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ASSISTED REPRODUCTION TECHNOLOGIES

Live birth in a complete zona-free patient: a case report

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Abstract

Objective To report a live birth from a patient with complete zona-free oocytes due to abnormal zona production and to reveal full time-lapse blastocyst development footage of its originating embryo.

Methods A 34-year-old woman presented with a history of failed fertilization via standard in vitro fertilization insemination and a potential absence of zona pellucida. A total of 3 intracytoplasmic sperm injection cycles were undertaken with all oocytes collected being zona-free. Embryos created in the initial 2 cycles were cultured in the G1+/G2+ sequential media in a benchtop incubator. During the final successful cycle, the culture strategy was shifted to single step media (G-TL) in an Embryoscope+ incubator.

Results The first 2 attempts led to a biochemical pregnancy or no blastocyst available for transfer. In the third cycle, 13 out of 24 collected oocytes were subjected to injection, with 4 being normally fertilized. Two blastocysts were subsequently formed, in which one was cryopreserved and the other transferred. A live baby girl (1570g) was subsequently delivered at 34 weeks of gestation by cesarean section.

Conclusion Live birth can be achieved for patients with zona production deficiency. Adjustment in ovarian stimulation and subsequent embryo culture strategies may have potentially contributed to the success of the 3rd cycle.

Keywords Zona-free · Time-lapse · Single stage culture · Live birth · Case report

Introduction

The zona pellucida (ZP) is an extracellular transparent glycoproteinaceous matrix that surrounds the oocyte and has been considered an important component for subsequent embryo development. The function of this structure is to provide a physical support enclosure for dividing blastomeres, to inhibit tubal implantation and to provide protection from the maternal immune system [1]. In addition, it offers a critical role during sperm binding, fertilization, and prevention of polyspermy [2]. The human ZP is comprised of four glycoproteins which are encoded in genes *ZP1*, *ZP2*, *ZP3*, and *ZP4* [3]. It has been shown in the human using native or recombinant ZP proteins that ZP1, ZP3, and ZP4 primarily bind capacitated sperm and induce acrosome reaction, whereas ZP2 binds acrosome-reacted sperm [4].

ZP-free oocytes may be present in the assisted reproductive laboratory as a result of either ruptured ZP during in vitro manipulation [5], purposefully removed ZP [6], or ZP production issues in a rare patient population [7]. In all of these patients, the oocyte is very fragile due to the lack of a protective surrounding shell and requires very delicate handling. There are a limited number of reports in the literature regarding live birth outcomes originating from such ZP-free oocytes and, in particular, the subgroup with intrinsic ZP production defect. In this case study, we report a live birth coupled with full time-lapse footage of its originating embryo from a patient who displayed complete ZP production failure.

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Case report

Patient history

Approval to perform this case report was obtained from an internal institutional review board (Repromed Scientific Advisory Committee-APP: 5.7 F2019 and the Monash IVF Queensland Medical Advisory Committee). A 34-year-old female and her same-age male partner presented with a history of failed fertilization via standard IVF insemination and a possible absence of the ZP. The recommendation by their previous clinic was to consider accessing donor oocytes. The patient was referred for a second opinion to a clinical geneticist who subsequently referred her to our clinic (Monash IVF Gold Coast). The patient had a normal female karyotype (46XX) with a BMI of 21.7 kg/m2, heterozygous for Factor V Leiden and Prothrombin 20210 G>A mutation and an anti-Mullerian hormone level in the normal range for her age (28.5 pmol/L). The male partner's semen analysis was normozoospermic.

Ovarian stimulation

The patient underwent a total of 3 intracytoplasmic sperm injection (ICSI) cycles, with all cycles utilizing an FSH + GnRH antagonist protocol. Briefly, stimulation began on day 3 of the cycle using highly purified urinary gonadotrophin Menopur 150-250IU/day (Ferring Pharmaceuticals, North Ryde, Australia) coupled with gonadotropin-releasing hormone (GnRH) antagonist (Ganirelix 250, MSD, Macquarie Park, Australia). Follicle development was monitored by ultrasound in conjunction with serum estradiol. For cycles 1 and 2, an hCG trigger (Ovidrel 500mg; Merck, Macquarie Park, Australia) was administered when two or more follicles ≥17 mm in diameter were present. Cycles 1 and 2 demonstrated a significant reduction from number COCs collected to number oocytes able to be inseminated. Therefore, for cycle 3, FSH dosage was increased to maximize number of COCs collected. A GnRH agonist trigger was utilized (Decapeptyl Depot; Ferring Pharmaceuticals, North Ryde, Australia) to minimize the risk of ovarian hyperstimulation, following which hCG (Pregnyl 500, Organon, Mansfield, Australia) was administered on the morning of oocyte retrieval to assist with luteal phase rescue. For all 3 cycles, luteal support was provided by using progesterone pessaries (Crinone, Merck, Macquarie Park, Australia) commenced daily on the evening after oocyte retrieval.

Gamete preparation

Oocytes were collected 36 h after trigger via transvaginal follicle aspiration under ultrasound guidance using a 16-gauge double lumen needle (Cook Medical, Eight Mile Plains, Queensland, Australia). The suction pressure was lowered to 60 mmHg to minimize damage to the cumulus oocyte complexes (COCs) during aspiration. Due to the absence of the ZP, it was difficult to identify COCs with viable oocytes at time of egg collection. All structures resembling COCs were isolated from the follicular fluid, washed gently in G-IVF+ (Vitrolife, Gothenburg, Sweden) and incubated at 37°C in 6% CO_2, 5% O_2, and 89% N_2.

The male partner produced the semen sample at the same time of the oocyte retrieval. Sperm preparation was undertaken using swim-up with 1ml of semen being placed at the bottom of a 14ml tube and overlaid by G-IVF+ (Vitrolife, Gothenburg, Sweden) and incubated for 30 min at 37°C, followed by isolation of the top layer of media (containing motile sperm) for subsequent insemination.

Denuding occurred at 39 h post trigger using a dilute hyalase solution of 37.5IU/ml (Hyalase®; Sanofi Aventis, Sydney, Australia) in G-IVF+ (Vitrolife, Gothenburg, Sweden). The cumulus cells were gently removed using a glass Pasteur pipette followed by a fine denuding pipette with a diameter of 200μm (RI EZ Strip, Cooper Surgical, Målov, Denmark). Very gentle manipulation was used to ensure that the fragile oocytes were not lysed and to prevent separation of the polar body from the oocyte.

ICSI and embryo culture

All oocytes where a polar body was observed underwent microinjection using gentle suction on the oolemma to ensure stability (this is due to the oolemma being much weaker than the ZP). For cycle 1 and 2, after microinjection oocytes were cultured in G-1+ (Vitrolife, Gothenburg, Sweden) under Ovoil (Vitrolife, Gothenburg, Sweden) at 37°C in 6% CO_2, 5% O_2, 89% N_2 in a MINC incubator (Cook Medical, Eight Mile Plains, Queensland, Australia). In the subsequent morning, fertilization was assessed, and unfertilized oocytes were removed from the dish, and embryos were cultured for a further 48 h (embryo corral dish®, Life Global, Målov, Denmark). On the morning of day 3, the embryos were carefully and extensively flushed with G-2+ culture media (Vitrolife, Gothenburg, Sweden) while remaining in the culture dish (to minimize any embryo handling) and cultured for a further 48 h to the blastocyst stage.

For cycle 3, to further minimize any disruption of these gametes/embryos, post-microinjection oocytes were cultured in G-TL single-step culture media (Vitrolife, Gothenburg, Sweden) supplemented with 10% human serum albumin under Ovoil (Vitrolife) in an Embryoscope+ time-lapse incubator (Vitrolife, Gothenburg, Sweden). In the subsequent morning, fertilization was assessed, and unfertilized oocytes were removed from the dish, and embryos were cultured for a further 48 h. On the morning of day 3, a media refresh was undertaken, and embryos were cultured for a further 48 h to

the blastocyst stage. Embryo development up to the day 6 was continuously monitored via time-lapse videography, with 10-min imaging intervals across 11 focal planes. Timing parameters were expressed as hours to reach different developmental milestones [8]. Abnormal biological events as reported by previous time-lapse studies, such as direct cleavage [9], reverse cleavage [10], less than 6 intercellular contacts at the 4-cell stage [11], and multinucleation at the 2- and 4-cell stages [12], were also assessed.

Embryo transfer

The embryo selected for transfer was incubated for a minimum of 10 min prior to transfer in EmbryoGlue (Vitrolife, Gothenburg, Sweden). Embryo transfer was undertaken on day 5 using a K-JET catheter (Cook Medical, Eight Mile Plains, Queensland, Australia) under ultrasound guidance.

Embryology and clinical outcomes

Embryology details in 3 cycles are summarized in Table 1.

Cycle 1: The first cycle resulted in 6 COCs being collected, 2 of which were suitable for insemination via ICSI. One oocyte was normally fertilized and subsequently formed a blastocyst, which was transferred on day 5. A low positive β-hCG was confirmed at 12mIU/mL 10 days following transfer, but level subsequently dropped indicating a biochemical pregnancy only.

Cycle 2: The second cycle resulted in 5 COCs being collected, 2 of which were suitable for insemination via ICSI. Two oocytes were normally fertilized; however, both arrested at day 3 due to cell disassociation and degeneration.

Cycle 3: The third cycle resulted in 24 COCs being collected, 13 of which were suitable for insemination via ICSI. Four oocytes were normally fertilized leading to the development of 2 blastocysts. One blastocyst was transferred and the other cryopreserved on day 5. Time-lapse observation demonstrated the delicate nature of cell-cell interaction during culture as shown in the video clip and still images (Supplemental Movie 1 & 2). Morphokinetic features of the transferred and cryopreserved blastocysts are presented in Table 2. The patient demonstrated a positive β-hCG level (163 mIU/mL) 8 days following transfer, and clinical pregnancy was confirmed via ultrasound (singleton) at 6 weeks and 4 days of gestation. A live delivery via cesarean section (due to intrauterine growth restriction) at 33.5 weeks of gestation was performed resulting in a healthy live female (1570g). Subsequent to birth, a blood clot was identified as present in the placenta. The patient was on clexane therapy throughout the pregnancy due to increased risk of thrombolytic events due to Factor V Leiden and Prothrombin 20210 G>A gene mutation. Despite small weight at birth, the baby girl did not require oxygen post-delivery and was discharged from hospital prior to due date. Follow-up of this live birth at 15 months post-delivery ascertained that the healthy baby girl has since met all developmental and growth milestones expected for age.

Discussion

The complete absence of ZP in oocytes is a rare event in IVF treatment; however, oocytes with damaged or partially absent zona due to laboratory manipulations are not uncommon [1, 5, 7, 13]. Consequently, much of the published experiences of ZP-free patients are cases of ZP damage and loss during handling, rather than a complete absence due to ZP production failure. The ZP forms during the early stages of follicle development and has been identified as serving an important role in protection of the oocyte, facilitation of sperm-egg interaction and fertilization, prevention of polyspermy, and maintenance of cell-cell contact prior to compaction [1]. While zona-free oocytes have been demonstrated to fertilize after ICSI, it is a challenge to maintain their cell-cell contact in the subsequent cleavage stages without the ZP restraint being present. It is interesting to observe that the embryo leading to the live birth in this case report managed to establish a relatively compact tetrahedral arrangement at the 4-cell stage, although the intercellular fragments appeared to have limited physical contacts between cells (Supplemental Movie 1). This is not commonly seen as was described in a previous study, where a spatial gap between 2 blastomeres at the 4-cell stage was found to adversely affect an embryo's implantation potential [11]. The approach for embryo culture in the third cycle of our reported case study was optimized to promote cell-cell contact,

Table 1 Treatment cycle summary

Cycle	Embryo culture approach	Number of oocytes collected	Number of oocytes injected	Number of oocytes normally fertilized	Number of embryo transferred (day)	Number of embryo frozen	Clinical outcome
1	G1+/G2+ in MINC	6	2	1	1 (D5)	0	Biochemical pregnancy
2	G1+/G2+ in MINC	5	2	2	NIL	0	Nil suitable to transfer
3	G-TL in Embryoscope+	24	13	4	1(D5)	1	Healthy live birth

Table 2 Morphokinetic features of the 2 normally fertilized zona-free oocytes that subsequently formed blastocysts in the cycle leading to live birth

	Blastocyst transferred	Blastocyst cryopreserved
tPNa	5.0	5.2
tPNf	22.6	23.0
t2	25.4	25.5
t3	35.8	35.4
t4	36.0	36.0
t5	47.9	47.8
t6	48.6	48.6
t7	49.9	48.8
t8	50.9	49.3
t9	66.0	66.3
tSC	76.5	78.4
tM	79.7	80.4
tSB	94.0	88.5
tB	105.1	94.7
MN@2c	1 out of 2	1 out of 2
MN@4c	Nil	Nil
RC	Nil	Nil
DC	Nil	Nil
<6 ICCPs at the 4-cell stage	Yes	Nil

Timing parameters were expressed as hours post insemination. *MN* multinucleation, *RC* reverse cleavage, *DC* direct cleavage, *ICCP* intercellular contact point

particularly during the pre-compaction stages. The design of microwells with a reduced diameter in the Embryoslide (Vitrolife, Gothenburg, Sweden) was believed to have assisted in this regard. Time-lapse footage elucidates the degree of cellular contact for the transferred and frozen embryos, which is maintained throughout cell divisions despite the absence of zona pellucida. The cells appear to have tenuous links immediately after cleavage, and the blastomeres quickly reassociate to maintain close contact. Development milestones observed between the two developing embryos is synchronous with little variation between the embryos, ultimately leading to two blastocysts which were utilized.

The physical stress of oocyte retrieval was also considered in our approach; in particular, the aspiration of follicles was identified as a potential risk to the fragile COCs. The suction pressure applied to aspirate follicles in this case was reduced by 50% to ensure more gentle aspiration, which did increase the time taken to undertake the oocyte retrieval however was necessary to ensure minimal damage to the COCs. Likewise, all handling procedures were carefully assessed and modified as required. The concentration of hyalase was also reduced by 50%, and the use of larger diameter pipettes was undertaken to minimize physical stress to the oocyte. In addition, the use of single-step culture medium in a time-lapse incubator enabled developmental milestones to be observed without disruption to the culture environment. Single-step culture media coupled with the unique Embryoslide design enabled an in situ media refresh without the need to handle embryos. Once embryos had developed to the blastocyst stage with the presence of tight cell-to-cell contact, transfer and vitrification could take place in accordance with routine procedure. One potential limitation in cycle 3 is the media refresh of G-TL performed at day 3, as this is not suggested by the manufacturer. Further improvement could be achievable via maximizing the full benefits of such single-step media without the need to refresh, as demonstrated previously [14].

In comparison to a previous report regarding successful live birth of complete zona-free patients due to ZP production issue [7], this case study reported a set of modified protocols in detail along with full time-lapse footage of blastocyst development. Due to the rare occurrence of such case, statistical analysis with a reasonable sample size to demonstrate which culture environment is best for these oocytes is deemed to be too difficult to acquire. While the early developmental footage of such embryos is considered novel, further data is required to determine the morphokinetic variation in the early development of such embryos. Recent time-lapse study has described morphokinetics of embryos with mechanically removed ZP [15], but there is little evidence demonstrating their similarity to the currently reported case. Further data is warranted in order to better understand the role of ZP production during early embryogenesis and morphokinetic parameters.

The mechanism behind complete absence of ZP as a result of production failure is unclear. It is reported that genes

expressing ZP proteins are highly conserved between mice and humans and the functionality of ZP1, ZP2 and ZP3 is purported to be similar between the two species [2]. Studies undertaken in mice that perturb the ZP3 gene have explored the impact of ZP3 upon ZP formation. Female mice without ZP3 demonstrated complete absence of the ZP, disorganized coronal radiata and granulosa cells, and subsequent infertility [16]. This suggests that mutation to the ZP3 gene in humans may cause total absence of the zona pellucida as observed in this case study. Exploration of potential changes to ZP3 gene in this patient may offer further insight into the cause. The preterm delivery of the baby girl was noted in this case; however, whether or not it is related to the ZP production issue is yet to be identified, given the only other report of a similar case led to a twin delivery which is not considered comparable. Neonatal outcomes (if applicable) following transfer of the cryopreserved blastocyst in this reported patient may offer additional data in this regard.

In conclusion, this case report has demonstrated that an optimized approach to ovarian stimulation, oocyte retrieval, handling, and embryo culture may promote successful blastulation and healthy live birth in patients with failed ZP production. In particular, the utilization of single-step media coupled with specially designed Embryoslide to maintain intercellular contact seemed to be advantageous and yielded successful outcome.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-021-02114-3.

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