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*Original Research*

# Factors Associated with the Occurrence of 0PN Fertilization in *in Vitro* Fertilization and its Effects on Embryo Development and Reproductive Outcomes

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**Abstract**

**Background**: To investigate factors associated with the occurrence of nonpronuclear (0PN) fertilization and to determine its effects on embryo development and reproductive outcomes in conventional *in vitro* fertilization (cIVF). **Methods**: This retrospective cohort study included 1116 IVF cycles and the corresponding 535 fresh transfer cycles performed during 2016–2022. Patients were divided into 0PN (+) (n = 279) and 0PN (–) (n = 837) groups based on whether they had embryos with 0PN fertilization. A multiple logistic regression model was used to determine confounders that could affect 0PN fertilization. Additionally, embryo development and reproductive outcomes were compared between the 0PN (+) and 0PN (–) groups, as well as between the 0PN embryo (n = 563) and two pronuclear (2PN) embryo (n = 1976) groups. **Results**: The number of mature oocytes was significantly correlated with the occurrence of 0PN fertilization (odds ratio: 1.15; 95% confidence interval 1.04–1.27; $p = 0.007$). The top-quality embryo rate was significantly higher in the 0PN (+) group than in the 0PN (–) group (52.9% *vs*. 49.9%; $p = 0.024$). Clinical outcomes were similar between these groups in terms of clinical pregnancy rates (43.1% *vs*. 45.5%), implantation rates (35.0% *vs*. 34.1%), miscarriage rates (15.3% *vs*. 17.1%), and live birth rates (33.6% *vs*. 33.9%). The top-quality embryo rates (45.3% *vs*. 52.9%, $p = 0.001$) and blastulation rates (57.7% *vs*. 63.8%, $p = 0.023$) were significantly lower in 0PN embryos than in 2PN embryos. The clinical outcomes were also similar between these groups. **Conclusions**: The number of mature oocytes is significantly correlated with the occurrence of 0PN fertilization in cIVF. 0PN fertilization does not affect reproductive outcomes during fresh transfer cycles.

**Keywords:** nonpronuclear fertilization; *in vitro* fertilization; embryo development; embryo transfer; pregnancy

## 1. Introduction

Normal and abnormal fertilization may occur in *in vitro* fertilization (IVF). Normal fertilization is characterized by the presence of two pronuclear (2PN) and two polar bodies (PB) in zygotes 16–20 h after fertilization, and abnormal fertilization may be nonpronuclear (0PN), monopronuclear (1PN), or polypronuclear. The occurrence of abnormal fertilization reduces the oocyte utilization rate during IVF-embryo transfer (IVF-ET) cycles, leading to the absence of transferable embryos. This imposes a negative effect on patients' treatment outcomes. The diploid rate of 1PN-derived embryo chromosomes is significantly higher in IVF than in intracytoplasmic sperm injection (ICSI) [1–3]. Additionally, measuring the pronucleus diameter [4,5] or establishing a morphological predictive model of blastocysts [6] can facilitate the selection of 1PN-derived embryos with developmental potential and clinical outcomes comparable to those of 2PN-derived embryos.

However, determining whether an 0PN oocyte has undergone fertilization at the time of fertilization assessment is challenging. The appearance of bipolar bodies indicates that the oocytes have undergone second meiosis to a certain extent. However, 37% of metaphase II (MII) oocytes, on average, have fragmented PB [7], thus creating a challenging scenario. Therefore, the utilization of 0PN embryos that have a similar division pattern and morphology as those of 2PN embryos in subsequent cultures is a concerning issue. Most analyses have confirmed that blastocyst culture is a noninvasive option for 0PN and 1PN embryo selection [8–11]. It is also possible to analyze the chromosomal and ploidy status of 0PN/1PN embryos by preimplantation genetic testing (PGT) and select normal diploid blastocysts for transfer to achieve healthy live births [12,13].

Currently, most studies have focused on the utilization of 0PN embryo transfer, with little attention paid to the causes of the occurrence of 0PN fertilization during treatment with assisted reproductive technology. Therefore, the present study investigated factors associated with the occurrence of 0PN fertilization and compared embryo development and clinical reproductive outcomes of cycles between 0PN (+) and 0PN (–) fertilization and between 0PN and 2PN embryos in conventional IVF (cIVF).

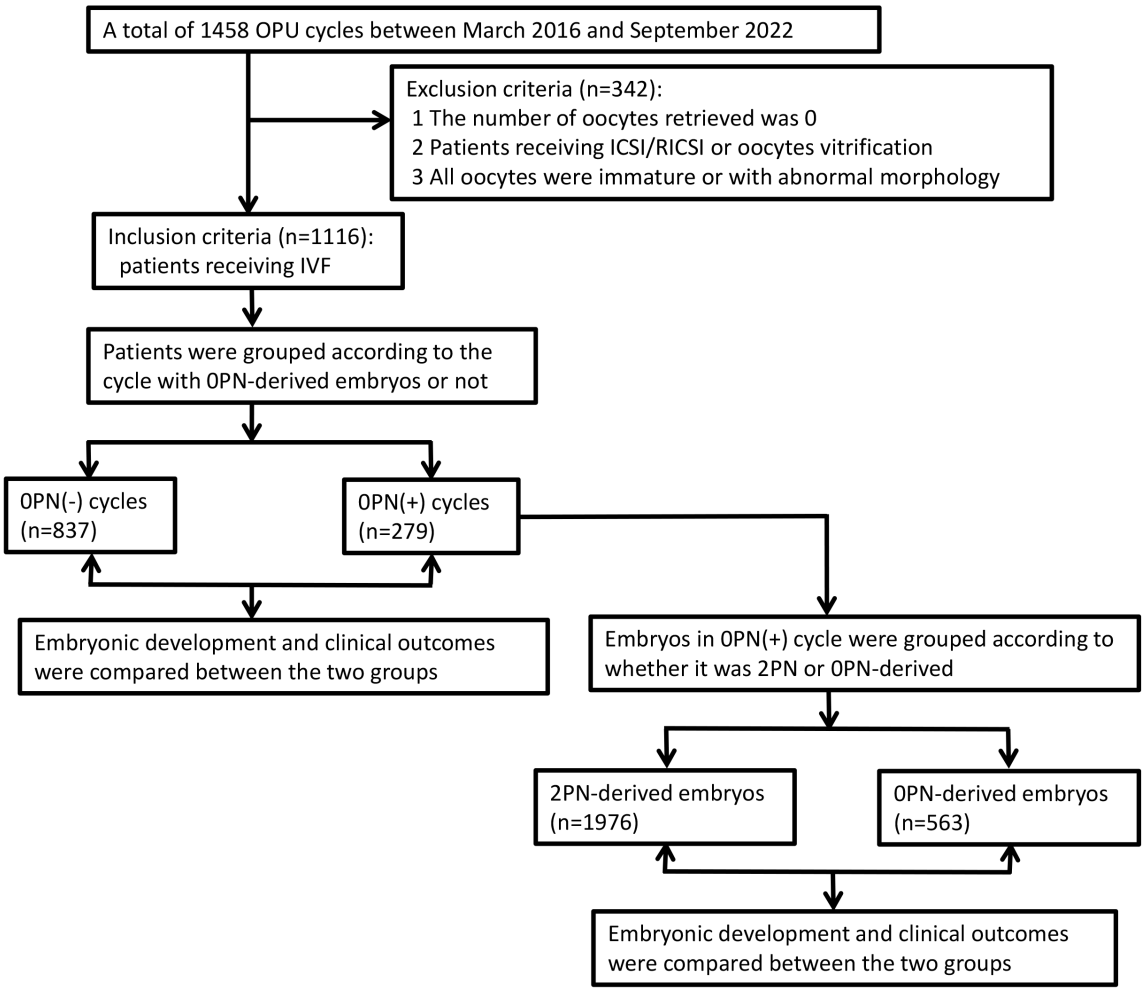


**Fig. 1. Flowchart of the patient selection process in this study.** IVF, *in vitro* fertilization; 0PN, nonpronuclear; 2PN, two pronuclear; OPU, oocyte pick-up; ICSI, intracytoplasmic sperm injections; RICSI, rescue intracytoplasmic sperm injections.

## 2. Materials and Methods

### *2.1 Patients and Definitions*

This retrospective cohort study included patients who underwent IVF-ET treatment at our reproductive center between March 2016 and September 2022. Patients who received IVF were included in this study, and patients whose retrieved oocytes were all immature or had any anomalies such as refractile bodies, dense central granulation, vacuoles, aggregation of smooth endoplasmic reticulum, and shape anomalies were excluded from the study.

0PN fertilization was defined as embryos without any pronuclei when observed within 18–20 hours post-insemination (hpi) but with cleavage when observed at 42–44 hpi. Patients were divided into two groups, namely, 0PN (+) cycles and 0PN (–) cycles, based on whether they had embryos with 0PN fertilization (Fig. 1).

### *2.2 Ovarian Stimulation and Oocyte Retrieval*

All patients underwent a standard controlled ovarian stimulation (COS) and monitoring procedure [14]. The standard COS procedure included both the agonist and the antagonist protocol. The gonadotrophin dosing regimen and the type of COS protocol used were chosen based on the patient characteristics such as age, body mass index (BMI), antral follicle count, and response to ovarian stimulation. The nomogram was used for calculating the starting dose of follicle-stimulating hormone (FSH) based on age and serum Anti-Mullerian hormone (AMH) and FSH levels [15]. When 2–3 dominant follicles reached a mean diameter of $\geq$18 mm, patients received human chorionic gonadotropin (hCG; 6000–10,000 IU; Merck Serono, Feltham, UK) to trigger ovulation. Oocytes were retrieved 36–38 hours after the hCG injection.

### *2.3 Fertilization and Embryo Culture*

The oocytes were fertilized with cIVF 38–40 hours after the hCG trigger, after which they were mechanically denudated (using denudation pipettes; Research Instruments Ltd, Falmouth, UK) and checked for pronuclei in the embryo at 18–20 hpi. The embryos were cultured in G-1 PLUS (Vitrolife, Gothenburg, Sweden) drops until day 3 and then

selected for embryo transfer (ET) or blastocyst culture. The embryos were cultured in G-2 PLUS (Vitrolife, Gothenburg, Sweden) drops for an extended period and observed until they reached the blastocyst stage up to day 6. One or two embryos are selected for fresh ET on day 3 or 5, and luteal support was initiated after retrieval.

Cleavage-stage embryos and blastocysts were graded according to the Istanbul consensus [16] and the Gardner and Schoolcraft system [17], respectively.

### *2.4 Outcomes Definitions and Measures*

Embryonic outcomes included the rate of oocyte maturation (number of MII oocytes per retrieved oocyte), rate of fertilization (number of fertilized oocytes per retrieved oocyte), rate of 2PN fertilization (number of 2PN fertilized oocytes per retrieved oocyte), rate of top-quality D3 embryos (number of grade I and II embryos with $\geq$6 blastomeres per normal cleavage embryo), rate of blastocyst formation (number of formed blastocysts per embryo cultured for an extended period), rate of top-quality blastocysts (number of grade $\geq$3BB blastocysts per formed blastocyst), and rate of available embryos ([ET embryos + frozen embryos] per [0PN + 1PN + 2PN] fertilized oocyte).

Clinical outcomes included implantation rate (gestational sacs visible on ultrasound per number of embryos transferred), clinical pregnancy rate (heartbeat detected on ultrasound per transfer cycle), miscarriage rate (clinical intrauterine pregnancy that occurs $<$12 weeks of amenorrhea), and live birth rate (delivery of a live fetus after 24 completed gestational weeks).

### *2.5 Statistical Analysis*

Data for categorical variables, continuous variables with a normal distribution, and continuous variables with a non-normal distribution were statistically presented as frequency and percentage, mean $\pm$ standard deviation, and median (interquartile range), respectively. The chi-square or Fisher's exact test was used to compare the differences in categorical variables between the groups. Based on whether the distribution of variables was normal, analysis of variance or Mann–Whitney test was used to determine statistically significant differences in continuous variables between the groups. A multiple logistic regression model was used to assess the confounders influencing the occurrence of 0PN fertilization. Statistical analyses were performed using IBM SPSS statistical software, version 23.0 (IBM Corp., Armonk, NY, USA), for Windows. $p < 0.05$ was considered to indicate statistical significance.

## 3. Results

A total of 1116 IVF cycles and the corresponding 535 fresh transfer cycles were assessed, with 279 classified as 0PN (+) cycles and 837 as 0PN (–) cycles. The number of 0PN embryos in the 0PN (+) group ranged from 1 to 18. Baseline data showed significant differences between the two groups ($p < 0.05$) in terms of age, basal FSH, infertility type, number of oocytes retrieved, and number of mature oocytes (Table 1). Patients' age (32.6 $\pm$ 4.8 *vs.* 34.4 $\pm$ 5.2 years, $p < 0.001$) and basal FSH levels (5.7 *vs.* 6.4 IU/L, $p < 0.001$) were significantly lower in the 0PN (+) group than in the 0PN (–) group. In contrast, the proportion of patients with primary infertility (36.2% *vs.* 26.8%, $p = 0.003$), the number of oocytes retrieved (12.3 $\pm$ 7.5 *vs.* 7.7 $\pm$ 6.2, $p < 0.001$), and the number of mature oocytes (11.4 $\pm$ 7.0 *vs.* 7.0 $\pm$ 5.7, $p < 0.001$) were significantly higher in the 0PN (+) group than in the 0PN (–) group.

The selection criteria for the covariables entered into the model in multivariate analysis were as follows: (1) Variables with $p < 0.1$ when comparing baseline data between the two groups (age, basal FSH level, type of infertility, gonadotropin (Gn) days, number of retrieved oocytes, and number of mature oocytes). (2) Patients' (woman) age and BMI were included. After adjusting for confounding factors, the number of mature oocytes was significantly correlated with the occurrence of 0PN fertilization (odds ratio 1.15; 95% confidence interval 1.04–1.27, $p = 0.007$; Table 2).

We further compared embryonic development and clinical outcomes between the two groups (Table 3). The results showed that the oocyte maturation rate (92.5% *vs.* 90.5%, $p = 0.001$) and the top-quality embryo rate (52.9% *vs.* 49.9%, $p = 0.024$) were significantly higher in the 0PN (+) group than in the 0PN (–) group, whereas the fertilization rate of 2PN embryos (58.2% *vs.* 71.5%, $p < 0.001$) and the available embryo rate (58.1% *vs.* 66.3%, $p < 0.001$) were significantly lower in the 0PN (+) group than in the 0PN (–) group. However, there were no significant differences in the implantation rate, clinical pregnancy rate, miscarriage rate, and live birth rate between the two groups.

Embryonic development and clinical outcomes were compared between the 0PN and 2PN-derived embryo groups in the 0PN (+) cycle group. The rate of top-quality day-3 embryo (45.3% *vs.* 52.9%, $p = 0.001$) and the rate of blastocyst formation (57.7% *vs.* 63.8%, $p = 0.023$) of 0PN embryos were significantly lower than those of 2PN embryos (Table 4). The rate of available embryo (46.5% *vs.* 63.5%, $p < 0.001$) and the rate of available D3 embryo (5.0% *vs.* 22.6%, $p < 0.001$) were significantly lower in 0PN embryos than in 2PN embryos. However, the rates of clinical outcomes between the 0PN and 2PN embryos were similar.

## 4. Discussion

The present study investigated the factors associated with the occurrence of 0PN fertilization in cIVF and its effects on embryonic development and clinical outcomes. The results showed that the number of mature oocytes is significantly correlated with the occurrence of 0PN fertilization in cIVF. In other words, the higher the mature oocytes are, the higher the likelihood of having 0PN fertil-

**Table 1. Demographic characteristics of the study population.**

| Items | 0PN (−) cycle | 0PN (+) cycle | *p* value |
|---|---|---|---|
| No. of cycles | 837 | 279 | |
| Age (years) | 34.4 ± 5.2 | 32.6 ± 4.8 | <0.001 |
| BMI (kg/m$^2$) | 24.5 ± 3.9 | 24.8 ± 4.5 | 0.274 |
| Basal FSH (IU/L) | 6.4 (5.0, 8.0) | 5.7 (4.4, 6.9) | <0.001 |
| Basal LH (IU/L) | 2.9 (2.1, 4.3) | 3.2 (2.1, 4.9) | 0.149 |
| Duration of infertility (years) | 3.0 (1.7, 5.0) | 3.0 (1.0, 5.0) | 0.974 |
| Type of infertility (%) | | | |
| Primary | 26.8 (224) | 36.2 (101) | 0.003 |
| Secondary | 73.2 (613) | 63.8 (178) | |
| Amounts of Gn (IU) | 2457.1 ± 936.9 | 2437.2 ± 903.4 | 0.756 |
| Duration of Gn (days) | 10.5 ± 2.6 | 10.8 ± 2.1 | 0.089 |
| Type of hCG (%) | | | |
| Recombinant | 39.4 (330) | 44.8 (125) | 0.114 |
| Highly purified | 60.6 (507) | 55.2 (154) | |
| COS protocol (%) | | | |
| Antagonist protocol | 47.1 (394) | 44.8 (125) | 0.510 |
| Agonist protocol | 42.5 (356) | 45.9 (128) | 0.329 |
| Others | 10.4 (87) | 9.3 (26) | 0.606 |
| No. of retrieved oocytes | 7.7 ± 6.2 | 12.3 ± 7.5 | <0.001 |
| No. of MII oocytes | 7.0 ± 5.7 | 11.4 ± 7.0 | <0.001 |

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; COS, controlled ovarian stimulation; Gn, gonadotropin; hCG, human chorionic gonadotropin; MII oocytes, metaphase II oocytes.

**Table 2. Multiple logistic regression analysis of potential factors associated with 0PN fertilization in IVF.**

| Items | OR | 95% CI | *p* value |
|---|---|---|---|
| Age (years) | 0.97 | 0.94–1.00 | 0.085 |
| BMI (kg/m$^2$) | 1.01 | 0.98–1.05 | 0.427 |
| Type of infertility | 0.86 | 0.62–1.21 | 0.400 |
| Basal FSH (IU/L) | 0.97 | 0.92–1.02 | 0.247 |
| Duration of Gn (days) | 1.02 | 0.97–1.09 | 0.435 |
| No. of retrieved oocytes | 0.96 | 0.88–1.05 | 0.377 |
| No. of MII oocytes | 1.15 | 1.04–1.27 | 0.007 |

BMI, body mass index; CI, confidence interval; FSH, follicle-stimulating hormone; IVF, *in vitro* fertilization; Gn, gonadotropin; MII, metaphase II; OR, odds ratio.

ization in cIVF. The occurrence of pronuclear fertilization is dynamic and transient; therefore, evaluating it at a fixed point in time is challenging. Considerable differences exist in the timing at which pronuclei become visible, which varies from 3 to 20 hpi [18–20]. A recent study based on time-lapse date suggested that the recommended fertilization assessment time of 17 ± 1 hpi should be revised to 16.5 ± 0.5 hpi, as the highest proportion of oocytes with visible pronuclei (98.3%) was observed at 16–16.5 hpi. With time delays, the proportion of visible pronuclei decreased [21]. Another study also showed that the microscopic assessment time was more significantly delayed in 0PN embryos than in 2PN embryos [22]. In our study, pronuclear assessment at 18–20 hpi may have caused some of the 0PN embryos to miss the visible timing for pronuclear fertilization. Additionally, variations existed in the maturation timing for oocytes obtained through COS; better oocyte maturation in the 0PN (+) group may have exacerbated the occurrence of this event because oocytes at the metaphase II stage have a higher fertilization competence than immature oocytes, which enables them to complete fertilization and subsequent cell cycle events earlier after insemination [23]. Moreover, no difference was observed in the total fertilization rate between the two groups, but the 2PN fertilization rate was significantly lower in the 0PN (+) group than in the 0PN (–) group, which further indicated that the time difference in oocyte maturation caused the asynchronous presence of pronuclei. However, as the time-lapse incubator was not used for embryo culture in our study, the possibility of pronuclei formation later than the observation time cannot be excluded. Moreover, the top-quality D3 embryo rate was significantly higher in the 0PN (+) group than that in the 0PN (–) group, consistent with the findings of previous studies [24]. Patients in the 0PN (+) group were younger and had a better ovarian response to gonadotrophin stimulation, which could also explain their better oocyte maturity and embryo quality in this study.

When comparing the 2PN and 0PN embryos of the 0PN (+) group, the rates of top-quality D3 embryo and the blastocyst formation of 0PN embryos were significantly lower than those of 2PN embryos, consistent with the re-

**Table 3. Comparison of embryonic and clinical outcomes between the 0PN (+) and 0PN (–) cycles groups.**

| Items | 0PN (–) cycle | 0PN (+) cycle | *p* value |
|---|---|---|---|
| Oocyte maturation rate (%) | 90.5 (5862/6476) | 92.5 (3174/3430) | 0.001 |
| Fertilization rate (%) | 84.1 (5446/6476) | 84.5 (2900/3430) | 0.556 |
| 2PN fertilization rate (%) | 71.5 (4628/6476) | 58.2 (1995/3430) | <0.001 |
| Top-quality D3 embryo rate (%) | 49.9 (2270/4549) | 52.9 (1046/1976) | 0.024 |
| Blastocyst formation rate (%) | 62.8 (1877/2988) | 61.5 (1178/1916) | 0.346 |
| Top-quality blastocyst rate (%) | 59.4 (1115/1877) | 59.9 (706/1178) | 0.772 |
| Available embryo rate (%) | 66.3 (3185/4801) | 58.1 (1564/2691) | <0.001 |
| No. of embryos transferred | 1.5 ± 0.5 | 1.5 ± 0.5 | 0.115 |
| Implantation rate (%) | 34.1 (209/613) | 35.0 (70/200) | 0.815 |
| D3 transfer | 29.5 (147/498) | 32.7 (49/150) | 0.462 |
| D5 transfer | 53.9 (62/115) | 42.0 (21/50) | 0.160 |
| Clinical pregnancy rate (%) | 45.5 (181/398) | 43.1 (59/137) | 0.624 |
| D3 transfer | 42.1 (120/285) | 43.7 (38/87) | 0.795 |
| D5 transfer | 54.0 (61/113) | 42.0 (21/50) | 0.158 |
| Miscarriage rate (%) | 17.1 (31/181) | 15.3 (9/59) | 0.737 |
| D3 transfer | 15.8 (19/120) | 18.4 (7/38) | 0.708 |
| D5 transfer | 19.7 (12/61) | 9.5 (2/21) | 0.502 |
| Live birth rate (%) | 33.9 (135/398) | 33.6 (46/137) | 0.942 |
| D3 transfer | 30.9 (88/285) | 33.3 (29/87) | 0.666 |
| D5 transfer | 41.6 (47/113) | 34.0 (17/50) | 0.360 |

**Table 4. Comparison of embryonic and clinical outcomes between the 0PN and 2PN embryos in the 0PN (+) cycle group.**

| Items | 0PN embryos | 2PN embryos | *p* value |
|---|---|---|---|
| Top-quality D3 embryo rate (%) | 45.3 (255/563) | 52.9 (1046/1976) | 0.001 |
| Blastocyst formation rate (%) | 57.7 (248/430) | 63.8 (877/1375) | 0.023 |
| Top-quality blastocyst rate (%) | 62.9 (156/248) | 59.7 (524/877) | 0.370 |
| D3 cell number | 7.9 ± 3.6 | 7.9 ± 2.4 | 0.665 |
| Available embryo rate (%) | 46.5 (262/563) | 63.5 (1254/1976) | <0.001 |
| Available D3 embryo rate (%) | 5.0 (28/563) | 22.6 (446/1976) | <0.001 |
| No. of embryos transferred | 1.6 ± 0.5 | 1.5 ± 0.5 | 0.373 |
| Implantation rate (%) | 35.7 (5/14) | 35.9 (65/181) | 0.988 |
| D3 transfer | 36.4 (4/11) | 33.6 (45/134) | 1.000 |
| D5 transfer | 33.3 (1/3) | 42.6 (20/47) | 1.000 |
| Clinical pregnancy rate (%) | 41.7 (5/12) | 43.2 (54/125) | 0.918 |
| D3 transfer | 44.4 (4/9) | 43.6 (34/78) | 1.000 |
| D5 transfer | 33.3 (1/3) | 42.6 (20/47) | 1.000 |
| Miscarriage rate (%) | 20.0 (1/5) | 14.8 (8/54) | 0.577 |
| D3 transfer | 25.0 (1/4) | 17.6 (6/34) | 1.000 |
| D5 transfer | 0.0 (0/1) | 10.0 (2/20) | 1.000 |
| Live birth rate (%) | 33.3 (4/12) | 33.6 (42/125) | 1.000 |
| D3 transfer | 33.3 (3/9) | 33.3 (26/78) | 1.000 |
| D5 transfer | 33.3 (1/3) | 34.0 (16/47) | 1.000 |

sults of another study which found that rapidly developing embryos derived from 0PN have better developmental potential than intermediately or slowly developing embryos, while 2PN embryos with a medium cleavage speed have the highest blastocyst rate and a good-quality blastocyst rate[25]. Meanwhile, a positive correlation exists between the formation and quality of blastocysts and the number of cells in D3 embryos [26,27]. All these findings indicate that rapidly developing embryos at the cleavage stage have better developmental potential. However, in this study, no significant difference existed in the number of D3 cells between 0PN embryos and 2PN embryos. Based on these observations, this study postulates that embryos with premature disappearance of pronuclei in IVF indicate only the acceleration of the first cell cycle, and whether 0PN embryos continue to have accelerated development in subsequent cell cycles is a manifestation of their developmental potential.

In this study, both the 0PN (+) group and 0PN embryos showed a significant decrease in embryo utilization. One important reason is that the present study used blastocyst culture to screen for transplantable 0PN embryos, and 0PN embryos are transferred only when patients lack available 2PN-derived embryos. The results of fresh transfer also showed that both the 0PN (+) group and 0PN embryo ET can achieve clinical outcomes similar to those of the 0PN (–) group and 2PN embryos, consistent with the findings of some studies that blastocysts derived from 0PN embryos can be transferred and demonstrate acceptable pregnancy outcomes [28–30]. These results once again indicate that early disappearance of the pronuclei is a difference in cell cycle time, and 0PN embryos can be screened and used for transplantation through blastocyst culture.

The present study has a few limitations that should be considered. Because of the routine operation of overnight insemination in cIVF, our judgment about mature oocytes comes only after the removal of granulosa cells on the morning of D1, which does not fully reflect oocyte maturation of COS but rather the maturation after a period of *in vitro* cultivation. Furthermore, as 0PN embryos are generally not considered as the preferred transfer option, the number of transfer cycles is limited; larger scale data analysis should be conducted on this topic to gain a better understanding.

## 5. Conclusions

Our study suggests a significantly correlated between the number of mature oocytes and the occurrence of 0PN fertilization in cIVF. The presence of 0PN fertilization reduces both the normal fertilization rate and the embryo utilization rate during the IVF-ET treatment cycle. Additionally, the embryo development potential of 0PN embryos is significantly lower than that of 2PN embryos. However, the occurrence of 0PN fertilization and the transfer of 0PN-derived embryos did not have a significant effect on the reproductive outcomes of IVF-ET treatment.

## Availability of Data and Materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

## Author Contributions

JD designed the research study. HG and XS wrote and revised manuscripts, proposed methodology and conceptualization. YJ conducted data collection and analysis. All authors contributed to editorial revisions in the manuscript. All authors read and approved the final manuscript. All authors have contributed sufficiently to this work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

All subjects provided written informed consent in this study. The rights and interests of the subjects were protected well in the whole process. The research was approved by the Ethics Committee of Liaocheng People's Hospital (NO. 2023076).


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## Conflict of Interest

The authors declare no conflict of interest.

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