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ARTICLE

Pregnancy and live birth following the transfer of vitrified–warmed blastocysts derived from zona- and corona-cell-free oocytes

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Abstract This study reports two clinical pregnancies and one live birth following the transfer of vitrified blastocysts developed from oocytes with neither zona pellucida nor corona cells. Two zona-free oocytes obtained from two patients of advanced maternal age undergoing minimal stimulation were normally fertilized after intracytoplasmic sperm injection. In case 1, all four blastomeres of the zona-free embryo were loosely associated and inserted back into ruptured zona on day 2. Zona-free embryo from case 2 had tight contacts between blastomeres and was cultured without zona. Both embryos derived from zona-free oocytes progressed to blastocyst stage and were cryopreserved by vitrification. When patients came back for a cryopreserved embryo transfer, both vitrified blastocysts survived warming. In case 1, transfer of a warmed blastocyst with reconstructed zona resulted in a clinical pregnancy that ended in a spontaneous abortion at 22 weeks. In case 2, live birth was achieved with a normal healthy baby (male) weighing 2381 g at 40 weeks' gestation. This report emphasizes the importance of maintenance of normal cell arrangement on the subsequent embryonic development for a zona-free oocyte. Zona-free oocytes may provide a valuable source of embryos for infertility patients, especially for those with a limited number of oocytes.

KEYWORDS: cell arrangement, live birth, minimal stimulation, reconstructed zona, vitrification, zona-free oocyte

Introduction

The zona pellucida is a glycoproteinaceous surrounding the mammalian oocyte and early embryo. *In vivo*, zona pellucida has been shown to play a critical role in species-specific spermatozoa–egg interaction, induction of acromosome reaction and avoidance of polyspermy, maintenance of the integrity of a pre-compacted embryo and protection of the early embryo against the maternal immune system (Barnea, 2004; Henkel et al., 1998). Zona-free oocytes are

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accidentally encountered in IVF when cumulus–oocyte–complexes are mechanically removed by repeat pipetting at the time of mechanical removal of cumulus. To maximize the number of embryos available for transfer, zona-free oocytes were sometimes injected. Although it has been reported that a zona-free human oocyte had the ability to be normally fertilized and further develop to the blastocyst stage (Ding et al., 1999; Hsieh et al., 2001; Stanger et al., 2001; Takahashi et al., 1999), developmental fate of the zona-free oocyte is still unclear.

The presence of zona pellucida allows for the normal course of preimplantation embryonic development without deviation of cell association. As shown in a mouse study, the absence of zona pellucida before compaction occurs affects the cell connection between blastomeres (Suzuki et al., 1995). So far there is only one case report describing a pregnancy that was achieved following the transfer of embryos derived from zona-free, coronal-cell-intact oocytes (Stanger et al., 2001). In their study, the intact corona cells surrounding the zona-free oocytes effectively achieved the same function of the zona pellucida, providing sufficient support to maintain blastomere interaction and embryo viability. No pregnancy has been reported following the transfer of an embryo without any support from neither zona pellucida nor corona cells.

The study clinic has injected a total of 30 zona-free oocytes during the last 4 years. Because embryos originating from zona-free oocytes were chosen for transfer only when there are no other good-quality embryos from zona-free oocytes, there were only four exclusive transfers of embryos derived from zona-free oocytes. This communication reports two successful pregnancies and one live birth following the transfer of vitrified blastocysts following intracytoplasmic sperm injection of zona- and corona-cell-free oocytes.

Case 1

A 43-year-old patient was referred to the centre for fertility treatment due to advanced maternal age and unobstructive azoospermia in her 46-year-old husband. They had two failed prior cycles. Testicular sperm extraction was preformed before the initiation of the cycle. Retrieved testicular spermatozoa was cryopreserved using a 1:1 dilution with an egg yolk freezing medium containing 12% glycerol (v/v) (Irvine Scientific, Santa Ana, CA). The mixture was then refrigerated at 4°C for 60 min and cooled in nitrogen vapour for 30 min before being plunged into liquid nitrogen tank. For thawing, the frozen vial was removed from storage and immersed in 37°C water bath for 5 min.

Ovulation induction was performed by minimal stimulation protocol, using clomiphene citrate (Serophene; Serono, Norwell, MA) and low-dose human menopausal gonadotrophin (Menopur, 75 IU LH and 75 IU FSH; Ferring Pharmaceuticals, Suffern, NY). Stimulation was started with clomiphene citrate 50–100 mg a day from day 3 to day 7, followed by 1–2 ampoules of human menopausal gonadotrophin for 4 days. Human chorionic gonadotrophin (HCG) injection (10,000 IU) (Novarel; Ferring Pharmaceuticals) was performed when the dominant follicle reaches 18 mm. Oocyte retrieval was performed 36 h after HCG administration. A total of three oocytes were retrieved. Cumulus–oocyte–complexes were exposed to 80 IU/ml hyaluronidase (SAGE In-Vitro Fertilization, Trumbull, CT, USA) for 1 min and cumulus cells were repeatedly aspirated through a finely pulled glass pipette. Unfortunately, one oocyte escaped from the ruptured zona pellucida during the oocyte denudation and became zona-free without the first polar body being attached to the oocyte. In view of the limited number of oocytes available and advanced maternal age, both zona-intact and zona-free oocytes were injected using frozen testicular spermatozoa. Two oocytes were normally fertilized, of which one was from the zona-free oocyte (**Figure 1A**). Oocytes and embryos were cultured in 20-μl microdrops of Quinn's Advantage Cleavage Medium supplemented with 10% (v/v) serum substitute supplement (SSS; SAGE In-Vitro Fertilization) until the morning of day 3. After that, embryos were transferred to 20-μl microdrops of Quinn's Advantage Blastocyst Medium (SAGE In-Vitro Fertilization) supplemented with either 10% SSS for extended culture.

Both fertilized oocytes developed to 4-cell stage on day 2. Fresh embryo transfer was performed on day 2, with priority being given to a 4-cell-stage embryo derived from a zona-intact oocyte. As shown in **Figure 1B**, the 4-cell-stage embryo developed from a zona-free oocyte has all four blastomeres in line and there were loose contacts between blastomeres.

To prevent the zona-free embryo from falling apart, all four blastomeres were inserted back into the ruptured zona pellucida using a medium-sized Blastomere Biopsy Micropipet (33–37 μm; Humagen, Charlottesville, VA) under inverted microscope. The reconstructed embryo was then further cultured for cryopreservation (**Figure 1C** and D). On day 6, a hatching blastocyst with clear inner cell mass and trophectoderm was obtained and vitrified using Cryotop as described by Kuwayama et al. (2005) and summarized as followed. HEPES-buffered TCM 199 supplemented with 20% SSS (Irvine Scientific) was used as base medium. The equilibration solution contained 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethylsulfoxide (DMSO; both from Sigma Chemical Co., USA). The vitrification solution was composed of 15% (v/v) ethylene glycol, 15% (v/v) DMSO and 0.5 mol/l sucrose (Sigma Chemical Co.). Prior to the cooling steps of vitrification, artificial shrinkage of the blastocoele was performed by drilling a hole with one single laser pulse (200 ms) generated by laser system ZILOS-tkTM (Hamilton Thorn Bioscience, Beverly, MA, USA). To avoid herniation of embryonic cells, the laser pulse targeted at the cellular junction of the trophectoderm epithelium opposite to the inner cell mass. Shrinked blastocyst was first equilibrated in equilibration solution for 15 min, after which it was transferred to vitrification solution, incubated in vitrification solution and loaded on to the tip of the Cryotop (Kitazato BioPharma) with 0.1 μl or less of vitrification solution within 1 min. The Cryotop was then immediately plunged into liquid nitrogen and inserted into a pre-cooled cover straw with the aid of a forceps.

Two months later, the patient came back for a frozen embryo transfer after a failed fresh embryo transfer. The vitrified blastocyst derived from a zona-free oocyte was then warmed on the morning of day 5 after ovulation. The protective cover was removed from the Cryotop while it was still submerged in liquid nitrogen. Vitrified embryos were warmed by directly immersing the polypropylene strip

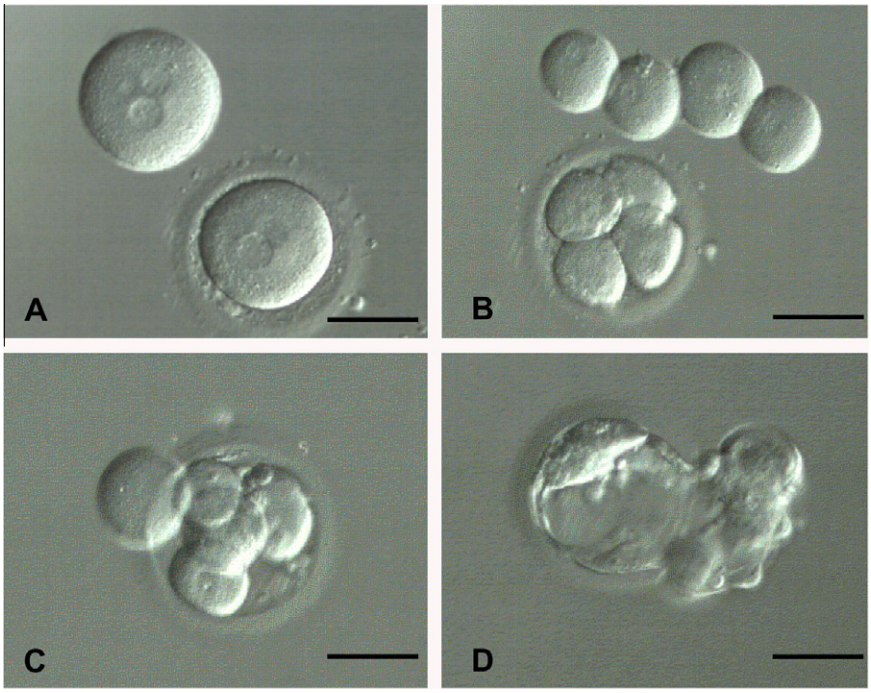

Figure 1 Embryonic development after zona pellucida reconstruction in case 1. (**A**) Normally fertilized zona-intact and zona-free oocytes. (**B**) A 4-cell-stage zona-intact embryo and a zona-free embryo with loosely associated blastomeres on day 2. (**C**) Reconstructed embryo after insertion of blastomeres into a ruptured zona pellucida. (**D**) Hatching blastocyst on day 6. Bars = 50 μm.

of the Cryotop into 5 ml pre-warmed 1.0 mol/l sucrose solution maintained on a warm stage at 37°C for 1 min. Embryos were then transferred into 5 ml 0.5 mol/l sucrose solution for 3 min, after which the warmed blastocyst were washed twice in TCM 199 with 20% SSS for 5 min each before being returned to culture in Sage Blastocyst Medium (Cooper Surgical, Trumbull, CT, USA).

The vitrified blastocyst derived from a zona-free oocyte survived the warming and showed full blastocoele re-expansion 2 h after warming. Positive HCG was obtained 10 days after embryo transfer and pregnancy was confirmed by ultrasound observation of fetal heart beat 4 weeks after the positive HCG. Unfortunately, the patient experienced a spontaneous abortion at 22 weeks.

Case 2

A 42-year-old female presented with advanced maternal age and high FSH (14 IU/l). Clomiphene citrate (Serophene) was given two tablets (50 mg/tablet) by mouth daily, starting from day 5 to day 9 of the menstrual cycle. Only one oocyte was retrieved. On the day of oocyte retrieval sperm analysis of the husband gave the following results: sperm concentration 9×10^6/ml with 61% motility. After the removal of cumulus cells, the oocyte was found to be zona-free with the first polar body being attached. Intracytoplasmic sperm injection (ICSI) was performed on the zona-free oocyte and two distinct pronuclei were present 16 h following sperm injection (**Figure 2A**). The fertilized zona-free oocyte developed to 2-cell stage with tight cell contact between blastomeres on day 2, underwent compaction on day 3 and progressed to the blastocyst stage on day 5 (**Figure 2B—D**). To accumulate more embryos for future embryo transfer, the zona-free blastocyst was cryopreserved using the protocol described in case 1.

One month later, the patient presented again for another treatment cycle. However, no oocyte was retrieved and fresh embryo transfer was cancelled. Vitrified blastocyst obtained from the zona-free oocyte from last retrieval cycle was then warmed on the fifth day after ovulation after gaining informed consent from the couple. Two hours post warming, the blastocoele re-expanded and the warmed blastocyst was replaced. Positive HCG was obtained 2 weeks after embryo transfer. Fetal heartbeat was confirmed by transvaginal ultrasound 6 weeks after embryo transfer. The pregnancy progressed normally with the patient giving birth to a normal healthy baby (male) weighing 2381 g at 40 weeks' gestation.

Discussion

As far as is known, this report documents the first pregnancy and live birth following the replacement of a vitrified—warmed blastocyst derived from a zona- and corona cell-free oocyte. In case 1, the clinical pregnancy was established by replacing a blastocyst which developed from a 4-cell-stage zona-free embryo where zona pellucida reconstruction was performed. It is also interesting to note that the zona-free embryo in case 1 was generated by ICSI using frozen testicular spermatozoa.

In light of the fact that the oolemma is much weaker than the zona pellucida, very gentle aspiration was used to immobilize the zona-free oocyte during the ICSI procedure. Similar to a zona-intact oocyte, a zona-free oocyte is injectable as long as the first polar body is attached. Loss of the first polar body happens occasionally at the removal

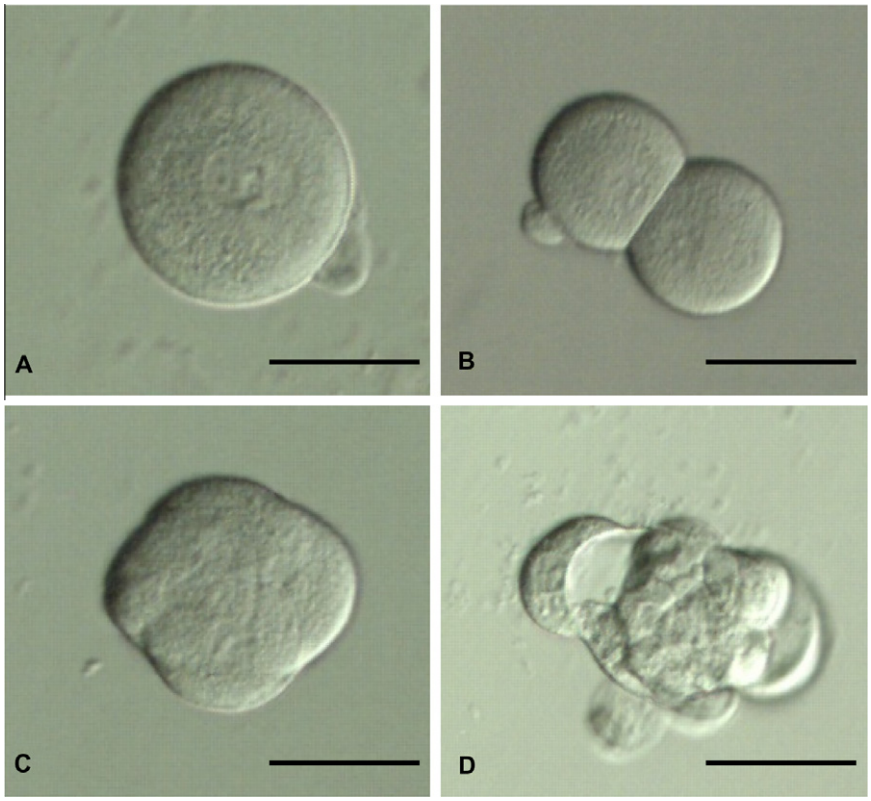

Figure 2 Embryonic development of a zona-free oocyte (case 2). (**A**) Fertilized zona-free oocyte. (**B**) A 2-cell-stage zona-free embryo with tight contact between blastomeres on day 2. (**C**) Compacted zona-free embryo on day 3. (**D**) Zona-free blastocyst on day 5. Bars = 50 μm.

of cumulus cells, making it difficult to tell the oocyte maturational status and to detect the location of meiotic spindle. This and other published reports (Ebner et al., 2004) show that a zona-free oocyte without a polar body could also be normally fertilized and initiate embryo cleavage in vitro. Due to the limited number of zona-free oocytes in published reports, it is still unclear whether a zona-free oocyte is more prone to damage from the ICSI procedure. Since the vitelline membrane of a zona-free oocyte is easily disrupted, special care should be taken to avoid lysis caused by adhesion of a zona-free oocyte to the dish and transfer pipette.

In addition to its important role in protecting the loss of blastomeres from early embryos, the zona pellucida plays a critical role in maintaining the appropriate cell arrangement. In a mouse model, Suzuki et al. (1995) evaluated the developmental ability of 4-cell-stage zona-free embryos according to the total points of contact among the four blastomeres. Embryos with three, four, five or six points of contact were classified as A, B, C and D, respectively. Their results showed that the majority of zona-intact embryos (87%) had six points of contact between the four blastomeres whereas only 15% of zona-free embryos had six points of contact. Although the cell number of blastocysts did not differ, blastocysts obtained from type A embryos had fewer inner cell mass than did either the zona-intact control or type D embryos. In addition, transfer of blastocysts derived from type A embryos resulted in significantly lower live fetuses than those from type D. This study demonstrated that while a fertilized zona-free oocyte could undergo cleavage and form a blastocyst *in vitro*, the absence of zona pellucida did affect the cell association, successful cell lineage and implantation potential.

It has been demonstrated that zona pellucida was no longer essential for continued normal embryonic development *in vitro* once compaction has occurred (Nichols and Gardner, 1989; Trounson and Moore, 1974). Based on the assumption that embryo hatching may be disturbed in some patients, zona pellucida was enzymically removed prior to embryo transfer. Pregnancies resulting from transfer of zona-free embryos have been documented in several published reports. In a prospective, randomized study performed by Urman et al. (2002), zona-free blastocyst transfer increases the success of blastocyst-stage transfer in patients with poor-quality blastocysts. It should be emphasized that the zona-free embryos in above study were originally from zona-intact oocytes and the removal of zona pellucida was performed at a more advanced stage when the embryos had initiated compaction. Events of the preimplantation embryonic development and cellular association of embryos derived from zona-free oocytes may very well be different in comparison with those from zona-intact oocytes.

A zona-free embryo provides a good model for studying the mechanism underlying early embryonic development. The capability of a fertilized zona-free human oocyte to

form a blastocyst *in vitro* has been observed (Ebner et al., 2004; Hsieh et al., 2001; Stanger et al., 2001); however, zona-free embryos tended to have abnormal early embryo development due to the abnormal cell—cell contacts (Liu et al., 2004). As demonstrated by Suzuki et al. (1995), the cell association/orientation of zona-free mouse embryos at the 4-cell stage influences differentiation into the inner cell mass and subsequent embryonic development to term. Artificial zona pellucida has been suggested as a strategy to rescue a zona-free embryo by increasing cell—cell contacts and restoring normal blastomere polarization. Results from different groups showed encapsulation of mouse embryos with sodium alginate improved embryonic developmental competence (Elsheikh et al., 1997; Liu et al., 2004), suggesting the importance of maintenance of normal cell arrangement before compaction occurs. In human, the embryo does not develop surface polarity before the 8-cell stage and all blastomeres are totipotent at this stage (Dale et al., 1995), indicating that 4-cell stage is an appropriate stage for zona pellucida reconstruction.

Successful pregnancies were achieved following the transfer of embryos derived from zona-free oocytes in both case 1 and case 2; however, significant differences existed in the cell association of zona-free embryos on day 2. The divided zona-free embryo in case 2 had tight blastomere contact and initiated compaction on day 3, which behaved similarly to normal early embryos derived from oocytes with intact zona. In case 1, cell arrangement of the 4-cell-stage embryo from the zona-free oocyte belongs to type A, with all the four blastomeres forming a flattened array. This type, according to Suzuki et al. (1995), has significant lower cell number of the inner cell mass. Insertion of blastomeres back into zona pellucida not only prevents cell loss during embryo culture and micromanipulation, but more importantly, it rescued the deviated embryonic structure, enabling the early embryo to initiate its normal course of preimplantation development. It is therefore assumed that it is not the zona pellucida *per se* but the cell association that determines blastocyst formation and further embryonic developmental potential.

The high efficiency of vitrification technique has allowed it to enter more and more into the mainstream of human assisted reproductive technology in recent years. Partially hatching and completely hatched blastocysts have been reported to successfully survive cryopreservation (Hiraoka et al., 2004a; Quintans et al., 2001; Zech et al., 2005). A recent report showed transfer of a vitrified day-7 spontaneously hatched blastocyst yielded a live birth (Hiraoka et al., 2009). In this study, viability of vitrified/warmed blastocysts developed from zona-free oocytes was verified by the successful pregnancies following embryo transfer, suggesting that a blastocyst obtained from a zona-free oocyte is able to successfully tolerate the vitrification—warming process. Beneficial effects of artificial shrinkage prior to cryopreservation on survival and pregnancy outcome of expanded blastocysts have been documented in previously reported reports (Hiraoka et al., 2004b; Mukaida et al., 2006). For this reason, the study centre performed artificial shrinkage by laser on the expanded blastocyst derived from a zona-free oocyte in case 1. Artificial shrinkage was not performed in case 2 because the embryo was still at the expanding blastocyst stage without an increased diameter.

In summary, this study demonstrates that a zona-free oocyte may be used as a source of oocytes for sperm injection in ICSI cycles, especially for those patients with a limited number of oocytes. While a zona-free oocyte could be normally fertilized and cleave and develop to the blastocyst stage *in vitro*, this report emphasizes the importance of normal cell arrangement on the subsequent development of a zona-free embryo. A further study is required to investigate the effects of cell—cell contacts and spatial positions of blastomeres on preimplantation development of a zona-intact human embryo.

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