Embryos from "0PN" zygotes may be most likely 2PN embryos in L-R-ICSI

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Abstract

Although some zero pronuclei (0PN) zygotes cleave and develop into good embryos, they are usually discarded because they lack two distinct pronuclei (2PN). In this case report, we followed four couples to determine whether 0PN embryos could be used in late rescue intracytoplasmic sperm injection (L-R-ICSI). Here, we report five healthy infants, including one set of twins, derived from "0PN" embryos from four frozen–warmed embryo transfer (FET) cycles in L-R-ICSI between 2015 and 2017. Of nine infants born from L-R-ICSI cycles in our center, five were "0PN" embryos, and all remained healthy. Embryos from "0PNs", such as 2PN, can develop into healthy babies using L-R-ICSI. This finding suggests that embryos from "0PNs" in L-R-ICSI may be different from traditional 0PN gametes and more likely to originate from 2PN gametes, and they may be used in infertile couples who lack 2PN embryos instead of discarding them. The use of "0PN" embryos increases the cycles reaching embryo transfer, allowing some infertile couples to have healthy children and to avoid mental anguish and wasted time and money.

Introduction

Assisted reproductive technology (ART) procedures include conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). In a conventional IVF cycle, approximately 3–5% of fertilization failures occurred with normal sperm parameters examined [1]. This result is consistent with data from our center, but some scholars have reported that the rate of complete fertilization failure was 10%-25% [2]. This discrepancy may be related to different statistical ranges, such as obtaining a low number of eggs and having abnormal semen parameters. Although the incidence of complete fertilization failure decreases as the second pole for fertilization increases at 4–6 hours after insemination, the failed process carries a high emotional and financial burden for the infertile couple. Accordingly, to avoid the catastrophe of fertilization failure, some embryologists advise ICSI on day one of unfertilized mature oocytes, applying the so-called “late rescue ICSI” (L-R-ICSI).

Until now, morphological evaluation has been widely used as a relatively simple and harmless method by which to predict embryo development, including prokaryotic observation, embryo and blastocyst grading. Here, we mainly introduce prokaryotic observations. Normally, fertilized zygotes from IVF and ICSI show two pronuclei (2PN) at 16–20 hours after insemination [1, 3, 4], and embryos from 0PN, monopronuclear (1PN) and three or more PN are considered to provide evidence of failed or abnormal fertilization. Guidelines do not recommend transferring embryos that do not have two pronuclei at the time of visual assessment [5]. However, the findings of Yao et al. reported that no significant differences were shown in euploid rates between 0PN and 2PN in blastocyst cycles of PGT-M (preimplantation genetic testing for a monogenic disorder) [6]. Therefore, it remains controversial whether 0PN embryos should be used.

In L-R-ICSI, that is, ICSI performed the day after conventional IVF because no or little fertilization has been achieved, the time course of 0PN formation is complicated and still unclear. It has been reported that the birth of healthy twins results from the transfer of embryos found to be without 2PN in the cytoplasm of the oocytes 16–18 hours post-insemination, when L-R-ICSI had been performed on day one in a fresh

cycle [1]. Here, we describe the results achieved—four successful full-term pregnancies and five infants—after the transfer of 0PN embryos in L-R-ICSI in frozen–warmed embryo transfer (FET) cycles.

Methods

Ethics approval

This study was approved by the Ethical Committee of Guangdong Provincial People's

Hospital, Guangdong Academy of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations, and the approved protocol was followed throughout the study period.

Informed consent

For each couple entering the treatment cycle, we signed an informed consent form to state the success rate and possible complications of IVF/ICSI and informed them that their data might be used for scientific research, but their personal information is completely confidential.

Differences between conventional 0PN and "0PN" in this paper

Conventionally, mature oocytes with no distinct two pronuclei (2PN) in the cytoplasm of any oocytes associated with extrusion of the second polar body at 16–20 h postinsemination are documented as 0PN. Accordingly, the time of the fertilization check is defined of 16–20 hour after insemination.

The observation point is delayed to more than 22 hours in L-R-ICSI because the time of reinsemination in L-R-ICSI is 5-6 hours earlier than normal insemination. Different from the traditional fertilization check, the observation point in this paper is more than 22 hours after insemination, therefore, "0PN" embryos in this article include but are not limited to 0PN embryos in the traditional sense, and we use quotation marks to distinguish them. That is to say, the "0PN" embryo in this article is compared to the traditional definition, which includes an embryo with prokaryotic disappearance between 20 hours after insemination and the observation point. This is also inconsistent with the standards of other articles on 0PN.

Clinical data

In L-R-ICSI of our center, 8 patients were successfully conceived, and 9 babies were born, among which 5 were from 0PN between 9 January 2015 and 23 August 2017. This paper mainly describes the data of 4 0PN cases, including a pair of twins.

Four female patients and their domestic partners with unexplained infertility for 3.4-7 years and aged 26 to 32 years were scheduled for fertilization (fresh cycle data are shown in Table 1). Three patients used the standard long stimulation GnRH agonist protocols, and the other protocol used the GnRH antagonist protocol. Human chorionic gonodotrophin at a dose of 5000-10000 IU was administered after at least two follicles of 18 mm or greater in size were visualized by means of transvaginal ultrasound scanning. Fifty cumulus oocyte complexes (COCs) were retrieved by ultrasound-guided needle aspiration in the four

infertile couples. The COCs were collected and washed fourfold and transferred into a Center-Well Organ Culture Dish (353037, 60×15 mm; Becton Dickinson, USA) containing 1 mL of G-IVF medium (Vitrolife, Sweden) for preinsemination culture. Oocytes were inseminated in the same medium with approximately $3\sim4\times10^5$ motile spermatozoa/mL after hCG injection 40–42 hours later and incubated at 37°C in a mixed atmosphere of 6% CO_2 and 5% O2 with high humidity. A fertilization check was performed 18-20 hours later (approximately 8 a.m.) on the next morning (D1) after insemination, and only five oocytes were fertilized. After patients provided appropriate signed informed consent, L-R-ICSI as an alternative treatment was performed on thirty-five metaphase II stage oocytes with sperm from D0 (day of oocyte pick-up, OPU) insemination at approximately 9.a.m. Embryos were cultured in G-1 medium drops (Vitrolife, Sweden) and covered with mineral oil in Falcon tissue culture dishes (353001, 35×10 mm, Becton Dickinson, USA). Approximately 8 a.m. On the D2 morning after OPU, only seven of these oocytes had two distinct pronuclei (2PN) in the cytoplasm, and the other 23 oocytes had no pronuclei but showed cleavage on the following culture.

All rescued embryos were observed on D3, D5 or D6 after L-R-ICSI. Here, we introduced two systems for classifying embryos. On day 3, the blastomeres and fragments were evaluated and classified into four types separately by means of the following scoring system. Blastomere: grade I-even sized blastomeres with regular morphology; grade II- slightly uneven sized blastomeres with regular morphology; grade III-asymmetrical blastomeres and irregular morphology; grade IV-severely asymmetrical blastomeres and irregular morphology or significant cytoplasmic particles. Embryo fragments were classified into grades I, II, III and IV according to fragmentation <5%, 6%-20%, 21%-50% and >50%. The number of cells 7-9, embryos and fragments were classified as I and II, but those with different levels of II were defined as top-quality embryos. Cell numbers >4, blastomeres grade I-III and fragments classified as I-II were considered suitable embryos for transfer, excluding embryos with blastomeres as III and fragments as II. The blastocyst grading system proposed by Gardner and Schoolcraft was based on blastocyst morphology parameters [7]; that is, blastocyst development was divided into 6 stages according to the size of blastocoele expansion degree, blastocyst cavity and whether it hatched or not. The inner cell mass (ICM) and trophoblast ectoderm (TE) were classified into three levels: A, B and C according to cell number and intercellular adhesion (A: a good number of cells and good intercellular adhesion; B: small cell numbers and good intercellular adhesion; C: almost no cells). The blastocysts with blastocoeles greater than or equal to 3, ICM and TE scores of A, B and C, but not both C, were considered eligible and transferred, and those defined as good blastocysts for ICM and trophoblast ectoderm (TE) were graded A or B.

Avoiding dyssynchrony between the endometrium and embryonic development, fresh cycles of embryo transfers were cancelled. The couples were informed of the uncertainty associated with the transfer of 0PN-derived embryos in terms of the health of any resulting babies. All embryos and blastocysts that reached the standard of transfer, either from 2PN or 0PN, were frozen in vitrification. The freezing process between embryos and blastocysts differed in that the blastocysts needed to be shrunk artificially with a laser pulse prior to vitrification by the embryologist. Embryos/blastocysts were first transferred to an equilibration solution (VT101-□, KITAZATO, Japan) for 10 minutes and subsequently transferred to a

vitrification solution (VT101-□, KITAZATO, Japan) for 1 minute. Then, one or two embryos/blastocysts were/were placed into a cryotop (Cryotop ®, KITAZATO, Japan), which was quickly plunged into liquid nitrogen, covered with a tube and stored in liquid nitrogen.

Approximately two months after oocyte pick-up, FET cycles were started, in which one or two cryopreserved embryos/blastocysts were warmed and transferred. To thaw, the embryos/blastocysts loaded into the cryotop were immersed in the first thawing solution (VT102-□, KITAZATO, Japan) at 37°C for 1 minute and then transferred to the second and third thawing solutions (VT102-□, □, KITAZATO, Japan) for 3 and 5 minutes at room temperature. Finally, the embryos/blastocysts were washed in the fourth solution (VT102-□, KITAZATO, Japan) at 37 °C for 5 minutes. After warming, embryos/blastocysts with more than 50% intact blastomeres were regarded as viable and were transferred to the recipient uterus of all four patients.

Ten embryos/blastocysts of five FET cycles were transferred to the four patients. One patient received two transplants, the first of which had two 2-prokaryotic embryos and was not a pregnancy. The remaining eight fresh cycles of 0 prokaryotic embryos were transplanted four times, and four clinical pregnancies resulted (FET data

cycles, as shown in Table 2). Then, 6-8 weeks after FET, four clinical pregnancies were confirmed using transvaginal ultrasonography. Among them were two twin pregnancies with four sacs but three fetal heartbeats.

Results

The four recipients of the "0PN" embryos were offered prenatal diagnosis and karyotype ploidy analysis to verify fetal normality at 16-20 weeks of pregnancy. Fortunately, the fetuses have normal ploidy, and four deliveries at 36-41 weeks resulted in the births of five normal healthy babies, including a pair of live twins between 28 December 2015 and 24 May 2018. One of the four pregnant women had a natural birth, and the other three women underwent lower segment cesarean sections. The detailed data of the 3 boys and 2 girls are shown in Table 2. All the children had regular physical examinations by their pediatricians and were growing normally. The oldest baby is four and a half years old, and the youngest twins are more than 2 years of age. All children are healthy with normal developmental milestones.

Discussion

To the best of our knowledge, this is the first report of live healthy infants of "0PN" embryos in FET cycles in L-R-ICSI. The results described in the present case report indicate that "0PN"-derived embryos may be most likely 2PN embryos in L-R-ICSI cycles, and the conventional discard of embryos in c-IVF and ICSI cycles that originated from 0PN zygotes at fertilization check-up may no longer apply to L-R-ICSI. Since "0PN-derived" embryos are shown to result in live and healthy babies, these "0PN" zygotes bring good

hope to infertile couples and increase the chances of having a successful pregnancy for infertile patients without 2PN embryos.

The use of 0PN zygotes in IVF has been a global topic of debate for the past 20 years because of uncertain ploidy. Some experts believe that 0PN-derived embryos should not be recommended based on the results of chromosome tests using FISH technology, in which only 3%-4.5% of the 0PN embryos presented euploid karyotypes [5,8]. Hence, in some IVF centers, it has become standard clinical policy to discard 0PN embryos. However, other scholars have reported different FISH results, including that 57%–62% of embryos developing from 0PNs were diploid and that 66% of diploid 0PN embryos were thrown away at the embryo stage due to erroneous classification of ploidy based on pronuclear number scoring[3,9]. Lee et al. also reported that 0PN-originated embryos had a similar euploidy rate (23.1% vs. 30.0%) by array comparative genomic hybridization (aCGH) [10]. Because the results reported by different investigators and institutions are quite different, the method of chromosome detection has been questioned, and whether any embryo (2PN or otherwise) can be guaranteed [11]. Therefore, in different IVF centers, transferring 0PN-derived embryos may or may not be considered in those treatment cycles in which 2PN-derived embryos are not available .

In brief, embryos derived from 0PN zygotes create a dilemma for embryo selection in clinical practice. Some studies, however, have reported that 0PN-derived embryos resulted in the birth of healthy babies [3,12,13]. Liu et al. also reported that 13 healthy infants resulted from 0PN-originated embryos with an implantation rate of 17.0% in conventional IVF cycles, and those authors believed that the source of embryos transferred did not have a significant impact on clinical pregnancies and live birth rates[4]. Capablo et al. reported the analysis of eight zygotes scored as 0PNs in ICSI cycles by PGT-A and found that all were diploid biparental[14]. Destouni et al. also reported that genome-wide haplotyping embryos developing from 0PN zygotes increased transferrable embryos in PGT-M[15]. These results suggest that 0PN- embryos should not be eliminated from further studies since they are probably misclassified zygotes due to the prokaryote form, or they may disappear too early or too late and miss the prokaryotic observation point [3, 4, 16].

In conventional IVF and ICSI, a fertilization check is usually performed 16-20 hours after insemination on day 1 after oocyte pick-up (OPU) [1, 3, 4]. Therefore, in Guangzhou, China, insemination is often performed at approximately 1:00-3:00 p.m. on the OPU day, and prokaryotic observation was conducted at 7:00-8:00 a.m. on the next morning. Thus, L-R-ICSI is carried out after the discovery of non-fertilization, usually at 8:30-9:00 a.m., so the time of reinsemination in L-R-ICSI is 5-6 hours earlier than normal insemination. The observation point is delayed to more than 22 hours in L-R-ICSI, making it difficult to observe the prokaryotes at regular fertilization checks (approximately 2-3 o'clock midnight) during work time, since going to work 5-6 hours earlier than normal work is unrealistic. Therefore, we speculate that the origin of the "0PN" zygote is primarily due to the disappearance of the prokaryote in L-R-ICSI, and the criteria for the available embryos of conventional IVF and ICSI that must be derived from 2PN may need to be changed. Based on this deduction, we required patients' signed informed consent for the use of 0PN embryos and used it as a backup, which not only increased the embryo reserve for patients but also

brought substantial benefits to the patients. The birth of five healthy babies confirmed the feasibility of this decision. Chian et al. (2003) also reported successful healthy live births from 0PN embryos after L-R-ICSI following failed fertilization. Their results combined with our cases presented in this report suggest that "0PN"-derived available embryos can produce healthy babies in L-R-ICSI cycles.

Another highlight of our work was the transfer of embryos during thawing cycles. The pregnancy rates after L-R-ICSI are lower than those after normal ICSI due to either poor quality of embryos or asynchrony between the endometrial secretory pattern and embryo development. The aging of oocytes in *in vitro* culture and the long interval between oocyte retrieval and L-R-ICSI may result in poor quality embryos [17,18]. Additionally, an asynchrony between the endometrial secretory pattern and embryo development may influence implantation and pregnancy. Although there had been previous reports of live births of fetuses in fresh embryo transfer cycles in L-R-ICSI [1, 19], prior to 2013, we had not yet had a successful pregnancy with 10 fresh transplants despite the embryo appearing to meet the requirements of good embryos, either D3 or D4, after egg retrieval. Therefore, we considered that dyssynchrony of the developmental stage of the embryo and endometrial receptivity may be the main cause. All available embryos were frozen during the fresh cycle and thawed and transplanted 2-3 months later. Since then, eight clinical pregnancies and live births have been derived through FET in our center between 2015 and 2017. The results of the present case study emphasize the importance of synchronization of embryonic development and endometrium for embryo transfer and that the FET cycle is the preferred strategy in L-R-ICSI.

In this case report, we describe four successful full-term pregnancies and five births after the transfer of L-R-ICSI embryos derived from "0PN". This result indicates that we should pay attention to "0PN" embryos in L-R-ICSI and consider that they are probably misclassified and zygotes are discarded due to the absence of pronuclei. We suggest that the conventional method of discarding embryos because zygotes originally lacked pronuclei at the fertilization check should be reconsidered in L-R-ICSI. Transferring "0PN-derived" embryos/blastocysts in the FET cycles with subsequent expected pregnancies can benefit families where the number of transferrable embryos is very limited and can be performed as the last ray of hope in case of failed fertilization. Therefore, it is reasonable to suggest that "0PN" embryos are more likely to originate from 2PN in L-R-ICSI. Certainly, confirming this viewpoint requires more cases. In addition, it is worth considering that our successful patients were only 26-32 years old. Whether 0PN embryos from older women have developmental competency after L-R-ICSI remains unknown.

It is worth emphasizing that there are multiple publications on apronuclear and unipronuclear embryos in which better technologies have been utilized to assess these embryos, such as the time lapse system (noninvasive) and chromosomal/ploidy analysis (invasive) [20-21]. In this instance, incubation in a time lapse incubator may have provided additional important information regarding the true origin of these abnormal zygotes, but that is currently missing from this study because of having no time lapse incubator. We checked the PN morphology more than 22 hours post-insemination and classified the "0PN" zygotes at this time. It is most likely that syngamy and PN fading already occurred, and the transferred embryos could be regular 2PN. Our center does not have the qualification of PGT, so we

cannot perform biopsy to analyse chromosomal/ploidy. The PN status of these '0PN' embryos may be wrongly classified due to late fertilization checks in L-R-ICSI zygotes. For this reason, we appeal that "0PN" embryos can be reserved for those patients who do not have 2PN embryos in L-R-ICSI.

Actually, the PN state is time-limited, while the prokaryotic observation is a point in time, so the zygotes will be misclassified as 0PN, which 2PN are present but disappeared before or after the fertilization check. A report showed that pronuclei could disappear before 15 h after insemination by a time-lapse [22]. Hence, even at traditional fertilization sites, some embryos may misjudge because their prokaryotes have disappeared or have not yet appeared at the time of the fertilization check . True 0PN embryos may be defined as those without prokaryotes between fertilization and cleavage but develop to the two or more cell stage. Since human embryos need a stable culture environment, the time and frequency of observation outside the incubator should be reduced, so the closest real data relies on real-time monitoring by time-lapse.

Conclusions

The PN status of these '0PN' embryos may be wrongly classified due to late fertilization checks in L-R-ICSI zygotes, since the observation point is delayed to more than 22 hours in L-R-ICSI. Embryos from "0PNs", such as 2PN, can develop into healthy babies in L-R-ICSI. This finding indicates that "0PN"-derived embryos may be most likely 2PN embryos in L-R-ICSI cycles, and the conventional discard of embryos in c-IVF and ICSI cycles that originated from 0PN zygotes at fertilization check-up may no longer apply to L-R-ICSI. We suggest that embryos from "0PNs" may be used in infertile couples who lack 2PN embryos instead of discarding them.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Guangdong Provincial People's

Hospital, Guangdong Academy of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations, and the approved protocol was followed throughout the study period. For each couple entering the treatment cycle, we signed an informed consent form to state the success rate and possible complications of IVF/ICSI and informed them that their data might be used for scientific research, but their personal information is completely confidential.

Consent for publication

We all agree to submission of the manuscript.

Availability of data and material

All data and material of this study are true and reliable.

Competing interests

All authors have no conflicts of interest to report.

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Authors' contributions

CL and DH conceived and designed the study. JC and YL carried out female patients' ovulation plans, counselled the patients and followed pregnancy outcomes. DH and C Z performed ICSI and embryo freezing procedures. CL and DH performed thawing and embryo transfer procedures. JL and YZ performed semen processing and embryo/blastocyst scoring. DL performed male physical examinations and recording. CL and JC performed the data and statistical analysis. CL drafted the original version of the article. C L, JC, DL and YL revised the article. All coauthors read and approved the final version of the article.

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Tables

Table 1 Data of fresh cycles

Characteristic	Case1	Case2	Case3	Case4
Age (years)	30	32	28	26
Duration of infertility (years)	7	6	6	3.4
Type of infertility	Primary	Secondary	Primary	Primary
Cause of infertility	Pelvic Inflammatory disease	Ovulation factor	Pelvic inflammatory disease and tubal factor	Polycystic ovary syndrome (PCOS)
Protocol	long-acting GnRH agonist	long-acting GnRH agonist	long-acting GnRH agonist	GnRH Antagonist
Number of oocytes	14	10	14	12
Oocytes of L-R-ICSI	6	7	10	12
Number of frozen D3 Embryos	5(0PN)	1(2pn)	2(0PN)	2(2pn)
Number of frozen blastocysts	0	2(0PN,D5)	2(0PN,D6)	2(0PN,D5)

GnRH gonadotropin-releasing hormone

Table 2 Data of of FET cycles

Characteristic	Case1	Case2	Case3	Case4	
Protocol for endometrial preparation	HRT	NC	NC	HRT	
Endometrial pattern	8	8.5	12	7.6	
Endometrial thickness (mm)	A	A-B	A-B	A-B	
Number of embryo transferred	2	2	2	2	
Day of embryo transferred	3	5	3	5	
Grade of embryo or blastocyst	8.21 /13.21	4AC/4CB	7.21/7.21	4BC/4CB	
Sac	2	1	1	2	
fetal heartbeat	1	1	1	2	
Weeks of delivery	41+3	39+6	40+2	35+6	
Type of delivery	Cesarean section	Cesarean section	uneventful delivery	Cesarean section	
Number of infant	1	1	1	2	
Gender	female	female	Male	male	female
Weight(kg)	3.75	3.45	3.77	2.95	2.30
Length(cm)	50	50	52	48	46

FET frozen–warmed embryo transfer ;
HRT hormone replacement treatment
NC natural cycle