CLINICAL STUDY

Intracytoplasmic Sperm Injection in Zona-free Oocytes

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Abstract

Excessive manipulation of cumulus-oocyte –complexes during preparation for ICSI can result in zona-free oocytes. Using a modified ICSI technique such gametes may be considered for injection. After checking six nude oocytes for loss of ooplasm intracytoplasmic injection was performed resulting in four zygotes, of which three embryos derived. Blastomeres showed a atypical elongated appearance but on day 6 two out of three embryos reached full blastocyst stage. To conclude, ICSI of zona-free oocytes and subsequent culture to blastocyst stage can be performed successfully and for ethical reasons affected gametes should not be disposed any longer.

Keywords: blastocyst stage, embryo, ICSI, zona-free oocyte, zona pellucida

Özet

Zonasız Oositlerde İntrasitoplazmik Sperm İnjeksiyonu

Kumulus-oosit kompleksinin ICSI hazırlığı esnasında fazla manipülasyonu zonasız oositlerin oluşumuna neden olabilmektedir. Modifiye bir ICSI tekniği kullanılarak bu gametler injeksiyon için düşünülebilir. Bu çalışmada altı oosite, ooplazm kaybı olup olmadığı değerlendirildikten sonra intrasitoplazmik sperm injeksiyonu uygulanmış ve dört zigot elde edilmiştir. Bu dört zigottan da üç embriyo elde edilmiştir. Blastomerlerin atipik uzanım göstermelerine rağmen altıncı günde üç embriyodan ikisinin blastokist safhasını tamamladığı izlendi.
Sonuç olarak, zonasız oositlere ICSI uygulaması ve bunların blastokist safhasına kültüre edilmeleri başarılı bir şekilde yapılabilmektedir. Bu nedenle, zonasız gametlerin gözden çıkarılması etik açıdan uygun bulunmamaktadır.

Anahtar sözcükler: blastokist evresi, embriyo, ICSI, zonasız oosit, zona pellucida

Introduction

In primordial follicle oocyte is only surrounded by a monolayer of granulosa cells. As the follicle develops, the granulosa cells multiply and establish extensive processes towards the oocyte. In the cleft between the two zona pellucida (ZP) forms. Despite considerable speculation about the origin of this acellular glycoprotein layer (15-20 μm) it has now been accepted that all zona proteins are synthesized exclusively by the oocyte in a coordinate manner (8). Experiments, primarily in the mouse, have led to the conclusion that zona pellucida in mammalian oocytes consists of three zona proteins. In detail, filaments are constructed of repeating ZP2-ZP3 units which are cross-linked by ZP1 (27), thus, contributing to the structural integrity of the zona matrix. Recently, characterization of involved genes demonstrated that there are in fact four ZP genes (15) and, consequently, four ZP glycoproteins are expressed in the human (17).

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However, around fertilization zona pellucida has several functions including species-specific sperm binding (ZP3), inducing acrosome reaction in order to prevent polyspermy (ZP2). After fertilization zona plays a role in protecting the integrity of the developing embryo and it also assists its oviductal transport. Developing embryos show a gradual thinning of the zona pellucida during culture (2, 28). Closely associated with expansion this thinning reaches its climax prior to rupture which seems to be mediated by zona lysins (22) and/or uterine enzymes (20).

In view of the importance of the zona pellucida it is likely that any irregularity in composition, thickness, colour or shape may hinder optimal function and result in reduced outcome. In extreme, oocytes of certain patients can be characterized by expression of very thin zonae (23) or complete absence of ZP (26). This phenomenon is more likely to have a genetic rather than environmental cause (18), e.g. a problem in ZP1 expression (23). However, in lack of the protective matrix of the ZP such oocytes tend to give multinucleated zygotes in conventional IVF and higher degeneration rates after ICSI. In the latter case, leaving the coronal cell layer intact may prevent oocytes from damage (23).

A similar situation is found in ICSI if excessive enzymatical and mechanical removal of cumulus cells (and/or suboptimal follicle puncture) occasionally may lead to partial or total extrusion of ooplasm out of the zona. Though data from mouse zona-free zygotes indicate that blastocyst development *in vitro* can happen and may give live offspring (24) only few case reports are published dealing with the invasive injection in complete zona-free human oocytes and subsequent culture to blastocyst stage (4,25).

This manuscript has been prepared in order to share our experience on this topic with the reader and to encourage embryologists not to discard such oocytes, especially in those cases in which all gametes are affected.

Materials and Methods

Within a 15 month period five ICSI patients out of 752 (0.7%) showed at least one oocyte completely denuded from cumulus cells and zona pellucida. The mean age of this small cohort was 36.4±4.5 years, the basal FSH was 6.0±4.5 mU/ml. All men suffered from male subfertility, with three women showing an additional factor, e.g. endometriosis or tubal blockage.

In all cases stimulation was performed according to a long protocoll using a combination of GnRH agonist (Suprecur®; Hoechst, Frankfurt, Germany) and an individually adjusted dose of hMG (Menogon®; Ferring, Kiel, Germany) or recombinant FSH (Puregon®; Organon, Vienna, Austria). Prior to ultrasound-guided follicle aspiration (36h) 5000 to 10000 IU of hCG were administered (Pregnyl®; Organon, Vienna, Austria) to induce ovulation. Follicle punction was done vaginally under ultrasonographic control.

After collection oocyte-cumulus complexes were incubated for 3 hours in BM1 medium (NMS Bio-Medical, Praroman, Switzerland). In preparation for ICSI cumulus cells were carefully removed by a two-step approach. In detail, denudation was started with hyaluronidase (SynVitro Hyadase®, MediCult, Jyllinge, Denmark) and finished with mechanical removal of persistent cells using hand-drawn glass pipettes. Once it turned out that the actual oocyte has left the zona pellucida special care was taken to exactly measure the ooplasm. Thus, partial loss of cytoplasm could be excluded, in case that a suspected breach in ZP strangled the ooplasm (Figure 1).

ICSI was performed as described elsewhere (5) but due to the fragility of the denuded ooplasm some slight modifications were introduced. It has to be mentioned that oocytes could not be positioned according to the first polar body since it had been lost during preparation (Figure 2a). No negative pressure was applied with the holding pipette, otherwise distorsion of the ooplasma would have happened at the area of contact. However, holding pipette was used to passively fix the MII-oocyte. Though no injection funnel could be observed a small amount of cytoplasm was aspirated in order to facilitate oocyte activation.

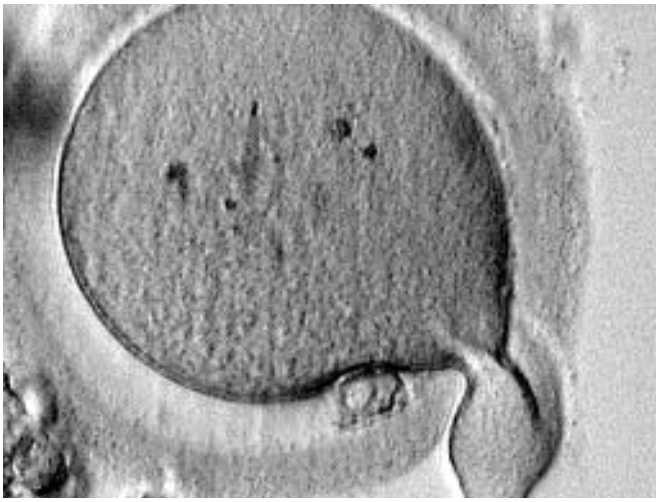

Figure 1. MII-oocyte with a small breach of the zona pellucida resulting in total escape from the zona pellucida.

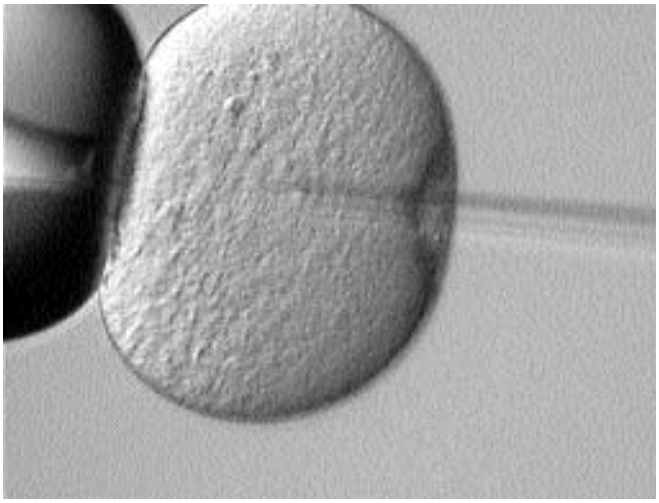

Figure 2a. ICSI in zona-free oocyte.

On day 1 (16-18 hours after injection) fertilization was assessed and only zygotes presenting two pronuclei were considered as normal. At zygote stage medium was changed from BM1 medium (NMS Bio-Medical, Praroman, Switzerland) to Blastassist System Medium 1 (MediCult, Copenhagen, Denmark).

On day 2 embryos were checked for cleavage and the number of blastomeres as well as percentage of fragmentation was documented. Culture (10 µl per embryo) was prolonged in Blastassist System Medium 2 (MediCult, Copenhagen, Denmark) until day 5. Medium was changed daily and it was tried not to loose blastomers during transfer of zona-free embryos.

Only one women had a zona-free blastocyst transferred on day 6 because it was the only conceptus available. The other surviving zona-free embryo was frozen at blastocyst stage and not considered for transfer.

Results

A total of six oocytes lost their zona pellucida after denudation treatment. This represented a percentage of 0.12% of all

collected gametes considered for ICSI. Women susceptible for artificial zona breaching did not differ from women without this problem in terms of hormonal parameter, stimulation response or demographic data (p>0.05).

All six oocytes did not show a germinal vesicle and were considered mature. Accurate measurement of gamete revealed that no ooplasm was lost or remained in the empty zona pellucida.

On day 1 of *in vitro* culture it could be seen that all gametes survived the invasive procedure, however, only four oocytes were fertilized regularly (66.7%) as indicated in Figure 2b. Of these, three zygotes showed signs of cleavage the following day (Figure 2c). Two embryos were of best quality, one showed minor fragmentation (<10%). In one embryo a single blastomere (out of five) was damaged irreversibly. All embryos reached day 3 (Figure 2d) and 4 (Figure 2e) but the slightly fragmented embryo stopped development prior to

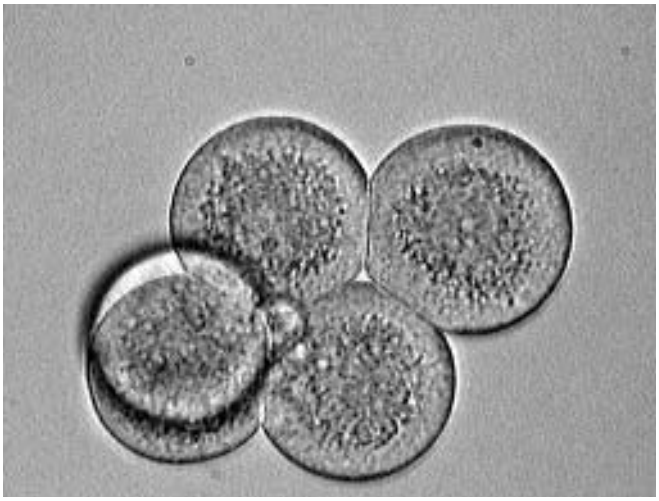

Figure 2d. Five-cell embryo on day 3 of *in vitro* culture. Note central granulation of blastomeres.

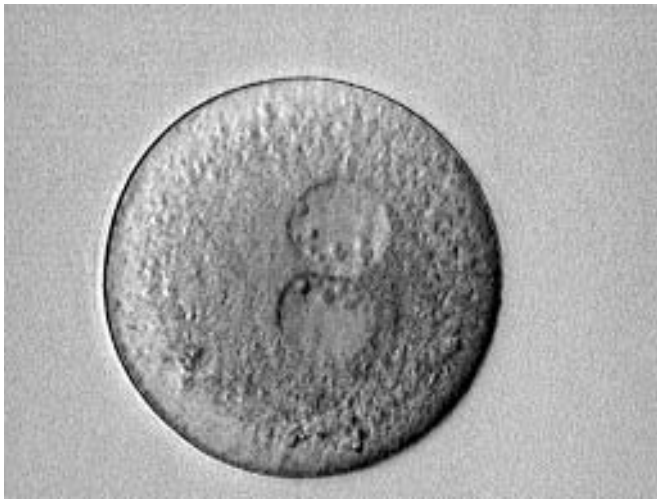

Figure 2b. Fertilization of zona-free oocyte showing pronuclear pattern 3 and a distinct halo.

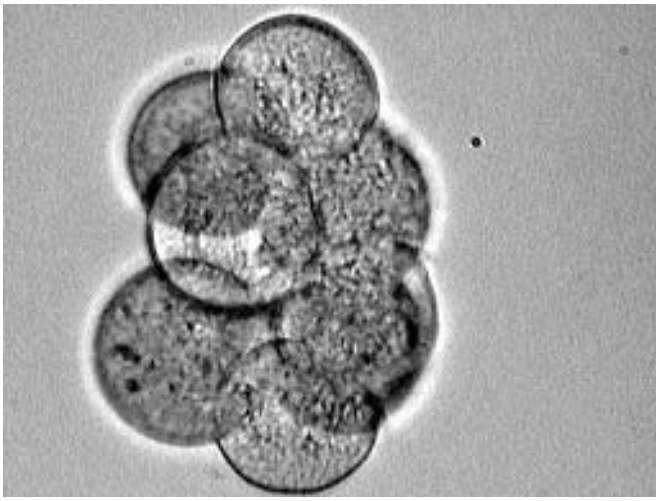

Figure 2e. Eight-cell embryo of best quality on day 4 of development.

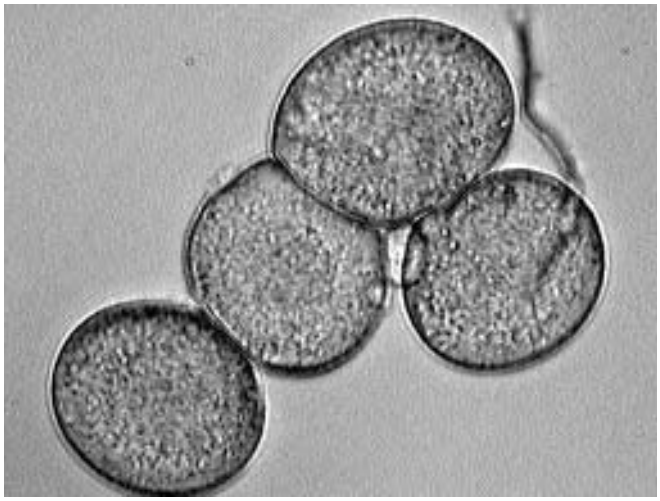

Figure 2c. Four-cell embryo without a zona pellucida on day 2 of preimplantation development. Note pitting appearance of cytoplasm.

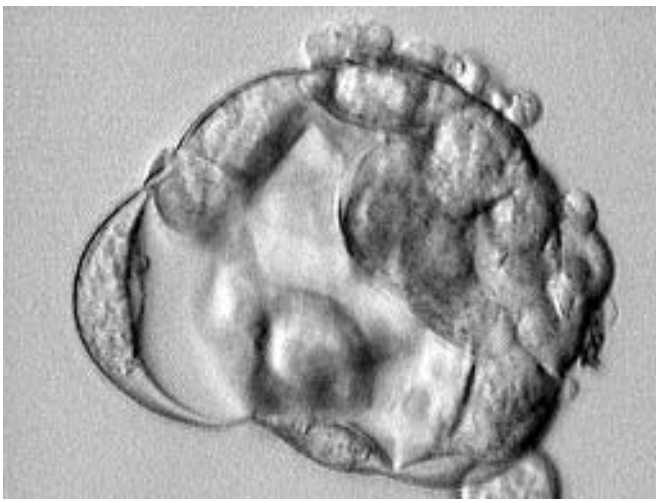

Figure 2f. Early blastocyt stage was reached on day 5.

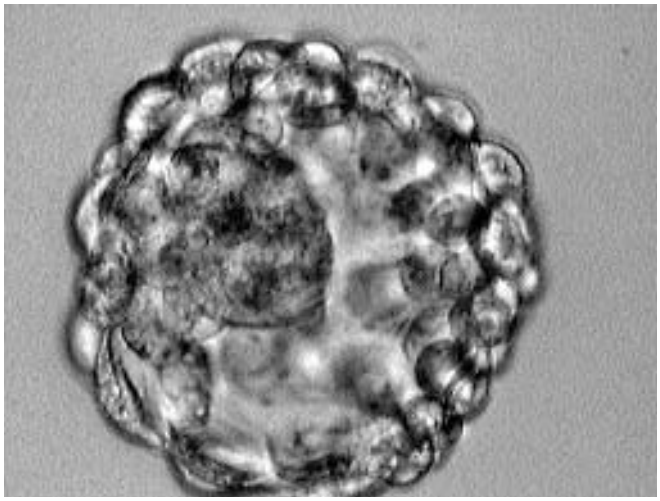

Figure 2g. Full development on the sixth day of preimplantation development.

compaction stage, whereas two conceptireached blastocyst stage (Figure 2f and 2g) on day 6 (50% of zygotes).

Since no alternatives were present in one case of low response (one oocyte) one zona-free blastocyst had to be transferred, but, unfortunately, no pregnancy could be achieved in this 43-year-old woman. The second blastocyst was cryopreserved and is still stored since a twin pregnancy occurred in this patient after transfer of two zona-intact embryos on day 3.

Discussion

ICSI is thought to be a highly invasive micromanipulation technique (6). In fact, even oocyte collection or pre-treatment of cumulus-complexes for denudation can cause minimal injuries of the oocyte, especially of the zona. Through this artificial gap (Figure 1) it may happen that the ooplasm is released from the ZP. In order not to loose a certain volume of ooplasm empty zonae and surrounding medium have to be searched for cytoplasmic droplets. Accurate measuring (or comparison with zona-intact counterparts) should guarantee that only those nude oocytes are injected which actually represent the full volume of cytoplasm containing the whole chromosomal and cytoskeletal material. Luckily, it is likely that incomplete nude oocytes are not prone to be fertilized (n=2, data not shown).

Beside a considerable mechanical stress which is applied to zona-free oocytes the most severe problem seems to be that the first polar body is lost during denudation. This indicator of metaphase spindle has no cytoplasmic connection to the oocyte and its exact localization is rather a rare event (26). Theoretically, it might happen that immature MI-oocytes are injected accidently which should result in lower fertilization rates. Facing this lack of information there is a much higher risk to irreversibly damage the spindle apparatus during injection though in vitro not all polar bodies are situated in close proximity to the meiotic spindle (3,13,19). As a result, failed fertilization or impaired cleavage might be observed, however, in our limited number of cases only one oocyte could have been affected due to an arrest at zygote stage.

Interestingly, not only first polar bodies but also anuclear cytoplasmic fragments seemed to disappear from the embryo. This is obviously a result of absent cytoplasmic bridges to the blastomeres and, consequently, did lead to exclusively high quality embryos in our study. In parallel, there is also a certain threat of the blastomeres to get lost or damaged (4). Thus, zona-free cleaved embryos may not be suitable for transfer into the uterus, since blastomeres may fall apart during or after transfer (4). This led us to cultivate all candidates until blastocyst stage. Promising pregnancy rates from transfer of zona-free blastocysts (9-11,16, 21) further promote day 5 transfer.

Two interesting obervation could be made during in vitro culture which are worth mentioning. First, atypical elongated appearance of the blastomeres was recognized in two out of three cleaved embryos (Figure 2c) which is supported by previously published images (4) of zona-free concepti. This deviation from normal morphology of human four-cell embryos (7,12), which is characterized by "crosswise" appearance of the blastomeres (with three blastomeres lying side-by-side), is presumably the result of an absent zona pellucida. This shaping function of the zona pellucida should not be underestimated since it supports maximal contact of the blastomeres. In the mouse a higher number of contact points (n=6) was a prerequisite for an optimal development of the fetus (24). Any increase in cell-to-cell contact will facilitate compaction due to a higher number of tight junctions available (4). This hypothesis may be closely related to the second phenomenon which was observed; delayed formation of the blastocoel and full expansion of the blastocyst not prior to day 6. Though our theory is based on a very small number of cases it is supported by literature (4).

To conclude, it has to be emphasized, that, though rare in incidence, ZP-free gametes should not be disposed, much more they can develop to high quality blastocysts (Figure 2f) which might be transferred or frozen (1,14).

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