

CASE REPORT

Intracytoplasmic sperm injection into zona-free human oocytes results in normal fertilization and blastocyst development

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Zona-free human oocytes are frequently encountered in in-vitro fertilization (IVF) laboratories. The oocytes escape out of the zona pellucida, following zona fracture, which can occur during oocyte retrieval or manipulation, but occasionally may be the result of increased zona fragility. Some of the zona-free oocytes are mature and morphologically healthy; nevertheless, all are typically discarded. In this report, we demonstrate that zona-free oocytes can be fertilized normally using intracytoplasmic sperm injection (ICSI) and can subsequently develop without zona to the blastocyst stage *in vitro*. We therefore suggest that those mature and morphologically normal zona-free oocytes may be rescued, fertilized with ICSI and then cultured to the blastocyst stage for subsequent transfer or cryo-preservation.

Key words: blastocyst formation/ICSI/zona-free oocytes/zona-free zygotes

Introduction

Intracytoplasmic sperm injection (ICSI) has become a common approach to the management of male factor infertility. Before performing ICSI, cumulus cells surrounding the oocyte are removed using hyaluronidase and gentle pipetting with a fine pulled, flame polished glass pipette. During the procedure, zona pellucida occasionally can be damaged, resulting in the partial or total extrusion of the ooplasm out of the zona pellucida. This can also happen during conventional in-vitro fertilization (IVF), when cumulus cells are being mechanically removed. Such zona-free oocytes are generally discarded. It has been suspected that the majority of these oocytes are degenerated. However, it is likely that at least some are healthy and only their zonae pellucidae have increased fragility or were mechanically disrupted. If so, such zona-free oocytes may be fertilized by ICSI and the resulting embryos may develop normally and produce normal pregnancies. This is supported by the finding that mouse zona-free zygotes can develop to the blastocyst stage *in vitro*, then implant *in vivo* and produce live offspring (Suzuki *et al.*, 1995). Human blastocysts with artificially removed zonae can also implant after transfer and produce normal pregnancies (Fong *et al.*,

1997). Here we report two cases of zona-free human oocytes successfully fertilized by ICSI, which subsequently developed to blastocysts *in vitro*.

Case reports

Case 1

A 33-year-old woman underwent an IVF/ICSI cycle because of male factor infertility. A total of 26 oocytes was retrieved. Of these, 12 were subjected to ICSI. One of them partially escaped from the zona pellucida during removal of the cumulus using hyaluronidase (~80 units/ml, type VIII, from bovine testes; Sigma, St Louis, MO, USA) and followed by repeated pipetting with a flame pulled and polished pipette. Morphologically, the oocyte appeared normal and it was judged mature since all the sibling oocytes were mature. The oocyte was injected successfully with one spermatozoon. All sperm-injected oocytes were then co-cultured with an autologous, expanded cumulus in 30 µl drop IVF-50 (Scandinavian IVF SciAB) medium covered with mineral oil (OvaOil 150, Scandinavian IVF SciAB) in an organ culture dish (double-well, Falcon 3037). The zona-free oocyte fertilized successfully and cleaved to the 2-cell stage on day 2 and to a 4-cell pre-embryo on day 3. At that stage, 30 µl of Hatch-50 medium (Scandinavian IVF SciAB) was added into the culture drop (1:1 ratio mix). Although one blastomere degenerated and broke away at the 4-cell stage, the rest of the blastomeres continued to divide and reached the 8–10 cell stage on day 4. On that day, 30 µl of culture medium was removed from the culture drop and replaced with 30 µl of fresh Hatch-50 medium. The pre-embryo was compacted by day 5 and formed an early blastocyst by day 6. At that stage, the pre-embryo was cryopreserved without zona using glycerol as cryoprotectant.

Case 2

This case involved a donor-recipient cycle. Eleven eggs were retrieved from a 24-year-old donor. Four eggs were degenerated and seven were normal. During removal of cumulus, one egg escaped from its zona pellucida (Figure 1a). This egg was judged mature since all the sibling oocytes were mature. Again, this zona-free egg was injected with one spermatozoon (Figure 1b) from the recipient's husband and cultured in 30 µl IVF-50 medium covered with oil. The egg was normally fertilized (Figure 1c), and cleaved normally to the 2-cell (Figure 1d), 3-cell (Figure 1e), 4-cell (Figure 1f), 6-cell (Figure 1g), and 8-cell (Figure 1h) stages. At the 8-cell stage (day 3),

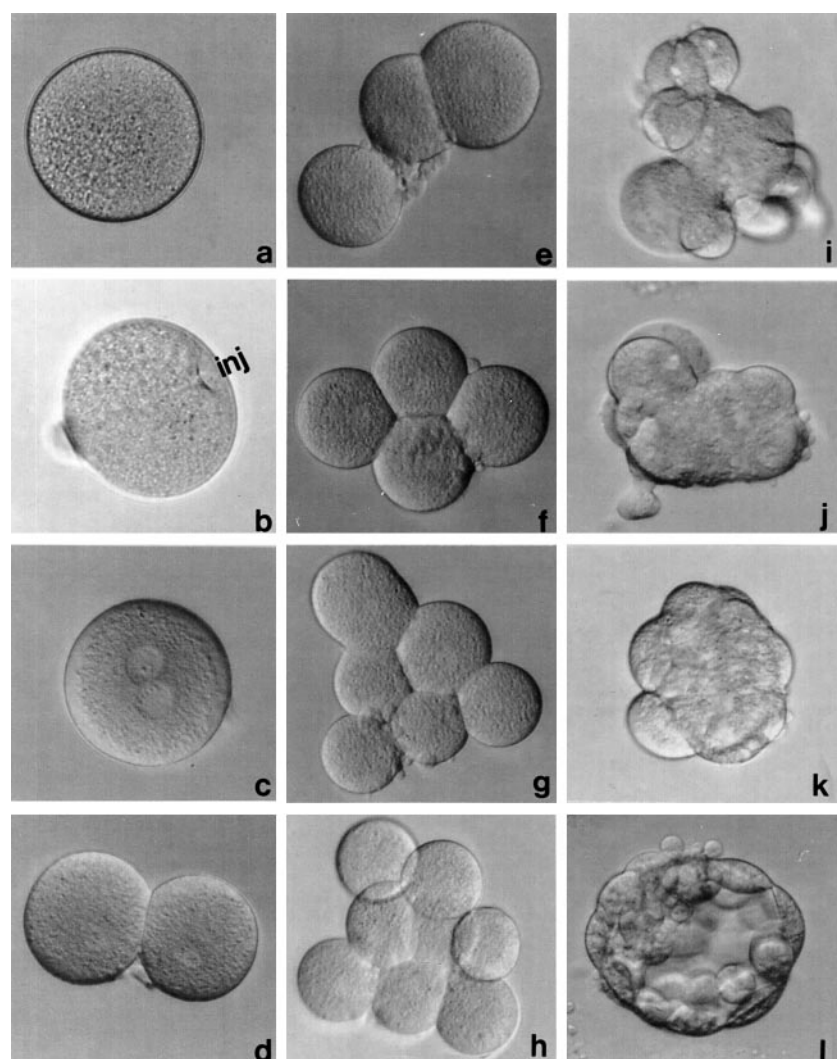


Figure 1. Case 2. (a) Zona-free oocyte; (b) following ICSI (inj: injection site); (c) zona-free zygote at 16 h post-ICSI (hpi); (d)–(h): zona-free pre-embryo at 2-cell (33 hpi), 3-cell (42 hpi), 4-cell (48 hpi), 6-cell (66 hpi) and 8-cell (70 hpi) stages; (i)–(k): compaction process on day 4 (88, 92 and 96 hpi respectively); (l): zona-free blastocyst on day 5 (112 hpi).

IVF-50 medium was replaced with S2 medium (Scandinavian IVF SciAB). On day 4, the embryo underwent a dramatic compaction process and formed a compact morula (Figure 1 i–k). By day 5, it developed to an early blastocyst (Figure 1l), which was transferred together with a zona-intact, expanded blastocyst to the recipient. Unfortunately, implantation did not occur.

Discussion

This report demonstrates that (i) zona-free human oocytes can be successfully fertilized by ICSI and (ii) fertilized zona-free human eggs can undergo normal cleavage, compaction and blastocyst formation *in vitro*. To our knowledge, this is the first report of two such cases.

It is well established that the zona pellucida plays an important, primarily protective role during fertilization and early embryonic development. First, it prevents cross species and polyspermic fertilization (Edwards and Brody, 1995). Sperm receptors on the zona pellucida (presumably ZP3), which facilitate sperm–egg binding, are highly species specific.

After sperm binding, ZP3 induces the acrosome reaction, which is essential for bound spermatozoa to penetrate zona pellucida. Subsequent to sperm penetration and oolemma binding, oocytes release cortical granules into the vitelline space, causing zona reaction that prevents polyspermic fertilization. Removal or fracture of the zona pellucida will result in a dramatic increase in polyspermic fertilization. Second, zona pellucida plays a protective role during early embryonic development. Early pre-embryos cleave inside the zona pellucida when blastomeres are loosely attached to each other and can easily be dissociated. The zona holds blastomeres together before compaction occurs. Thus zona-free early cleavage embryos may not be suitable for transfer into the uterus, since the blastomeres may fall apart during manipulation and/or after transfer. During compaction, however, tight junctions develop between outside cells of the embryo, sealing off the inside of the sphere and forming a compact ball. At this stage, zona-free embryos may survive the transfer procedure. Indeed, pregnancies resulting from transfer of zona-free blastocysts have been documented in humans (Fong *et al.*, 1997), cattle (Warfield *et al.*, 1987) and mouse (Suzuki *et al.*, 1995).

It has been suggested that blastomeres from early cleavage embryos are polarized (Edwards and Beard, 1997) and later polarization of microvilli and surface markers can be demonstrated at the 8-cell stage as inner and outer cells differentiate. Polarized cells allocate to the trophoctoderm lineage and apolar cells to the inner cell mass lineage (see Johnson *et al.*, 1986). Removal of zona pellucida may have an impact on the cell-cell contacts during early cleavage. As shown in Figure 1, before compaction, cells were arranged in a flattened array and cell contacts with neighbouring cells were different from those in the zona-intact embryos. This phenomenon is also seen in the mouse (Suzuki *et al.*, 1995). Four types of 4-cell mouse embryos which developed from zona-free fertilized oocytes were observed according to total points of contact between the blastomeres (Suzuki *et al.*, 1995). Types A, B, C and D had three, four, five and six points of contact respectively (all formed a flattened array except type D, which was the most common type in zona-intact embryos, representing 87%). It was noted that although blastocyst formation rate was not different between the four types of 4-cell zona-free mouse embryos, type A had fewer inner cell mass cells and had a significantly lower rate of embryos developing to live fetuses than type D embryos and the zona-intact embryos. This indicates that normal polarization of blastomeres was affected, probably because of the decrease in blastomere contacts. Nevertheless, even the type A embryos had the potential of developing into live fetuses. In the present report, at the 4-cell stage both zona-free human embryos belonged to type C (Figure 1f, five points of contact) and both underwent compaction and developed to the blastocyst stage, suggesting that such embryos have the potential of developing into live fetuses.

Edwards and Hollands (1988) reported that the individual separated human 2-cell blastomeres without zona pellucida, although capable of replication, failed to undergo compaction. Bolton (1991), on the other hand, observed the compaction process of zona-free human embryos, but noted that abnormal blastocysts were formed with multiple blastocoelic cavities. This, however, might have been caused by sub-optimal culture. This assumption is supported by the fact that the zona-intact blastocysts when transferred in the Bolton system resulted in a pregnancy rate of only 10% (3/29) with an implantation rate of 7% (Bolton *et al.*, 1991). This rate is low when compared to others (Menezo *et al.*, 1995: 31%; Gardner *et al.*, 1998: 63% with implantation rate of 45%) and ours (54.8% with an implantation rate of 28%, data not shown). However, further improvement of the culture system for zona-free zygotes should be considered. As suggested by Edwards and Hollands (1988), zona-free zygotes may be cultured in agar or viscous media, or even be replaced in recipient zona pellucida to hold the blastomeres together until compaction occurs.

It is difficult to evaluate the maturity of the zona-free oocytes, since they usually lose their first polar bodies during the escape from their zonae pellucidae. In the present report, the maturity of the zona-free oocytes was judged according to the maturity of their sibling oocytes, which may not be entirely accurate. It has been reported that immature human oocytes lacked the ability fully to decondense the penetrated

spermatozoa (Lopata and Leung, 1988). When checked 4 h post-insemination, the sperm heads located in MI oocytes were partially decondensed, while the sperm heads in germinal vesicle (GV) oocytes and germinal vesicle breakdown (GVB) oocytes had not undergone detectable decondensation. Therefore, if the zona-free oocytes were not mature, they most likely did not possess the fertilization and developmental ability. However, a recent report by Van Blerkom *et al.* (1994) showed that after 36 h of culture post-insemination, sperm penetrated human GV oocytes completed both the first and second steps of meiosis and formed both female and male pronuclei. This observation indicates that even if an immature (GVB or MI) oocyte is injected, the female nucleus is still able to complete its meiosis. But whether the 'fertilized' oocytes possessed the capability for further development was not tested. It is therefore necessary further to examine the karyotype of the embryos developed from fertilization of zona-free oocytes by ICSI.

Another problem associated with the ICSI of zona-free oocytes is not knowing the intracellular location of the metaphase spindle. If the spindle was to be disrupted by the ICSI procedure, abnormal fertilization would result. Therefore, in such cases, the zygotes should be carefully examined for the evidence of normal fertilization.

Collectively, we conclude that healthy zona-free mature eggs may be rescued by ICSI and in-vitro culture to the blastocyst stage for transfer. Such embryos may have a good chance for producing normal pregnancies, but more studies are required.

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