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ASSISTED REPRODUCTION TECHNOLOGIES

# Live births resulting from 0PN-derived embryos in conventional IVF cycles

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**Abstract**
*Purpose* The aim of this study was to (1) investigate the incidence of embryos derived from "unfertilized oocytes" i.e., oocytes not displaying pronuclei (0PN) at the time of the fertilization check and (2) determine the clinical pregnancy rates when transferring 0PN-derived embryos.
*Methods* In this retrospective study, 4424 IVF-ET cycles were reviewed.
*Results* In total, 11.3 % (4966/43,949) 0PN-derived embryos were observed. It was found that female age, number of oocytes, and the top-quality embryo rate were significantly correlated with 0PN-derived embryo occurrence. The source of embryos transferred did not impact significantly on clinical pregnancy and live-birth rates. Of the 183 cycles included in this study where 275 0PN-derived embryos were transferred in total, only 0PN-derived embryos were available in 70 of those cycles. It was noteworthy that 13 healthy infants resulted from 0PN-derived embryos with an implantation rate of 17.0 %.
*Conclusion* These results indicate that the traditional method of excluding embryos because of those oocytes originally lacking any sign of a pronucleus at the fertilization check should be reconsidered as transferring 0PN-derived embryos with subsequent expected developmental performance may be considered as an option for those patients where no other embryos are available.

*Capsule* Zygotes not demonstrating PN at fertilization check are capable of generating term pregnancies.

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In in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment cycles, a fertilization check is performed at 17–20 h post insemination to exclude oocytes that have fertilized abnormally or not fertilized at all. Normal fertilization of an oocyte is defined by observing two distinct pronuclei (2PN) and two polar bodies after insemination. Oocytes showing no pronucleus (0PN), one pronucleus (1PN), or three pronuclei (3PN) (or more) are deemed as having fertilized abnormally and may be discarded. However, this assumption may not always be accurate. On the one hand, cytogenetic analysis has revealed that a proportion of diploid embryos can develop from 1PN zygotes [1]. Healthy babies developing from such embryos have been reported [2]. On the other hand, 2PN zygotes are not necessarily euploid. Furthermore, there are reports of 0PN-derived embryos present in 12 to 20 % of day 3 embryos [3–5] which result in healthy babies [4, 6, 7]. Although to transfer such embryos in the absence of the signs of normal fertilization may have become an option in clinical policy, it is still a dilemma for the clinical team when it comes to carrying this out in practice.

In this study, fertilization results from conventional IVF were reviewed to (1) investigate the incidence of embryos derived from 0PN oocytes and (2) determine the clinical pregnancy rates (CPR) when transferring such 0PN embryos.

## Materials and methods

### Patients

From Jan 2007 to Jun 2014, all embryo transfers from conventional IVF treatment were included in this study.

### Ovarian stimulation

All patients used the standard long or short stimulation protocols by using GnRH analog (Diphreline, IPSEN) and gonadotropins (HMG, Li Zhu China and GONAL-F, Merck Serono, Swiss) for controlled ovarian hyperstimulation [8]. Human chorionic gonodotrophin (hCG, Lizhu China) at a dose of 6500 IU ~ 10,000 IU was administrated after at least two follicles of 20 mm or greater in size were visualized by means of transvaginal ultrasound scanning. Oocyte retrieval was performed 34 ~ 36 h later by transvaginal ultrasonography (TVS)-guided aspiration. Embryos were cultured in the Vitrolife series of culture medium drops covered with mineral oil in Falcon tissue culture dishes (353001 Becton Dickinson, Franklin Lakes, USA). In detail, 4 h after follicle retrieval, oocytes were inseminated (conventional IVF) and cultured in G-IVF medium (0.1 ml) (Vitrolife, Sweden) at 37 °C in humidified atmosphere of 6 % $CO_2$. Insemination was performed after hCG injection 40–42 h later. Oocytes were assessed at 16–20 h after insemination for evidence of pronuclei and then continuously cultured in G-1 medium (0.1 ml) (Vitrolife, Sweden). The number of pronuclei observed was recorded as 1, 2, 3 etc. Mature oocytes with no sign of pronuclei were documented as 0PN. Polar body (PB) status was documented as 1PB, 2PBs, or fragmented PB.

Embryos with more than 50 % intact blastomeres were considered viable. Each embryo was observed daily and assessed by means of the following scoring system: grade I—number of cells ≥4 on day 2, or number of cells ≥6 on day 3 with even sized blastomeres, regular morphology, integrated zona pellucida, clear cytoplasm, no particles and no indication of multinucleation, no debris, and embryo fragmentation <5 %; grade II—number of cells ≥4 on day 2, or number of cells ≥6 on day 3 with slightly uneven sized blastomeres, slightly irregular morphology, fragmentation between 6 and 20 %; grade III—significantly uneven sized blastomeres, significantly irregular morphology and fragmentation between 21 and 50 %; grade IV—abnormal rate of embryo development, severely unequal sized blastomeres, significant cytoplasmic particles, a high quantity of vacuoles and fragmentation >50 %. Grade I and grade II were defined as top-quality embryos; grade I, grade II, and grade III were deemed suitable embryos for transfer.

### Embryo transfer

One or 2 embryos were selected for transfer according to the embryo potential as determined by its score on day 3. Embryos with a quality better than grade IV derived from 0PN oocytes were selected for transfer when there were none or only one 2PN embryo available for transfer. Multiple embryos were transferred in situations where patients had only one 2PN embryo available for transfer. Embryos were transferred with the aid of ultrasound guidance, and luteal-phase support was prescribed by administrating progesterone and estradiol valerate daily for at least 2 weeks following embryo transfer. Serum hCG concentrations were measured 14 days after embryo transfer.

A clinical pregnancy was confirmed by the detection of a sac(s) by ultrasound at 6 weeks after embryo transfer. If the pregnancy test proved positive, patients were tracked with a series of ultrasound scans to determine fetal viability. A prescription of estradiol valerate (6 mg/day) and progesterone (60 mg/day) were continued until 10 weeks gestation.

### Follow-up

All pregnant women were followed up until 1 to 2 month(s) after parturition. All of the infants delivered were evaluated for any signs of complication.

### Statistical analysis

Statistical analysis was performed using the SPSS17.0 statistics software. The chi-square test was used for analysis of differences in the case of percentage comparison. Binary logistic was used for analysis of occurrence of 0PN embryos, clinical pregnancy, and live birth. Significant difference was defined as $p < 0.05$.

## Results

In the study period from 2007 to 2014, 4424 IVF-ET cycles were reviewed. It was noted that 11.3 % (4966/43,949) of embryos observed were scored as 0PN. Clinical features of cycles with and without the incidence of 0PN-derived embryos are summarized in Table 1. Cycles where 0PN-derived embryos did occur had significantly lower female age, FSH level, and higher number of eggs retrieved. Although a significantly lower 2PN rate was observed in the cycles where 0PN-derived embryos were present, the top-quality embryo rate was significantly higher than those cycles that lacked 0PN-derived embryos. A binary logistic analysis was then performed looking at the relationship of the occurrence of 0PN-derived embryos and the covariates. It was found that female age, number of oocytes, and the top-quality embryo rate were significantly correlated with 0PN-derived embryo (Table 2, $B = -0.29$, Wald = 88.92, $P = 0.00$).

Two hundred and seventy-five embryos that developed from 0PN oocytes with embryo grade III or better were transferred in 183 cycles. Of these 183 treatment cycles, 70 cycles had embryos transferred where 0PN-derived embryos only were available. Binary logistic analysis was performed to analyze the impact of cycle characters and the transferring of 0PN-derived embryos on CPR (Table 3) and live-birth rates

**Table 1** Clinical features in cycles with and without the incidence of 0PN embryos

| Characteristic | Patients with 0PN | Patients without 0PN | $\chi^2$/t | *P* value |
|---|---|---|---|---|
| Number of cycles | 1899 | 2525 | | |
| Age (years) | 31.44 ± 5.35 | 32.52 ± 5.77 | 6.39 | 0.00 |
| Basal FSH (IU/L) | 6.87 ± 2.79 | 7.39 ± 3.80 | 5.34 | 0.00 |
| BMI | 22.59 ± 2.86 | 22.54 ± 2.96 | −0.48 | 0.63 |
| Cause of infertility | | | | |
| Male factor | 122 | 185 | 1.37 | 0.26 |
| Ovulation factor | 102 | 129 | 0.15 | 0.70 |
| Tubal factor | 997 | 1326 | 0.00 | 0.99 |
| Unexplained infertility | 85 | 107 | 0.15 | 0.70 |
| Combined factor | 589 | 774 | 0.07 | 0.80 |
| Other | 4 | 4 | 0.16 | 0.69 |
| Primary infertility | 831 | 1079 | 0.47 | 0.50 |
| Secondary infertility | 1068 | 1446 | 0.47 | 0.50 |
| No. of oocytes/cycle | 11.38 ± 6.52 | 8.84 ± 6.27 | 13.15 | 0.00 |
| Rate of 2pn (%) | 57.3 (12402/21628) | 70.0 (15621/22321) | 759.67 | 0.00 |
| Rate of top-quality embryos (%) | 55.7 (6905/12402) | 50.4 (7876/15621) | 76.66 | 0.00 |
| No. of embryo transferred | 2.05 ± 0.42 | 2.01 ± 0.46 | 2.84 | 0.01 |
| Clinical pregnancy rate (%) | 45.7 (558/1222) | 45.8 (728/1591) | 0.00 | 0.96 |
| Miscarriage rate (%) | 8.8 (108/1222) | 10.9 (174/1591) | 3.37 | 0.07 |
| Live-birth rate (%) | 36.7 (449/1222) | 34.7 (552/1591) | 1.27 | 0.26 |

(LBR, Table 4). Adjusted by female age, BMI, the top-quality embryo rate and number of embryos transferred, the source of embryos transferred, i.e., 0PN-derived embryos or 2PN embryos, did not have a significant impact on CPR and LBR.

The CPR of the cycles where 0PN-derived embryos were exclusively transferred was 24.3 % (17/70). The implantation rate was 17.0 % (19/112) with 1 ectopic pregnancy, 2 miscarriages, and 1 stillbirth observed. Of the 13 healthy infants born from 13 cycles, there were no reports of deformities.

## Discussion

Embryos with two pronuclei are preferentially selected for embryo transfer in IVF treatments as these are assumed to be the only normally fertilized oocytes. However, some oocytes may display no pronucleus or only one at the time of the fertilization check, but they continue to cleave and form morphologically normal looking embryos [9]. In some IVF centers, it has become a routine clinical policy to transfer such embryos in instances where patients do not have other viable embryos. In such circumstances, a decision is made based more on logical deduction rather than scientific evidence.

It has been reported that the percentage of embryos from 0PN oocytes may vary from 12.7 to 20 % of day 3 embryos [6]. In this study, 11.3 % 0PN-derived embryos were observed with an average age of 32.8 comparable with our general patient population, suggesting that aging is not a significant contributory factor to the occurrence of 0PN-derived embryos. This finding is consistent with previous reports [6].

The rationale in allowing the transfer of 0PN-derived embryos is that 0PN oocytes might be bipronucleated and develop into euploid embryos, but due to differences in cell cycle timing, pronuclei were not visible at the time of the fertilization check [6]. In this study, 0PN-derived embryos were more likely to be observed in good prognosis patients, i.e., younger

**Table 2** Binary logistic analysis of factor related to the occurrence of 0PN embryos

| Variable | *B* | Wald | OR (95 % CI) | *P* value |
|---|---|---|---|---|
| Age | −0.02 | 5.821 | 0.99 (0.97, 0.99) | 0.02 |
| Basal FSH | −0.02 | 2.43 | 0.98 (0.96, 1.00) | 0.12 |
| No. of oocytes/cycle | 0.08 | 160.22 | 1.08 (1.07, 1.09) | 0.00 |
| Rate of 2pn | −2.90 | 354.94 | 0.06 (0.04, 0.07) | 0.00 |
| Rate of top-quality embryos | 0.99 | 42.16 | 2.69 (2.00, 3.63) | 0.00 |
| Constant | 1.01 | 16.29 | 2.74 | 0.00 |

**Table 3** Binary logistic analysis of factor related to clinical pregnancy per cycle

| Variable | Clinical pregnancy | | *B* | Wald | OR (95 % CI) | *P* value |
|---|---|---|---|---|---|---|
| | YES | NO | | | | |
| Age | 30.61 ± 4.68 | 32.09 ± 5.38 | −0.35 | 27.42 | 0.71 (0.62, 0.81) | 0.00 |
| BMI | 22.47 ± 2.93 | 22.88 ± 2.99 | −0.04 | 9.46 | 0.96 (0.94, 0.99) | 0.00 |
| Basal FSH | 6.94 ± 2.48 | 7.14 ± 2.98 | −0.01 | 0.29 | 0.99 (0.96, 1.02) | 0.59 |
| No. of oocytes/cycle | 9.89 ± 4.78 | 9.15 ± 5.02 | −0.00 | 0.02 | 1.00 (0.98, 1.02) | 0.90 |
| Rate of 2pn (%) | 66.6 (8438/12671) | 62.4 (8683/13922) | 0.53 | 8.16 | 1.69 (1.18, 2.43) | 0.00 |
| Rate of top-quality embryos (%) | 54.3 (4586/8438) | 53.9 (4516/8386) | 0.21 | 2.89 | 1.23 (0.97, 1.56) | 0.09 |
| No. of embryo transferred | | | | | | |
| ET2 | 1100 | 1146 | | 37.16 | 1.00 | 0.00 |
| ET1 | 46 | 191 | −1.12 | 27.00 | 0.33 (0.21, 0.50) | 0.00 |
| ET3 | 135 | 184 | −0.02 | 0.03 | 0.98 (0.75, 1.28) | 0.87 |
| The source of embryos transferred | | | | | | |
| 2PN ET | 1217 | 1411 | | 0.10 | 1.00 | 0.95 |
| 0PN + 2PN ET | 48 | 65 | 0.10 | 0.10 | 1.11 (0.58, 2.12) | 0.75 |
| 0PN ET | 17 | 53 | 0.08 | 0.05 | 1.09 (0.53, 2.23) | 0.82 |
| Constant | | | 0.82 | 2.67 | 2.27 | 0.10 |

patients with a good ovarian stimulation response. And cycles where 0PN-derived embryos occurred were associated with having a higher percentage of top-quality embryos. Based on these observations, we hypothesize that the appearance of good quality 0PN-derived embryos may be a sign of accelerated biological pace, i.e., the pronuclei were present but disappeared before the fertilization check, rather than development slowing down. A time-lapse study showed that pronuclei could disappear before 15 h after insemination [10]. Early pronuclear breakdown is considered a good indicator of embryo viability [11], which is consistent with the finding of this study that a higher percentage of top-quality embryos were observed in the cycles where 0PN-derived embryos were present (Table 1). Furthermore, the logistic regression analysis revealed that 0PN-derived embryos are more prone to appear in the patients with younger female age, higher number of oocytes, and higher percentage of good quality embryos (Table 2).

**Table 4** Binary logistic analysis of factor related to live birth per cycle

| Variable | Live birth | | *B* | Wald | OR (95 % CI) | *P* value |
|---|---|---|---|---|---|---|
| | YES | NO | | | | |
| Age | 30.38 ± 4.50 | 31.99 ± 5.35 | −0.47 | 40.37 | 0.63 (0.54, 0.72) | 0.00 |
| BMI | 22.45 ± 2.90 | 22.83 ± 3.00 | −0.04 | 6.73 | 0.96 (0.94, 0.99) | 0.01 |
| Basal FSH | 6.95 ± 2.44 | 7.11 ± 2.93 | −0.00 | 0.03 | 0.10 (0.97, 1.03) | 0.87 |
| No. of oocytes/cycle | 9.89 ± 4.79 | 9.27 ± 4.98 | −0.00 | 0.22 | 0.10 (0.98, 1.01) | 0.64 |
| Rate of 2pn (%) | 66.7 (6600/9899) | 63.0 (10521/16694) | 0.41 | 4.45 | 1.50 (1.03, 2.19) | 0.04 |
| Rate of top-quality embryos (%) | 55.8 (3686/6600) | 51.5 (5416/10521) | 0.35 | 7.52 | 1.42 (1.10, 1.82) | 0.01 |
| No. of embryo transferred | | | | | | |
| ET2 | 866 | 1380 | | 28.60 | 1.00 | 0.00 |
| ET1 | 33 | 204 | −1.17 | 23.61 | 0.31 (0.19, 0.50) | 0.00 |
| ET3 | 102 | 217 | −0.10 | 0.44 | 0.91 (0.69, 1.21) | 0.51 |
| The source of embryos transferred | | | | | | |
| 2PN ET | 952 | 1676 | | 0.10 | 1.00 | 0.95 |
| 0PN + 2PN ET | 37 | 76 | 0.09 | 0.07 | 1.10 (0.54, 2.22) | 0.80 |
| 0PN ET | 13 | 57 | 0.05 | 0.01 | 1.05 (0.48, 2.29) | 0.90 |
| Constant | | | 0.53 | 0.96 | 1.69 | 0.33 |

In addition to the possibility that 0PN-derived embryos result from an accelerated developmental rate, the presence of pronuclei may also be manifested asynchronously among a cohort of oocytes due to the variation in the timing of oocyte maturity. Assessment of pronuclei is typically carried out 16–18 h after insemination. Such a time frame is based on the assumption that all of the oocytes were mature and completed the first meiotic division at the time of insemination. The actual fertilization time for each oocyte may vary among its cohort due to differences in the timing of oocyte maturity as well as the interaction speed of the oocyte and sperm in conventional IVF. In turn, this will influence the scoring for pronuclei for any individual oocyte at the time of the fertilization check. There may be occasions where an oocyte that did not reveal pronuclei at the fertilization check shows 2PNs at a later stage, e.g., during a "re-fertilization" check or "an early cleavage" check which is typically at 20–23 h post insemination. In such cases, the fertilization status of such oocytes may have been delayed due to the oocyte maturation process. It is the finding of the authors, however, that in ICSI cycles where the insemination time is firmly controlled by the operators, very few 0PN oocytes result in normal embryo development (unpublished data).

The fertilization check, among other embryology assessments, is a highly operator dependent procedure. Pronuclei can be difficult to visualize due to granularity [12]. The presence of 2 PB is also a critical sign for confirmation of fertilization. It has been declared that the second polar body appears in ~–90 % of fertilized oocytes 6 h after insemination. Therefore, the appearance of two PB helps to efficiently exclude those oocytes that have failed to fertilize [13]. However, due to degeneration of polar bodies on the morning of day 1, it is practically difficult to differentiate the number of PBs from those fragmented PBs. In addition, the second polar body may not lie adjacent to the first polar body. The second polar body may not be visualized without rotating the oocyte. Therefore, in this study, the appearance of the second polar body was not included as an indicator of fertilization.

In most cases, embryos developed from 0PN oocytes will not cause a dilemma for embryo selections, because only normally fertilized embryos are preferable for ET. However, what if the only embryo or the best quality embryo from a morphological point of view was from a 0PN oocyte? It is not only a frustrating situation for embryologists in IVF labs, but it is also an added emotional and psychological stress for patients [14]. There are a number of studies regarding chromosome abnormalities of 0PN-derived embryos versus 2PN embryos. A fluorescent in situ hybridization (FISH) study found that 57 % (13/23) of embryos defined as 0PN at the fertilization check formed diploid embryos, six of which displayed a Y chromosome signal [6]. In contrast, Nicole Noyes et al. reported a FISH analysis for a number of chromosomes. Only 3–4.3 % of the 0PN embryos presented aneuploid karyotype. Therefore, 0PN-derived embryos were not recommended for transfer [15, 16]. However, those chromosome screening results were queried due to the questionable reliability of the FISH methods. Recently, array comparative genomic hybridization (aCGH) revealed that 0PN-derived embryos had a similar euploidy rate (23.1 vs. 30.0 %) and aneuploidy rate (73.1 vs. 64 %) with 2PN embryos [5]. But the methodology of detecting chromosome status is still questionable as diploidy, haploidy, and uniparental disomy are not distinguishable by aCGH. Although there is discrepancy between those studies regarding chromosome status, it is worth while bearing in mind that the chromosomal status of any embryo (2PN or otherwise) cannot be guaranteed [17, 18]. In addition to differences in detection methods, different outcomes may be due to differences in patient profiles, insemination methods, and the time of fertilization check. Firstly, those studies involved subjects with different maternal age groups and treatment history [6]. Secondly, the different insemination methods, i.e., ICSI versus IVF, may result in different outcomes.

In this study, embryos derived from 0PN oocytes that were subsequently transferred were following regular IVF insemination. The time of assessment for fertilization was 16–20 h after insemination, in contrast to the study by Noyes et al. (2008), where the assessment of the pronuclei was carried out 16–18 h after insemination. Timing of embryo development is a process which is influenced by numerous variables not only from the laboratory but presumably also from the patient. From a lab point of view, temperature and PH are the most obvious denominators, but culture medium does also play a role [19]. All the variables mentioned above may have an impact on the results of fertilization assessment and later on embryo progression and development. In practice, therefore, certain possibilities should be considered before deciding if an embryo from 0PN is suitable for transfer: (1) accelerated progression through the first cell cycle [3]; (2) displaying two PBs—the presence of diploid chromosome sets does not exclude parthenogenetic activation because of non-extrusion of the second polar body. Selecting zygote with 2PBs would be an indication that oocyte meiosis is complete and more likely to be diploid than oocytes with 1PB [3]; (3) morphology of embryos, i.e., direct three-way division [20]; (4) blastocyst formation [3, 20]. In comparison with embryo development, this study demonstrated that the source of embryos, whether derived from 2PN or 0PN, does not have significant impact on the success rates (Tables 3 and 4).

In this retrospective study, 70 embryos derived from 0PN oocytes were transferred in 45 cycles where normally fertilized embryos (2PN) were not available. The couples were informed of the uncertainty associated with the transfer of 0PN-derived embryos in terms of the health of any resulting babies. Given very limited data regarding the prognosis of babies resulting from 0PN-derived embryos [21], it was observed that these couples experienced more anxiety and

despair at the time of embryo transfer in comparison to what the couples experienced at enrollment for treatment. Consequently, the chance of success may be even reduced by decreased uterine receptivity from stress [22]. Such a finding suggests that those patients should be allowed the opportunity for a psychological consultation prior to embryo transfer to assess mood and anxiety symptoms as well as discuss potentially unrealistic treatment expectations. In addition, counseling services may be extended beyond the time a pregnancy ensues when such assistance usually ends and also around the time of the first trimester screening (if the couple opts for such testing). On the other side, although clinicians considered that it would be the least preferable form of transfer in clinical application to transferring 0PN-derived embryos [4], this application has been introduced into clinical practice without solid proof of safety. This study, which has been based on a large number of ART cycles, together with the associated clinical follow-up, is notable in not only giving insight into the potential of 0PN-derived embryos resulting in healthy offspring, but also valuable in helping clinicians and couples in their decision making and identifying the need for protracted counseling facilities for the well-being of the couple.

The data in this study implies that the appearance of 0PN-derived embryos in IVF can be considered as an indicator of a cycle with appropriate embryo development. Transferring 0PN-derived embryos should be considered in those treatment cycles where alternative embryos for transfer are not available.

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