Normal birth after microsurgical enucleation of tripronuclear human zygotes: Case report

Suresh Kattera¹ and Christopher Chen

Centre for Reproductive Medicine and Gleneagles IVF Centre, Singapore 258500
¹To whom correspondence should be addressed. E-mail: kattera@signet.com.sg

Microsurgical enucleation of a single pronucleus from each of three tripronuclear zygotes was performed and the embryos were transferred to a 38-year-old woman on day 3 after fertilization. A normal healthy baby boy was born at 38 weeks and 4 days gestation, demonstrating that with polyspermic fertilization, removal of the extra male pronucleus allows the zygote to develop normally.

Key words: embryos/enucleation/male pronuclei/oocytes/tripronuclear zygotes

Introduction

Polyspermic fertilization occurs in ~7% of cases in human IVF programmes (Ho et al., 1994). The microsurgical removal of one of the two presumed male pronuclei from human triploid zygotes to restore the diploid state was first reported in 1988 (Rawlins et al., 1988). In that study, the cytoskeletal relaxing agent, cytochalasin B, was used to assist pronuclear removal, but survival was poor. Using the same technique, Gordon et al. (1989) achieved a 55% survival rate after removal of a single pronucleus from human triploid zygotes. In the same year, a 36% survival rate was reported after pronucleus removal in the absence of cytoskeletal relaxants (Malter and Cohen, 1989). In another study, a single pronucleus was removed by puncture of the zygote’s plasma membrane with a sharp needle (Palermo et al., 1994); however, survival rates and whether or not cytochalasin was used were not reported. More recently, Feng and Gordon (1996) reported the birth of normal mice after removal of supernumerary male pronuclei from polyspermic zygotes treated with cytochalasin.

There is no known report of human pregnancy or live birth occurring after microsurgical correction of tripronuclear zygotes. We report the first case of a normal baby delivered after microsurgical correction of a tripronuclear zygote.

Case report

Clinical history

The patient, aged 35 years at the time of initial investigation in 1998, had been married for 4 years; she was healthy and had regular 28-day periods. Her Fallopian tubes were patent and her uterus normal. The patient’s husband had normal semen parameters (concentration 55 × 10⁶/ml, motility 60% and normal morphology 17% as per strict criteria).

She conceived normally in 1994 soon after her marriage but terminated the pregnancy. Subsequently she used oral contraception for 9 months followed by barrier contraception. She later attempted to become pregnant and underwent two failed cycles of intrauterine insemination elsewhere in May and July 1998 before attending our fertility centre in August 1998. She was treated for a total of five IVF cycles without success. The insemination number in all the five cycles was maintained at 50 × 10³ motile sperm.

(i) First IVF cycle (September 1998). Three oocytes were retrieved and fertilized, resulting in 4-cell grade I embryos on day 2. The patient did not become pregnant after the transfer of three embryos on day 2 (48 h) after oocyte retrieval.

(ii) Second IVF cycle (March 1999). Three oocytes were retrieved and fertilized, resulting in 8-cell grade I embryos on day 3. The patient did not become pregnant after the transfer of three embryos on day 2 (72 h) after oocyte retrieval.

(iii) Third IVF cycle (August 1999). In this cycle, three oocytes were retrieved (one germinal vesicle). Two oocytes were fertilized, and on day 3 both the embryos (five cells, grade I) were transferred into the uterus but did not result in pregnancy.

(iv) Fourth and fifth IVF cycle (August 2000 and May 2001). Three oocytes were retrieved during each attempt. After their fertilization and cleavage, 5-cell grade I embryos were transferred into the uterus on day 3. No pregnancy resulted.

After these failed attempts, the patient did not wish to undergo further oocyte recovery and expressed interest in using donor oocytes. The patient arranged a donor who agreed to donate only three oocytes as she herself was going through an ICSI cycle due to severe male factor infertility. Before the initiation of treatment, the patient was tested for β-hCG to rule out spontaneous pregnancy.
The sixth IVF cycle of treatment in October 2001 consisted of pituitary down-regulation with buserelin 0.5 mg given 8-hourly, followed by progynova (total 354 mg) and progesterone (total 1600 mg) in preparation for embryo transfer.

**Sperm preparation**

Semen samples in all the IVF cycles were processed as follows. Samples were collected in sterile containers by masturbation. After liquefaction, samples were assessed for sperm density, motility and morphology. Subsamples of 1 ml were placed in 5 ml tubes, overlaid with 1 ml of Medicult IVF medium (Medicult, Denmark) and then incubated at 37°C under 5% CO₂ for 30–60 min. The supernatant was washed twice at 600 g for 5 min and the sperm pellet was resuspended in 0.5–1.0 ml of culture medium for insemination.

**Insemination and fertilization**

The three donated oocytes were co-incubated with 50 × 10³ motile sperm for 3 h, after which the oocytes were separated from cumulus, washed in culture medium and placed in one well of a 4-well Nunc culture dish containing freshly equilibrated Medicult IVF medium. Oocytes were checked for fertilization after 18 h, when it was observed that all three were fertilized but with three pronuclei.

The patient was informed of the triploid zygotes and of the possibility of performing enucleation to remove one of the two presumed male pronuclei. The patient agreed that the procedure be carried out and gave her written consent. The hospital ethics committee was informed and it gave written permission to perform the enucleation procedure.

**Microsurgical removal of the additional male pronucleus**

The possible presence of pseudopronuclei in these triploid zygotes was ruled out before performing the enucleation procedure. True pronuclei differ from pseudopronuclei by the presence of nucleoli, which were very clear and prominent, and were polarized in all three triploid zygotes.

The holding pipette was obtained commercially (Cook IVF, Australia), while the enucleation pipette was fabricated in the laboratory using equipment from Research Instruments, UK. The inner diameter of the enucleation pipette measured ~13 μm with a spike, and the pipette was bent at an angle of 30°.

The zygotes were subjected to micromanipulation in microdroplets of Medicult IVF culture medium under oil. Each zygote was rolled over with the holding pipette to visualize the pronuclei clearly, ensuring that they did not overlap. When the pronuclei were seen clearly delineated by their nuclear membrane, the zygote was stabilized by applying negative pressure to the holding pipette. The extra male pronucleus was identified by its location, distal from the second polar body and its slightly larger size compared with the female pronucleus, which was usually located close to the second polar body (Rawlins et al., 1988; Palermo et al., 1994; Feng and Gordon, 1996).

The enucleation pipette was filled with culture medium up to the 30° bent angle of the pipette so as to see the movement of culture medium in the pipette. Before removal of the pronucleus, suction of the aspiration pipette was maintained at optimum pressure by prior movement of culture medium in the pipette. The pipette was then inserted through the zona pellucida and into the ooplasm. As the aspiration pipette approached the target male pronucleus, progressive suction pressure was applied gradually, which led to the gentle aspiration of the pronucleus without affecting the remaining two pronuclei. The aspiration of the target pronucleus was followed by aspiration of a small amount (~2 pl) of cytoplasm, taking care not to disturb the other two pronuclei. The enucleation pipette was withdrawn slowly from the zygote. The zygote was then released from the holding pipette and rotated again to confirm the complete removal of the pronucleus. Each zygote was then washed several times in Medicult IVF culture medium and cultured further for 72 h (day 3). All the manipulated zygotes reached eight cells on day 3, and were grade I with equal size blastomeres and without any fragmentation (Cummins et al., 1986). The embryos were hatched by means of zona thinning using acid Tyrode solution, and then transferred into the uterus of the patient.

Patient’s serum β-hCG was detected 10 days after embryo transfer. The patient later was scanned ultrasonically, and a single sac with pulsation was observed. The triple test for Down’s syndrome was performed at 16 weeks gestation and was negative. Obstetric ultrasound scanning revealed no major structural abnormality. The patient’s pregnancy continued uneventfully. A lower segment Caesarian section was performed on June 20, 2002 at 38 weeks and 4 days gestation, and a healthy normal baby boy was delivered. The baby weighed 4580 g with a normal apgar score. Cord blood was collected at the time of delivery for cytogenetic analysis and a normal male karyotype XY was reported. To date, the child has developed normally.

**Discussion**

It is two decades since the birth of the first baby through the process of IVF. During this period, thousands of embryos have been produced in embryology laboratories worldwide. Approximately 7% of these embryos are polyploid (Ho et al., 1994). Triploid zygotes due to dispermy are the most common form of polyspermy in human IVF (Kola et al., 1987). The majority of dispermic embryos will arrest prior to differentiation. These embryos are mosaic and may progress to hydatiform moles in rare instances (Palermo et al., 1994).

In the present report, during the previous five failed IVF attempts, the patient’s oocytes were fertilized normally after insemination with 50 × 10³ motile sperm from her husband. However, when the donor oocytes were inseminated with a similar concentration of sperm from her husband, the resultant zygotes were triploid. The oocyte donor underwent an ICSI cycle due to severe oligoasthenoteratospermia of her husband, and hence oocytes were not inseminated by her husband’s sperm. Thus it was not possible to know whether oocytes from the donor would have become polyploid if inseminated by her husband’s sperm. Polyspermy may be due to the presence of immature or post-mature oocytes, or to a high insemination concentration of sperm (Van Der Ven et al., 1985; Angell et al., 1986; Plachot et al., 1988). This was not the
case in the present study. Polyospermmy could also be due to the zona pellucida failing to block the simultaneous entry of two sperm (Gordon et al., 1989).

Attempts to remove the extra male pronucleus from polypronuclear zygotes have been made in both animals and humans. This has been performed with or without the use of a cytoskeletal relaxant such as cytochalasin B. Previously published enucleation techniques include microsurgical removal of single (Modliński, 1975; Hoppe and Illmensee, 1977) or multiple (McGrath and Solter, 1983) pronuclei from mouse zygotes. Reported survival rates ranged from 65% (Hoppe and Illmensee, 1977) to 97% (McGrath and Solter, 1983).

Attempts at enucleation of human triploid zygotes focused mainly on single pronucleus removal (Rawlins et al., 1988; Gordon et al., 1989; Malter and Cohen, 1989). First attempts to remove an extra male pronucleus were reported in 1988 (Rawlins et al., 1988); however, the enucleated zygotes failed to cleave. The following year, a 100% survival rate was reported using cytoskeletal relaxants on one group of oocytes, and in another group none survived micromanipulation without the use of cytoskeletal relaxants (Gordon et al., 1989). During the same year, a 36% survival rate was reported after enucleation of polypronuclear oocytes without the use of cytoskeletal relaxant (Malter and Cohen, 1989). In spite of better survival rates, intervention in cytoskeletal organization of the oocytes by exposure to cytochalasin can cause fragmentation, delayed cleavage, early developmental arrest (Modliński, 1975; Balakier et al., 1976; Hoppe and Illmensee, 1977) or instability of the meiotic spindle (Pickering et al., 1988). Nevertheless, live animals were born from such treatment (Feng and Gordon, 1996) and, more recently, 100% survival rate of human oocytes was reported after using cytochalasin for the enucleation procedure (Ivakhnenko et al., 1988). During the same year, a 100% survival rate was achieved (Ivakhnenko et al., 1989). First attempts to remove an extra male pronucleus were reported in 1988 (Rawlins et al., 1988); however, the enucleated zygotes failed to cleave. The following year, a 100% survival rate was reported using cytoskeletal relaxants on one group of oocytes, and in another group none survived micromanipulation without the use of cytoskeletal relaxants (Gordon et al., 1989). During the same year, a 36% survival rate was reported after enucleation of polypronuclear oocytes without the use of cytoskeletal relaxant (Malter and Cohen, 1989). In spite of better survival rates, intervention in cytoskeletal organization of the oocytes by exposure to cytochalasin can cause fragmentation, delayed cleavage, early developmental arrest (Modliński, 1975; Balakier et al., 1976; Hoppe and Illmensee, 1977) or instability of the meiotic spindle (Pickering et al., 1988). Nevertheless, live animals were born from such treatment (Feng and Gordon, 1996) and, more recently, 100% survival rate of human oocytes was reported after using cytochalasin for the enucleation procedure (Ivakhnenko et al., 1988). Because of concern over the detrimental effects of cytochalasin, we chose to avoid its use. We achieved 100% survival and cleavage after enucleation of all three tripolar zygotes. Although the number is small, this success could be attributed to our past experience in enucleation of tripolar zygotes. We previously have performed enucleation on 20 tripolar zygotes, of which two did not survive, two did not cleave, two each became morulae and blastocysts, and the remainder arrested at the 4- to 8-cell stage (unpublished data). Our experience showed that to perform a successful enucleation, the right microtool must be used, optimum suction pressure in the aspiration pipette must be maintained to aspirate the pronuclei gently and slowly, and the aspiration pipette must be withdrawn slowly. The pronuclei must be clearly seen delineated by a nuclear membrane, without any overlapping.

Identification of extra male pronuclei for removal from polypronuclear zygotes so far has been based on their slightly larger size and location further away from the polar body than female pronuclei (Rawlins et al., 1988; Palermo et al., 1994; Feng and Gordon, 1996). However, there is no evidence to prove that the larger pronuclei are always male pronuclei. Our own study on zygotes (unpublished) using a digital video camera (Nikon, Japan) with Image Pro-Plus analysis software (Media Cybernetics Inc., USA) showed that in ~70% of cases the pronucleus nearer the second polar body was slightly smaller; in 8% the pronucleus nearer the polar body was slightly larger and in 22% both the pronuclei were of equal size. Clearly, the enucleation of a pronucleus from a polypronuclear zygote on the basis of size may lead to the production of androgenic embryos. Recently, we performed biopsy and fluorescence in-situ hybridization (FISH) analysis of blastomeres from embryos derived from six enucleated tripolar zygotes, and all of them were shown to be normal for diploid chromosomal status (unpublished). Selection of the additional male pronuclei for enucleation in these zygotes was based only on their location distal to the polar body. Thus, it appears that the location of pronuclei is a more reliable criterion than size in identifying paternal origin. However, a diploid condition in an embryo derived from enucleated tripoolar zygotes might not indicate that a male pronucleus had been removed, as the removal of a female pronucleus would give a similar observation provided that the zygote was not fertilized by two Y-chromosome-bearing sperm. The only definitive conclusion is the demonstration of a Y-chromosome by FISH in the enucleated pronucleus.

It is also important to differentiate between pronuclei and pseudopronuclei, as ~40% of suspected polypronuclear zygotes have pseudopronuclei. The latter can be distinguished by lack of nucleoli or DNA (Van Blerkom et al., 1987). In this report, all the zygotes which had three pronuclei contained very prominent nucleoli. Thus, we were dealing with truly polypronuclear zygotes.

Within the zygote/embryo, a functional centrosome is essential for the formation of a normal bipolar spindle and subsequent development of the embryo. Early work with sea urchin eggs (Wilson and Mathews, 1895) demonstrated the role of the centrosome and showed that the active organelle is of paternal origin in this species. A similar role for sperm-derived centrosome has been shown for the pig (Szollosi and Hunter, 1973), rabbit (Longo, 1976), sheep (Crozet, 1990) and cow (Long et al., 1993). Of the mammals studied, the mouse is the only species in which spindle formation is controlled by a maternal centrosome (Schatten et al., 1986). The data from dispermie human embryos and tripolar zygotes strongly suggest that spindle formation in human embryos is determined by sperm-derived centrosomes and their centrioles (Sathanathan et al., 1996).

As more than one functional centrosome results in a multipolar spindle and abnormal embryo development, it is essential that the extra paternal centrosome be removed from tripolar zygotes with the extra male pronucleus. Because the centrosome is closely associated with the pronucleus, aspiration of a small amount (~2 pl) of cytoplasm may be adequate to remove the centrosome. We suggest that this has happened in the case reported here. It may be possible to examine the blastomeres after cleavage of enucleated tripolar zygotes for the presence of a centrosome (Sathanathan et al., 1996).

As it is not always possible to ensure removal of the extra centrosome with the extra male pronucleus, it is suggested that enucleation of a pronucleus from a tripolar zygote should be performed only as a last resort.
The case reported here demonstrates that microsurgical removal of one pronucleus from a tripronuclear zygote can result in the birth of a normal baby. Thus it is feasible to enucleate pronuclei from tripronuclear zygotes in patients that have only polyspermic zygotes available after IVF. Further, where normal diploid embryos are available for transfer, supernumerary polypronuclear zygotes may be frozen for future use after their correction to the diploid state. A further potential use of these microsurgically corrected zygotes is in stem cell research, as their use rather than use of normal embryos may allay ethical objections.

To conclude, tripronuclear zygotes in humans have the potential of developing into normal babies after their microsurgical correction to the diploid state.

References


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