J Assist Reprod Genet (2011) 28:879–883
DOI 10.1007/s10815-011-9610-0

ASSISTED REPRODUCTION TECHNOLOGIES

# Prognostic value of triploid zygotes on intracytoplasmic sperm injection outcomes

**Rita C. S. Figueira · Amanda S. Setti · Daniela P. A. F. Braga · Assumpto Iaconelli Jr. · Edson Borges Jr.**

Received: 23 February 2011 / Accepted: 11 July 2011 / Published online: 30 July 2011


**Abstract**
*Purpose* To evaluate the prognostic significance of triploidy incidence on the outcomes of embryos derived from normally fertilized oocytes from the same cohort.
*Methods* This study included 1500 ICSI cycles. Logistic regression models were used to study the influence of abnormal fertilization on the development and clinical outcomes of embryos derived from normally fertilized oocytes from the same cohort
*Results* We observed a negative influence of the percentages of triploid zygotes on fertilization (75.2% and 56.8%, $P<0.0001$), high-quality embryos (58.9% and 48.2%, $P=0.0001$), pregnancy (34.1% and 28.2%, $P=0.0540$) and implantation rates (20.0% and 13.3%, $P=0.0012$). When the 3PN zygote rate was >25%, the percentages of normal fertilization, high-quality embryos and implantation rates were significantly lower than in the control group.
*Conclusions* We observed an approximately 50% lower risk of pregnancy and a 3.5-fold higher risk of miscarriage in cycles with a 3PN incidence of >25%.

**Keywords** Abnormal fertilization · ICSI · Triploidy · 3PN

*Capsule* The degree of triploidy serves as a representative marker of oocyte competence and is independent of ovarian stimulation protocols.

R. C. S. Figueira · D. P. A. F. Braga · A. Iaconelli Jr. · E. Borges Jr. (✉)
Fertility–Assisted Fertilization Center,
Av. Brigadeiro Luis Antônio, 4545,
Zip code: 01401-002 São Paulo, SP, Brazil
e-mail: edson@fertility.com.br

A. S. Setti · D. P. A. F. Braga · A. Iaconelli Jr. · E. Borges Jr.
Sapientiae Institute–Educational and Research Center in Assisted Reproduction,
São Paulo, SP, Brazil

## Introduction

Two pronuclei normally form after fertilization in humans, with one pronucleus deriving from the oocyte and one from the penetrating spermatozoon in anticipation of syngamy. However, in vitro fertilization (IVF) and embryo culture routinely enable variations of this outcome, which are also presumed to occur in vivo [1]. Triploidy is one of the most frequent chromosomal abnormalities affecting human gestation. Its prevalence among all pregnancies has been estimated to be 1% to 3% [2], whereas it accounts for 15%–18% [3] of cytogenetically abnormal cases among spontaneous abortions. On the cellular level, triploidy is characterized by the presence of three (3n) instead of two (2n) haploid chromosome complements. Two alternatives can be distinguished: the combination of one maternal and two paternal sets (diandric triploidy or diandry) or the combination of one paternal and two maternal sets (digynic triploidy or digyny) [4–6]. Diandry can arise from the fertilization of a haploid oocyte by two independent spermatozoa (dispermy) or from fertilization by a diploid spermatozoon. Digyny requires the fertilization of a diploid oocyte by a single haploid spermatozoon. The participating diploid gametes may result either from a failure during the first or second meiotic division or from the reduction of tetraploid precursor cells. Data from recent studies applying molecular markers demonstrated that digyny is more frequent than previously thought. Although the use of intracytoplasmic sperm injection (ICSI) essentially eliminates dispermic triploidy, it does not prevent oocyte-induced 3PN formation [7].

By the time of birth, the mammalian ovary contains a pool of quiescent primordial follicles, each consisting of a small inactive oocyte, arrested at prophase of the first meiotic division, and a single layer of granulosa cells [8–10]. The maturation and resumption of meiosis occur in response to the luteinizing hormone (LH) surge, which leads to ovulation and is accompanied by the extrusion of the first polar body that contains a single chromosome of each pair [11, 12]. The mature oocyte is arrested again at the second metaphase (MII). On fertilization, the oocyte is activated and the second meiotic division is resumed, followed by the extrusion of the second polar body (PB2) that contains a single chromatid of each chromosome [13]. A correct meiotic division of germ cells resulting in haploid gametes is a prerequisite for normal fertilization.

Irrespective of the cause, the question remains as to whether the degree of triploidy predicts the quality of the resultant embryo cohort. Because the ICSI outcome is highly dependent on oocyte quality, a high incidence of 3PN zygotes in ICSI cycles may signal an occult oocyte factor that can serve as a surrogate marker of oocyte competence and can be used as an independent predictor of the IVF cycle outcome. In the current study, we evaluated the prognostic significance of triploidy incidence on the outcomes of embryos derived from normally fertilized oocytes from the same cohort.

## Materials and methods

### Patients

This retrospective observational study included 1500 fresh-nondonor ICSI cycles performed at a private assisted fertilization center from January 2008 to March 2010. All female patients were younger than 36 years old to eliminate possible age-related cycle characteristics. To exclude a possible sperm effect on the rate of 3PN zygote production, all cases of sperm concentration less than $1\times10^6$ M/mL and sperm motility less than 20% were excluded from the study. All patients provided written informed consent, in which patients agreed to share the outcomes of their cycles for research purposes. The study was approved by the local Institutional Review Board.

### Ovarian stimulation

Controlled ovarian stimulation was achieved by long pituitary down regulation using a gonadotropin-releasing hormone agonist (GnRH agonist, Lupron Kit™, Abbott S.A Societé Française des Laboratoires, Paris, France), which was followed by ovarian stimulation with recombinant follicle-stimulating hormone (FSH) (Gonal-F®, Serono, Geneve, Switzerland). When adequate follicular growth was observed, (until a minimum of two follicles reached an average diameter of 18 mm) recombinant human chorionic gonadotropin (rhCG, Ovidrel™, Serono, Geneve, Switzerland) was administered to trigger final follicular maturation. Oocyte retrieval was performed 35 h after the administration of rhCG, via transvaginal ultrasonography

### Preparation of oocytes

After retrieval, oocytes were incubated in Human Tubal Fluid culture medium (HTF, Irvine Scientific, Santa Ana, USA) and covered with mineral oil (Ovoil™, Vitrolife, Kungsbacka, Sweden) at 37°C and 6% $CO_2$ for 3–4 h. Cumulus cells were removed with a 30-s exposure to HEPES-Buffered medium (HEPES-HTF, Irvine Scientific, Santa Ana, USA) containing 80 IU/mL of hyaluronidase (Irvine Scientific, Santa Ana, USA), after which coronal cells were manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA). Denuded oocytes were assessed for nuclear status. Oocytes having released the first polar body were considered mature (MII oocytes) and were used for ICSI.

### Intracytoplasmic sperm injection procedure

Intracytoplasmic sperm injection was performed in MII oocytes according to the technique described by Palermo et al. [14]. Oocytes were placed individually into 4-μl droplets of HEPES-HTF covered under warm mineral oil. Sperm were placed in a central 4-μL droplet of polyvinylpyrrolidone solution (PVP, Irvine Scientific, Santa Ana, USA), and the procedure was performed on the heated stage of an inverted microscope (Eclipse TE 300; Nikon®, Tokyo, Japan).

### *Assessment of fertilization, embryo quality and embryo transfer*

Fertilization was assessed approximately 16–18 h after ICSI. Normal fertilization was declared when two clearly distinct pronuclei were present and the extrusion of the second polar body was detected. Embryos developing from normally and abnormally fertilized oocytes were kept in a 50-μl drop of HTF medium supplemented with 10% Human Serum Albumin (HSA, Irvine Scientific, Santa Ana, USA) under oil and in a 6% $CO_2$ humidified atmosphere at 37°C until transfer.

Embryo transfer was performed on the third day of development. High-quality embryos were defined as having all of the following characteristics: 7 to 10 cells, less than

15% fragmentation, symmetric blastomeres, the absence of multinucleation, colorless cytoplasm with moderate granulation with no inclusions, the absence of perivitelline space granularity and the absence of zona pellucida dysmorphism. Embryos lacking any of the above characteristics were considered to be of low quality.

### Clinical follow-up

A pregnancy test was performed 12 days after embryo transfer, and a positive pregnancy test was considered as a biochemical pregnancy. All women with a positive test had a transvaginal ultrasound 2 weeks after the positive test. A clinical pregnancy was diagnosed when the fetal heartbeat was detected. To calculate the implantation rate, the number of gestational sacs was divided by the number of embryos transferred. Miscarriage was defined as a spontaneous abortion before 20 weeks of gestation. Multiple births from 1 pregnancy were counted as 1 live birth and multiple-birth rates as the percentage of live births that were multiple.

### Statistical analysis

The results are expressed as mean ± standard deviation for numeric variables, while proportions (%) are used for categorical variables. Mean values were compared using a *Student's t* parametric test or the Mann–Whitney non-parametric test, as appropriate. Associations between abnormal fertilization occurrence and clinical outcomes were examined using a Chi-squared cross-tabulation test or the Fisher exact test, as appropriate. To study the influence of abnormal fertilization on the development and clinical outcomes of embryos derived from normally fertilized oocytes from the same cohort, we used logistic regression models. For continuous variables, we performed linear regression analysis, and the results are expressed as regression coefficients (RC) and $p$ values. We used binary logistic regression analysis for dichotomic variables, and these results are expressed as odds ratios (OR), 95% confidence intervals (CI) and $p$ values. The results were considered to be significant at the 5% critical level ($p<0.05$). Data analyses were carried out using Minitab (version 14), a statistical analysis program.

## Results

A total of 1500 ICSI cycles and 9644 embryos developing from normally and abnormally fertilized collected oocytes were analyzed. For the entire study, 559 cycles were characterized by the presence of zygotes with 3PN, whereas no occurrence of zygote triploids was observed in 941 cycles. The distribution of cycle-specific parameters between groups is described in Table 1, and no significant differences were observed between the two groups.

On the other hand, the percentages of normal fertilization (75.2% and 56.8%, $P<0.0001$), high-quality embryos (58.9% and 48.2%, $P=0.0001$), pregnancy (34.1% and 28.2%, $P=0.0540$), implantation (20.0% and 13.3%, $P=0.0012$) and multiple-birth (21.8% and 13.5%, $P=0.0375$) rates decreased significantly with 3PN occurrence. The miscarriage (15.5% and 14.3%, $P=0.7906$) and live-birth rates (29.2% and 26.5%, $P=0.2527$) were similar between the two groups. Our data also demonstrated a negative influence of the percentage of 3PN zygotes on normal fertilization (RC: −19.078, $P<0.0001$), high-quality normally fertilized embryos (RC: −8.6, $P<0.0001$) and implantation rates (RC: −3.072, $P=0.048$).

To detect the minimum percentage of triploidy in which the ICSI outcomes are impaired, the cycles were split into groups according to 3PN zygotes rate: no 3PN zygotes detected (3PN=0%, $N=944$); 3PN=1–25% ($N=469$) and 3PN >25% ($N=90$). No significant differences were observed between the three groups in terms of patient demographics, stimulation and cycle characteristics (Table 2).

When the triploidy rate was ≤25%, we observed that the ICSI outcomes were not impaired. However, when the 3PN zygote rate was >25%, the normal fertilization,

**Table 1** Characteristics of patients and stimulation cycles

| Characteristics of the study groups | 3PN = 0% ($N=941$) | 3PN >0% ($N=559$) | $P$ value |
|---|---|---|---|
| Female's age (y) | 31.1±2.9 | 30.8±3.3 | 0.4871 |
| Male's age (y) | 41.6±5.3 | 42.5±6.2 | 0.5358 |
| Total gonadotrophin dose (IU) | 2125±602 | 2099±1247 | 0.1810 |
| No. of retrieved oocytes/ no. follicles (%) | 67.7 | 70.3 | 0.0919 |
| MII oocyte/total number of retrieved oocyte (%) | 50.1 | 52.4 | 0.0814 |
| Sperm concentration (M/mL) | 24.5±5.8 | 26.4±5.3 | 0.3654 |
| Sperm progressive motility (%) | 42.1 | 40.9 | 0.4865 |
| Embryos transferred | 2.6±0.9 | 2.7±0.9 | 0.0814 |

**Table 2** Characteristics of patients and stimulation cycles

| Characteristics of the study groups | 3PN = 0% (*N*=941) | 3PN = 1–25% (*N*=469) | 3PN >25% (*N*=90) | *P* value |
|---|---|---|---|---|
| Female's age (y) | 31.1±3.0 | 30.8±3.3 | 31.2±3.0 | 0.5910 |
| Male's age (y) | 41.6±5.3 | 42.1±4.1 | 42.8±3.5 | 0.2487 |
| Total gonadotrophin dose (IU) | 2125±602 | 2153±1309 | 1853±550 | 0.0665 |
| No. of retrieved oocytes/ no. follicles (%) | 67.7 | 71.5 | 62.5 | 0.0786 |
| MII oocyte/total number of retrieved oocyte (%) | 50.1 | 52.9 | 49.5 | 0.0803 |
| Sperm concentration (M/mL) | 24.5±5.8 | 27.2±3.4 | 23.1±2.8 | 0.0958 |
| Sperm progressive motility (%) | 42.1 | 44.1 | 38.9 | 0.1356 |
| Embryos transferred | 2.6±0.9 | 2.8±0.9 | 2.5±0.9 | 0.0625 |

high-quality embryos, pregnancy, implantation, miscarriage and live-birth rates were significantly lower than in the control group (Table 3).

Our data demonstrated a negative influence of the high percentage of 3PN zygotes (>25%) on normal fertilization (RC: −32.645, $P<0.0001$), high-quality normally fertilized embryos (RC: −13.166, $P=0.005$) and implantation rates (RC: −6.951, $P=0.043$). Our results also demonstrated that the high percentage of 3PN zygotes determines the likelihood of both the pregnancy (OR: 0.46, CI: 0.27–0.78, $P=0.004$) and miscarriage rates (OR: 3.51, CI: 1.25–9.83, $P=0.017$).

## Discussion

Triploidy formation resulting from standard IVF insemination could be due to polyspermy (diandric causes). In the present study, we minimize diandric causes of triploidy by including only those cycles where ICSI was performed. By injecting a single sperm into a single oocyte, the ICSI procedure negates the potential for dispermic triploidy. Because the presence of diploid sperm is increased in abnormal sperm samples, and evidence suggests that these sperm could contribute triploidy, we excluded couples in which the male had a sperm concentration less than $1\times10^6$ M/mL and sperm motility less than 20%.

Macas et al. [15], evaluated 3PN formation in cases from ICSI in which the male had severe sperm abnormalities; they found that 33% of the triploids were due to diploid sperm. In addition, other studies demonstrated that the presence of diploid sperm is increased in oligozoospermic samples suggesting that these sperm could contribute to the cause of triploidy [16–19]. In the present study, the absence of a severe male factor emphasizes that 3PN formation likely illustrates a dysfunctional oocyte or cytoplasmic incompetence, which resulted in the retention of the second polar body. Spandorfer et al. [20], observed a significantly greater frequency of digyny with advancing maternal age suggesting a compromised ability to extrude the second polar body in oocytes from older women. Therefore, to exclude possible age-related oocyte abnormalities, we included only female patients who were younger than 36 years old.

Studies have previously suggested that the mechanism of 3PN formation after ICSI is mostly of digynic origin. Digynic triploidy occurs secondary to the failed meiosis II expulsion of the second polar body and the subsequent fertilization of a diploid oocyte. This situation may occur with a damaged metaphase plate or oocyte cytoskeleton after abnormal spindle formation or increased female age [21]. Investigators have suggested that IVF stimulation-specific parameters, such as total gonadotropin dose, $E_2$ levels and increased oocyte yield, may induce the incidence of triploid zygotes [22]. Using multivariate analysis, Rosen

**Table 3** Clinical outcomes according to triploidy incidence

| Characteristics of the study groups | 3PN = 0% | 3PN = 1–25% | 3PN >25% | *P* value |
|---|---|---|---|---|
| Normal fertilization rate (%) | 78.7 | 65.3 | 41.6 | 0.0001 |
| High quality embryo rate (%) | 58.9 | 56.4 | 42.3 | 0.0500 |
| Pregnancy rate (%) | 38.3 | 36.8 | 24.4 | 0.0407 |
| Miscarriage rate (%) | 10.1 | 14.3 | 20.7 | 0.0318 |
| Implantation rate (%) | 24.0 | 19.7 | 16.2 | 0.0396 |
| Live-birth rate (%) | 29.2 | 29.2 | 12.2 | 0.0024 |
| Multiple-birth rate (%) | 21.8 | 13.9 | 9.1 | 0.1065 |

et al. identified factors in ovarian stimulation that could be predictors of digynic 3PN formation [7]. The authors found that the start dose, total amount of administered gonadotropins, and the number of days of stimulation were each independent predictors of 3PN formation. They also observed a parabolic relationship between the total days of stimulation and 3PN formation. Most recently, a retrospective cohort study determined that the presence of 3PNs was not associated with hyperresponse. Dayal et al. demonstrated that couples with ≤20% triploid zygotes had higher E2 levels at the time of hCG trigger and contained more oocytes [23]. By contrast, we did not observe any significant differences between the triploidy rate and cycle stimulation characteristics, indicating that the presence of 3PN was not associated with hyperresponse but may be a reflection of a compromised oocyte cohort. We did not find a marker for a cohort of oocytes that is more likely to retain the second polar body.

The high percentage of 3PN zygotes after ICSI may reflect a globally dysfunctional oocyte cohort. Our results demonstrated an inverse correlation between triploidy incidence and ICSI outcomes. Cycles with a high proportion of triploid zygotes displayed lower percentages of normal fertilization, high-quality embryos and implantation rates. In addition, we observed an approximately 50% lower chance of pregnancy and a 3.5-fold higher risk of miscarriage in cycles with a 3PN incidence of >25%. Rosen et al. and Dayla et al. observed that higher proportions of affected embryos led to worse clinical outcomes (Rosen et al., 2006 and Dayla et al., 2009). Elaborating on Rosen's findings, Dayal established whether the degree of 3PN after ICSI fertilization predicts the clinical outcome. Similar to our findings, they reported 20% as the lowest degree of triploid fertilization beyond which IVF clinical outcomes were adversely affected. However, they demonstrated lower pregnancy and live birth rates even though morphologically high-quality embryos were available for transfer. In our study, triploidy incidence seems to influence oocyte competence reflected by not only clinical outcomes but also laboratory parameters, such as embryo quality.

## Conclusions

In conclusion, our results suggest that the degree of triploidy serves as a representative marker of oocyte competence. We believe that although future decisions regarding ovarian stimulation should not minimize 3PN formation, triploidy rate should be considered a relevant parameter, in conjunction with well-established laboratory and clinical aspects, to determine the number of embryos to be transferred.

## References


1. Porter R et al. Estimation of second polar body retention rate after conventional insemination and intracytoplasmic sperm injection: in vitro observations from more than 5000 human oocytes. J Assist Reprod Genet. 2003;20(9):371–6.
2. Jacobs PA et al. The origin of human triploids. Ann Hum Genet. 1978;42(1):49–57.
3. McFadden DE, Robinson WP. Phenotype of triploid embryos. J Med Genet. 2006;43(7):609–12.
4. Edwards JH et al. Three cases of triploidy in man. Cytogenetics. 1967;6(2):81–104.
5. Niebuhr E. Triploidy in man. Cytogenetical and clinical aspects. Humangenetik. 1974;21(2):103–25.
6. Rosenbusch BE. Mechanisms giving rise to triploid zygotes during assisted reproduction. Fertil Steril. 2008;90(1):49–55.
7. Rosen MP et al. Triploidy formation after intracytoplasmic sperm injection may be a surrogate marker for implantation. Fertil Steril. 2006;85(2):384–90.
8. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev. 1996;17(2):121–55.
9. Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol. 1991;124:43–101.
10. Richards JS et al. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. Recent Prog Horm Res. 1995;50:223–54.
11. Jones KT. Mammalian egg activation: from Ca2+ spiking to cell cycle progression. Reproduction. 2005;130(6):813–23.
12. Fortune JE. Selection and maintenance of the dominant follicle: an introduction. Biol Reprod. 2001;65(3):637.
13. Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote. 1994;2(4):371–2.
14. Palermo G et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340 (8810):17–8.
15. Macas E, Imthurn B, Keller PJ. Increased incidence of numerical chromosome abnormalities in spermatozoa injected into human oocytes by ICSI. Hum Reprod. 2001;16(1):115–20.
16. Egozcue S et al. Diploid sperm and the origin of triploidy. Hum Reprod. 2002;17(1):5–7.
17. Egozcue S et al. Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. Hum Reprod Update. 2000;6(1):93–105.
18. Aran B et al. Screening for abnormalities of chromosomes X, Y, and 18 and for diploidy in spermatozoa from infertile men participating in an in vitro fertilization-intracytoplasmic sperm injection program. Fertil Steril. 1999;72(4):696–701.
19. Bernardini L et al. Comparison of gonosomal aneuploidy in spermatozoa of normal fertile men and those with severe male factor detected by in-situ hybridization. Mol Hum Reprod. 1997;3 (5):431–8.
20. Spandorfer SD et al. Effect of parental age on fertilization and pregnancy characteristics in couples treated by intracytoplasmic sperm injection. Hum Reprod. 1998;13(2):334–8.
21. Tsuchiya K et al. A cytogenetic study of in-vitro matured murine oocytes after ICSI by human sperm. Hum Reprod. 2002;17 (2):420–5.
22. Sachs AR et al. Factors associated with the formation of triploid zygotes after intracytoplasmic sperm injection. Fertil Steril. 2000;73(6):1109–14.
23. Dayal MB et al. Effects of triploidy after intracytoplasmic sperm injection on in vitro fertilization cycle outcome. Fertil Steril. 2009;91(1):101–5.