Estimation of Second Polar Body Retention Rate After Conventional Insemination and Intracytoplasmic Sperm Injection: In Vitro Observations From more than 5000 Human Oocytes

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Purpose: Trippronucleate (3pn) development after conventional insemination (CONV) or ICSI was analyzed to estimate the rate of second polar body retention giving rise to 3pn formation. **Methods:** Data from 453 consecutive IVF cycles were reviewed during a 6-month period. Mature oocytes were monitored in ICSI ($n = 3195$) and CONV ($n = 2274$) groups by fertilization assessment 16–18 h post-insemination. Ovulation induction protocols and in vitro culture conditions remained constant during the study interval.

Results: Normal (2pn) fertilization occurred in 74.2% and 70.5% for CONV and ICSI groups, respectively ($p < 0.003$). 1pn formation was observed in 4.5% of CONV oocytes, and 2.5% of ICSI oocytes ($p < 0.001$); 3pn formation was 8.1% in the CONV group, and 2.5% in the ICSI group ($p < 0.0001$). We observed 4pn formation in 0.4% of oocytes in the CONV group, but in only 0.04% of oocytes fertilized with ICSI ($p < 0.007$). Cellular degeneration occurred in 2.4% of oocytes inseminated conventionally, and in 3.5% of oocytes fertilized by ICSI ($p = 0.02$). Maternal age did not impact pronuclear status.

Conclusions: We found the 3pn formation rate after ICSI to be approximately one-third that observed in the CONV group. Extrapolating the ICSI data to the CONV data, it may be inferred that 2.5% of 3pn development after CONV was due to second polar body retention. This suggests that 5.6% of CONV oocytes showed dispermic fertilization. Decreasing oocyte quality with increasing maternal age had no apparent influence on any of the fertilization outcomes.

KEY WORDS: Fertility; fertilization; oocyte; reproductive techniques.

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INTRODUCTION

Only two pronuclei normally form after fertilization in the human, with one pronucleus deriving from the oocyte and one from the penetrating spermatozoon in anticipation of syngamy. However, in vitro fertilization (IVF) and culture routinely enables observation of variants of this outcome that might be presumed to occur in vivo also. As these abnormally fertilized oocytes can undergo early embryonic development

(and in some cases fetal development) comparable with normally fertilized embryos, it has become a central feature of IVF therapy to monitor pronuclear status of zygotes during culture. This is to reduce the possibility of including abnormally fertilized embryos among those chosen for fresh transfer or cryopreservation.

Monopronuclear (1pn) and tripronuclear (3pn) zygotes are the most common aberrations observed following either conventional insemination (CONV) or intracytoplasmic sperm injection (ICSI). While most 1pn zygotes from CONV are diploid and normally fertilized with asynchronous formation of the pronuclei, the reverse seems true for 1pn zygotes derived from ICSI (1). Further, 3pn zygotes from CONV arise from both dispermic penetration and second polar body retention, while by definition ICSI 3pn zygotes can only occur following second polar body retention (1).

This investigation sought to describe 3pn formation after insemination by CONV or ICSI techniques, and to estimate the rate of second polar body retention and 3pn formation postfertilization on the basis of observations from a large urban infertility referral center. Data were stratified according to maternal age to discern if 3pn formation increased with increasing maternal age, as has been previously reported for ICSI cycles (2). In our study, all abnormal fertilization events were recorded to characterize more fully the factors influencing polar body retention during IVF.

METHODS

Patients and Stimulation Protocol

This study drew upon data obtained from a retrospective chart review of 453 consecutive patient cycles (IVF and ICSI) data over a 6-month period, when ovarian stimulation and in vitro culture conditions remained unchanged. Maternal ages of patients in CONV and ICSI groups were 35.4 and 34.6 years, respectively ($p < 0.025$). All women underwent controlled ovarian hyperstimulation with luteal phase downregulation followed by gonadotropins, GnRH-agonist "flare" plus gonadotropins, or luteal phase pituitary suppression with discontinuation of GnRH-a at the time of gonadotropin administration. There was no significant difference among these stimulation protocols as a function of insemination technique.

Assessment of Oocyte Maturation

If clearly visible or denuded of cells, oocytes were classified according to the presence of first polar bodies/germinal vesicles. *Mature oocyte*: Metaphase II (M II)—first polar body present, no germinal vesicle, and granulosa cells are large and well-dispersed. *Immature oocyte*: Metaphase I (M I)—no first polar body, no germinal vesicle, and granulosa cells are small and tightly packed. *Immature oocyte*: Prophase I (P I)—Germinal vesicle present, and granulosa cells are smaller and very tightly packed.

We utilized conventional assessments of maturity based upon expansion and radiance of the cumulus–corona complex surrounding the oocytes, an approach which generally approximates the nuclear status of the oocyte. In brief, oocytes were classified as "mature" (metaphase II) when an expanded–luteinized cumulus matrix and radiant (i.e., sunburst pattern) corona radiata was present. Oocytes with less-expanded cumulus–corona complex were regarded as demonstrating an intermediate stage of maturity (metaphase I), while absence of the expanded cumulus was considered evidence of cellular immaturity (prophase I) (3). The appropriation of oocytes of each maturational category was similar for CONV and ICSI subgroups. We monitored 3739 oocytes (mature = 3195) in the CONV group and 3059 (mature = 2274) in the ICSI group. Fertilization was evaluated 16–18 h post-insemination for all zygotes using an inverted microscope fitted with interference contrast optics (IX-70, Olympus, Tokyo, Japan). Comparison between both CONV and ICSI groups was made for 0pn, 1pn, 2pn, 3pn, and 4pn status. Additionally, the rate of complete cellular degeneration after insemination was also registered. Outcomes in each group were calculated (mean value and percentage) relative to the number of mature oocytes at the time of insemination.

Statistics

Data followed a normal distribution for all study parameters. Mantel–Haenszel χ^2 test and two-tailed unequal Student's t test were used to compare observed data, as appropriate. Computer-assisted analysis was by Microsoft Excel 97 SR-1 (Redmond, Washington, USA). Data were collected retrospectively from patient records in a nonidentifiable manner, and did not influence therapy of couples who had already completed treatment. Although all patient consent forms were developed in accordance with institutional

Table I. Clinical and Laboratory Features Observed During Monitoring for Polar Body Development Among Human Oocytes After CONV and ICSI

	CONV	ICSI	<i>p</i> ^a
Maternal age, mean [IQR 25;75]	35.4 [33;38]	34.6 [32;37]	0.025 ^b
Total oocytes retrieved	3739	3058	NS
Oocytes retrieved/patient	15	15	NS
M II oocytes (%)	3195/3739 (85.5)	2274/3059 (74.3)	0.012
Pronuclear configuration (%)			
1pn	143/3195 (4.5)	56/2274 (2.5)	0.001
2pn	2370/3195 (74.2)	1604/2274 (70.5)	0.003
3pn	258/3195 (8.1)	56/2274 (2.5)	0.0001
4pn	13/3195 (0.4)	1/2274 (0.04)	0.007
Oocyte degeneration (%)	77/3195 (2.4)	78/2274 (3.5)	0.02

Note. CONV = Conventional insemination; ICSI = intracytoplasmic sperm injection; M II = metaphase II/mature; pn = pronucleus; IQR = interquartile range; NS = not significant, *p* > 0.05.

^a By χ^2 test, except as noted.

^b By Student's *t* test.

review board policies, as this passive observational study involved no patient recruitment or other “active interventions,” ethics board approval was not required.

RESULTS

In this study, an average of 15 oocytes was retrieved in each group (CONV and ICSI). We observed normal (2pn) fertilization in 74.2% of oocytes in the CONV group, and 70.5% of oocytes in the ICSI group (*p* < 0.003) as depicted in Table I. The formation of a single pronucleus (1pn) was evident in 4.5% of the CONV oocytes, and 2.5% of the ICSI oocytes (*p* < 0.001).

Additionally, 3pn formation was significantly higher in the CONV group than in the ICSI group (8.1% vs. 2.5%, respectively; *p* < 0.0001). In addition, we noted 4pn formation in 0.4% of oocytes inseminated in the CONV group, and in 0.04% of the ICSI oocytes (*p* < 0.007). Degeneration occurred in 2.4% of oocytes in the CONV group, and in 3.5% of the ICSI oocytes (*p* = 0.02). When classified by patient age, CONV and ICSI subgroups were as follows: CONV: ≤29 (*n* = 26), 30–34 (*n* = 78), 35–39 (*n* = 112), and >39 (*n* = 35). For ICSI patients the patient age distribution was: ≤29 (*n* = 22), 30–34 (*n* = 77), 35–39 (*n* = 88), and >39 (*n* = 17). No deviations from the above trends were observed in the data when patients were stratified by maternal age (Fig. 1).

DISCUSSION

The union of human gametes during normal fertilization is characterized by the development of two

pronuclei (2pn) at syngamy to restore diploidy. Development of monopronucleate (1pn) and trippronucleate (3pn) zygotes is considered exemplary of abnormal fertilization mechanisms, as documented by previous investigators (4–11). Studies of the frequencies at which such anomalies occur suggest that 3pn formation (after CONV) develops at a rate of 1.0–6.6% (8,9,12), and at a rate of 3.2% following ICSI (10).

These earlier reports suggest that 3pn formation may occur at a slightly higher rate among conventionally inseminated oocytes as compared to those inseminated by ICSI. Such a finding is not unexpected, given that two mechanisms of 3pn formation may be operant in the setting of CONV: 1) dispermic penetration, and 2) second polar body retention. For oocytes fertilized with ICSI, a 3pn outcome would be plausible only following second polar body retention, since dispermic fertilization is obviated by single sperm microinjection. Hypothetically, cytoskeletal injury could follow ICSI and any associated microarchitectural disruptions might increase the frequency of postfertilization aneuploidy. Indeed, researchers fluent with ICSI technique (13) have postulated that easier breakage of the oolemma during ICSI could be associated with higher incidence of 3pn formation, yet the exact mechanism(s) by which this may occur remain unclear.

If it may be assumed that 3pn development after ICSI results from the retention of the second polar body, then a baseline frequency of this phenomenon could be estimated regardless of the form of insemination. In our study population, the actual (observed) 3pn frequency after ICSI was 2.5%. In contrast, the frequency of 3pn formation in the CONV group was

Fig. 1. Fertilization outcomes as a function of maternal age (years) and insemination technique (CONV [upper panel, $n = 3195$] vs. ICSI [lower panel, $n = 2274$]) observed in 5469 mature human oocytes.

8.1%. From prior calculation, it may be estimated that 2.5% of this derived from second polar body retention, with the remaining 3pn zygotes (5.6%) developing as a result of dispermic fertilization.

A 3pn outcome following ICSI could also result from two other unusual scenarios. Should ICSI be performed on a diploid oocyte, then a 3pn embryo would be expected to develop. However, such diploid oocytes are typically much larger than normal haploid oocytes and therefore are easily avoided during in vitro screening and culture (14). Additionally, in the infrequently observed setting of “pronuclear fragmentation” (abnormal splitting or break up of a single pronucleus) a diploid embryo may appear to be

3pn even though the 2pn status prevails (unpublished data). Such a dysmorphic pronuclear configuration would generally be recognized as abnormal and result in nonselection of the affected embryo for transfer.

The propensity to form higher-order pronuclei after CONV compared to ICSI was also supported by the observed rate of 4pn development in our study population. Specifically, we noted the frequency of 4pn formation occurred at a rate 10 times greater among oocytes inseminated conventionally, as compared to those fertilized via ICSI. It is possible that a subpopulation of the 4pn zygotes in the CONV group were in fact dispermic, where the second polar body had also been retained. An explanation for 4pn formation

after ICSI is less intuitive. Yet, when proper technique is followed so that only one sperm is injected, then either inappropriate pronuclear subdivision could occur, or the initial haploid status of either gamete may be in question. For example, injection of a diploid sperm during ICSI can yield a 4pn zygote (15). It should be noted that such diploid spermatozoa are generally larger than normal (haploid) sperm, and can therefore easily be avoided during selection prior to ICSI.

Although previous investigators found pronuclear configuration following ICSI to be influenced by oocyte quality and/or maternal age (2), this relationship was not evident from our study population. In the present investigation, mean maternal age was found to be higher among CONV patients ($p = 0.02$), although this statistic may be misleading. The actual clinical significance of an age difference of less than 1 year is difficult to ascertain, and we do not regard the higher mean age among patients undergoing CONV to be responsible for the higher observed rates of fertilization abnormalities noted in this subgroup.

The 1pn formation rate was lower in our ICSI group compared to the CONV group. In this circumstance oocyte activation might give rise to 1pn formation more frequently following the ICSI procedure itself. However, up to 80% of accurately recorded 1pn zygotes are in fact diploid and are normally fertilized embryos following CONV (5). Such embryos have been considered as suitable for transfer when no other embryos are available (15,16). Consequently this may account for a higher rate of 1pn formation in the CONV group (4.5% vs. 2.5%), and therefore it may not be accurate to infer that 2.5% of CONV 1pn zygotes were haploid activated oocytes (with the additional 2.0% of 1pn zygotes being diploid).

It should be noted that oocyte maturity was 85.5% (mean = 12.8) in the CONV group and 74.3% (mean = 11.2) in the ICSI group ($p < 0.012$), but this maturational difference may have been due to enhanced laboratory monitoring following cumulus-coronal stripping with hyaluronidase specifically for oocytes to be inseminated by ICSI. Such enzymatic processing of oocytes destined to undergo ICSI might also be a variable influencing second polar body retention in this subpopulation. Maturational status of the oocyte upon fertilization is yet another factor associated with polar body retention, such that triploid zygotes may develop more frequently after insemination of metaphase I oocytes. Interestingly, while oocyte maturity was significantly higher among conventionally inseminated oocytes in this study, it was

within this same subgroup that a higher polar body retention rate was noted.

In summary, our observations enable an approximation of the rate of second polar body retention occurring following fertilization of the human oocyte. In this population of more than 5000 oocytes, approximately 31% (80/258) of 3pn zygotes arising from CONV were digynic in origin, indicative of second polar body retention as the source of the second pronucleus. While our results are in general agreement with previous studies that found bipolar regular first mitotic division in 33.8% of 3pn zygotes developing after CONV (1), additional observational studies will be useful to characterize this phenomenon more precisely.

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