also shows

As shown in Table 2, the abnormal fertilization includes embryos with 0, 1, 3, 4, 5, 6, and 9 pronucleus/pronuclei. For the 0PN group, 13 out of 41 (32%) are euploid, and there is a 49% ((13+7)/43) diploidy rate. For the 1PN group, there are 23% (12/53) haploid rate, 15% (8/53).

Euploidy rate, and 38% ((8+12)/53) diploidy rate. For 3PN group, the triploid rate is 26% (21/80). The euploidy rate is 14% (11/80), and diploidy rate is 41% ((11+22)/80). For 4PN group, there is

more abnormality, i.e. chromosome misalignment, microtubule irregularities, with old women [12]. All of these observations may suggest a relationship between incidence of PPN and integrity of egg genetic component, i.e. spindle apparatus. As for ICSI group, it is only 1spermatozoon injected into an egg. It is not convincing that diandry exists in ICSI group. It is more likely originates from egg, i.e. retention of second polar body or fragmented of egg spindle apparatus. For IVF group, in addition to retention of second polar body or dis-integration of egg chromosome organization (spindle apparatus), it is possible that diandry happens. With this consideration, it is understandable that IVF group has higher incidence of PPN.

The incidence of PPN (in this study) and aneuploidy rate show similar pattern that increasing along with maternal age [13]. By examination of the incidence rate, it is less than 10% by PPN criterion while more than 50% by aneuploid criterion. Apparently, the mechanism for these errors are not the same. In mammalian oocytes, there is an age-related declining in spindle assembly components expression [14]. In addition, the partial loss of sister chromatids cohesion and another cellular organelle's dysfunction may further deteriorate the aneuploidy rate. All evidence indicates maternal age is a significant factor correlates with egg quality. In Table 1, the only age group does not show significant difference is the group with age at least 40-year-old. It is understandable that age is the dominant factor for oocyte quality. At this age group the quality of oocytes is too bad to differentiate the difference of insemination procedure.

The real 0 ploid means the ooplasm only. It is not in the consideration of this study. The 49% 0PN embryos are diploidy. It is more likely that the verification window for presence of pronucleus was missed. By cytogenetic analysis, Feenan and Herbert [15] claimed some oocytes had normal cytogenetic constitution despite the absence of pronucleus. The data matches with our observation that 32% embryos are euploid in 0PN group.

For 1PN group, there is a 23% chance that the number of pronucleus matches with ploid. The diploid rate (38%) is more than the haploid rate (23%). It might suggest de-synchronization of pronucleus formation, or one pronucleus contains more than

one set of chromosomes. Levron et al. [16] reported that male and female genomes could be associated in a single pronucleus and transfer of a single pronucleus embryo resulted in delivery with a normal baby [17]. Cytogenetic analysis also confirmed a proportion of 1PN zygotes are diploidy [18]. These reports match the euploidy incidence of 1PN embryos in this study.

For 3PN group, 26% of embryos are triploid, while 41% are diploid. The diploid rate is more than the triploid rate. The logical consideration is that one whole set of chromosomes can split to form more than one pronucleus. Partial set of chromosomes can form a pronucleus. The 41% diploidy rate in this study is like the 36% diploid rate of 3PN zygotes from Grossmann et al. study [8]. Pang et al. [19] reported 7 out of 32 3PN zygotes were pure triploid (22%), which is similar to the 26% triploid rate in this study. Rosenbusch [2] described hypotriploid and hypertriploid chromosome counts for tripronuclear zygotes, which also suggested one pronucleus could contain less or more than one set of chromosomes. Although microsurgical enucleation of 3PN zygotes can achieve pregnancy with a normal baby [6], the risk is real, as only about a quarter of 3PN embryos are real triploid. The majority of 3PN embryos do not correspond with triploid. Microsurgical enucleation could have only a quarter chances to be correct. Majority of situation is to introduce errors by the microsurgical procedure. To correct 3PN zygotes by microsurgical removal of 1 pronucleus is a good intention [5,6], but it has a good chance of removing the genetic component of 3PN diploid embryos. The consideration gives warning of restoring diploidy by aspiration out of the supernumerary pronucleus and suggests a need to closely examine the chromosome constituent of blastomeres after micro-enucleation.

Since number of embryos for more than 3PN are small, it is more likely a qualitative observation of “number of pronucleus does not reflect the number of ploidy”. In this study, 4 embryos show 9 small pronuclei in their pronuclear stage. Matt et al. [20] observed 3 to 8 pronuclei. Bisioli et al [21] reported 3 to 9 pronuclei from patients with Reynaud’s syndrome. These observations indicate that high number of pronuclei do exist. By examining the 4 9PN embryos, it reveals that 2 are diploidy, and the other 2 are chaotic. None shows higher than pentaploid. The logical assumption is that one set of chromosomes can form more than one pronucleus. For the 6PN embryos, 2 have no FISH signals. The one with signal is diploidy, not sextuploidy. The only embryo with 5 pronuclei is triploidy, not petaploidy. All these observations support the hypothesis that the pronuclear number does not correspond to the ploidy number.

By examining chromosomes X, Y, 13, 15, 16, 17, 18, 21, and 22, Munne et al. [22] have reported that about 43% of embryos from donor eggs were euploidy. If the eggs were from fertility patients, the euploidy rate was about 34% for the young group (age 18 to 34) and 21% for the old group (age 40 to 45). In this study, the euploidy rate for 2PN embryos is 31%. It is similar to the large cohort data from Munne’s report [22]. By considering both euploid and aneuploid embryos as diploid, Munne et al. [22] showed

about 68% diploid rate for donor egg cases. For patients’ age less than 40, the diploidy rate was about 65%. These observations are also similar to the diploid rate of 67% from 2 PN embryos in this study. As predicted, the 2PN embryos have significantly higher incidence of euploid (31.8% vs. 16.9%), and diploid rates (67.4% vs. 40.2%) ($P < 0.001$, chi-square test) than the non-2PN embryos do (Table 4). The 2PN group is the only group where the number of pronucleus mostly reflects the number of ploidy. The observations also indicate that the “normal” fertilization does not 100% reflect the diploid rate. About 5% of the 2PN embryos are haploid, 5.5% are more than diploid, and 12% are chaotic (Table 3). These observations and the results from the non-2PN embryos all suggest that the number of pronucleus does not fully reflect the number of ploidy. The diploid embryos obtained from more than 2PN zygotes suggest 1 set of chromosomes can split and form more than 1 pronucleus. In addition to missing the pronucleus formation window, the diploid embryos from 1PN “zygotes” could suggest that one pronucleus could contain more than 1 whole set of chromosomes [16]. There are 2 other pieces of evidence that support the study conclusion. One is by Mitalipov et al. [23] regarding parthenogenetic observation and the other one is by Macas et al. [24] regarding chromosomal complements observation. The parthenogenetic activation of an egg by ionomycin with di-methyl-amino-purine (DMAP) is expected to result in diploidy only but 3 and smaller pronuclei are observed in about 10% of parthenotes [23]. It is indirect evidence that diploid zygotes can form more than 2 pronuclei. By examination of 3PN zygotes, Macas et al. [24] found diploid zygotes with 2 pronuclei had complementary chromosomes that became one complete set of chromosomes while the 3rd pronucleus had one complete set of chromosomes. They concluded, “a small group of chromosomes split off from the maternal genome and formed one additional supernumerary pronucleus”.

By using confocal microscope, Li et al. [25] reported that immature oocytes exhibited a significantly higher incidence of abnormality with spindle (43.7%) and chromosomal configuration (33.3%) while the in vivo mature oocytes had rates of 13.6% and 9.1% respectively. Strassburger et al. [26] reported that immature oocytes had a significantly higher incidence of multipronucleated fertilization. These observations indirectly suggest that the integrity of the spindle apparatus associates with the abnormal number of pronucleus.

The conventional IVF inseminated the oocytes with tens of thousands of spermatozoa. It always has some spermatozoa attached to the zona pellucida. In some incidence, we have observed spermatozoa swimming within perivitelline space. For PGD with FISH cases, the spermatozoa surrounding the embryo could introduce erroneous signals. In order to avoid any non-specific signals, majority of PGD cases use ICSI as insemination procedure in our program. The ICSI procedure excludes possibility of diandry. Although it cannot totally exclude out diploid spermatozoon used for ICSI, majority of the data more likely reflect the egg factor and technical limitation (possible disturbance of oocyte spindle apparatus during ICSI procedure).

There are 2 limitations in the interpretation of the observations in this study. One is the criteria of sampling for FISH: the sampling date and number of blastomere(s) in the embryo. The second limitation is the inherit mosaic incidence of embryos. By using probes for chromosomes X, Y, and 18, Staessen and Steirteghem [27] reported a 31.2% haploid rate for 1PN embryos and a 68% triploid rate for 3PN embryos. Their numbers looked higher than the numbers (22.6% haploid rate for 1PN and 26.2% triploid rate for 3PN) in this study. By using a cytogenetic approach to examine 3PN at the zygote stage, Macas et al. [24] reported 17 out of 37 (45.9%) zygotes were triploid, and 11 of these 37 (29.7%) were diploid. By comparing the data of 26.2% triploid rate and 41.2% diploid rate for 3PN embryos, it seems that the diploid rate is higher in this study. These discrepancies might be due to the stage of examination. The usage of day 1 to day 3 embryos with blastomeres range from 1 cell to 8 cells is a wide range. It is possible that embryos with severe chromosomal abnormalities may not progress through cell cleavage timely. The later stage of development and the greater number of blastomeres in embryos might suggest fewer faults in the chromosomal constitution. In this study, the criteria for biopsy are day 3 and at least 6-cell stage. It is the latest stage and greatest number of blastomeres in the embryos among these 3 studies. That might explain the higher diploid rate and lesser haploid or triploid rates in this study. As to mosaics, by re-examination of monosomy embryos at the blastocyst stage on days 5-7, we realized that 18 of 51 (35.2%) blastocysts were mosaic [11]. Coskum et al. [28] reported that the excess day-3 embryos (not transferred or cryopreserved) had a 28% (35 out of 125) mosaic rate. Baart et al. [29] reported that after 2 blastomeres FISH analysis, 50% (61 out of 121 examined day 3 embryos) of embryos showed mosaics. By considering the 35.2% mosaic rate of study population, the study might inherit a 35.2% variation rate of our biopsy sampling.

In conclusion, maternal age and insemination method significantly correlate with abnormal fertilization phenomena. Those 0PN, 1PN, 3PN, and more than 3PN embryos could be diploid. Diploidy is the majority for those abnormal fertilization embryos. Those embryos with 2PN had only about a 2/3 chance to be diploid. All these observations indicate that the number of pronucleus does not precisely reflect the ploid status of embryos. It supports the conclusion that "Routine inspection of the number of pronuclei is not an absolutely reliable tool for excluding the development of triploid embryos" by Rosenbusch [2]. It also indirectly suggests that one set of chromosomes can form more than one pronucleus, and one pronucleus can contain more/less than one complete set of chromosomes. This study raises the safety concern of restoring diploid by removal of one pronucleus from 3PN zygotes.

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