

CASE REPORT

Birth from cryopreserved embryos following in-vitro maturation of oocytes and intracytoplasmic sperm injection

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This case report describes the birth of a baby following the transfer of cryopreserved embryos generated from intracytoplasmic sperm injection (ICSI) carried out on the second day after oocyte pick-up of in-vitro-matured metaphase I and germinal vesicle stage oocytes. The couple had a history of three failed intrauterine insemination attempts and reduced fertilization rates in two previous in-vitro fertilization (IVF) cycles. In the IVF–ICSI treatment cycle, 6/11 mature oocytes became fertilized following ICSI on the first day. However, the patient failed to conceive following the transfer of three embryos. Five oocytes were immature (two at metaphase I stage and three with a germinal vesicle) and these were cultured overnight. All had extruded a polar body by the following day and ICSI was therefore performed; four oocytes became fertilized, and were cryopreserved at the pronuclear stage in propane-diol. In the next treatment cycle, transfer of frozen embryos was planned. The pronuclear zygotes were thawed and cultured for 24 h prior to the transfer of two embryos in a cycle stimulated with low doses of follicle stimulating hormone. This resulted in a pregnancy and the delivery of a healthy baby boy. In-vitro maturation of metaphase I and germinal vesicle oocytes which are routinely collected in IVF–ICSI cycles, followed by second day ICSI fertilization, may provide a valuable source of embryos for infertile couples.

Key words: birth/cryopreservation/ICSI/immature oocytes/in-vitro maturation

Introduction

Due to the asynchrony of follicular growth and maturation in gonadotrophin-stimulated cycles, a proportion of the oocytes collected will be immature, and either at the metaphase I or the germinal vesicle (GV) stage. In intracytoplasmic sperm injection (ICSI), only mature (metaphase II) oocytes are injected with sperm and the immature oocytes are usually discarded. The number of immature oocytes collected is dependent on the individual, and the incidence of immature oocytes retrieved can vary from 0 to 50% in our experience.

A recent report on ICSI fertilization rates of metaphase I oocytes matured for 8 h (Bonada *et al.*, 1996) indicated a reduced fertilization rate of these oocytes when compared to oocytes that were at metaphase II at the time of oocyte retrieval. Veeck *et al.* (1983) reported two pregnancies following transfer of embryos obtained from in-vitro-matured oocytes in an in-vitro fertilization (IVF)–embryo transfer programme and recently Nagy *et al.* (1996) achieved a birth following ICSI carried out on in-vitro-matured GV stage oocytes. At present the data available on immature oocytes are limited.

In our ICSI programme we have attempted to mature metaphase I and GV oocytes in culture and to perform second day ICSI on matured oocytes. In this case report we present the first successful pregnancy we have achieved by transferring cryopreserved embryos generated from second day ICSI of metaphase I and GV stage oocytes matured *in vitro* for 24 h.

Case report

A 26 year old woman was referred to our clinic with a history of three unsuccessful intrauterine insemination (IUI) attempts with her husband's spermatozoa. The clinical investigations indicated that her menstrual cycles were irregular, varying from 40 to 60 days, and the laparoscopic appraisal of the pelvic organs appeared normal. The routine semen analysis carried out on the 27 year old husband indicated no abnormality; however, the acrosome reaction to ionophore challenge test showed a reduced response (an ARIC score of 2% and a repeat of 5%, Yovich *et al.*, 1994). Due to the past history of failed IUI attempts, IVF–embryo transfer was planned as their treatment option. The couple had two unsuccessful IVF attempts in which they achieved reduced fertilization despite the use of the sperm stimulant, pentoxifylline (3/6 and 4/12 oocytes fertilized respectively). Furthermore, in both attempts the embryo quality was poor (1–1.5 on a scale of 0–4, 4 being the best quality).

For the next IVF attempt the couple was offered ICSI in order to improve the fertilization rate. In the treatment cycle, superovulation was carried out using 20 IU of Lucrin (leuprolide acetate; Abbott, N.S.W., Australia) given from day 2 to day 5 on a flare-up regimen in combination with 3 ampoules per day of follicle stimulating hormone (Metrodin; Serono, N.S.W., Australia). An ovulation trigger injection of 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl; Serono) was given when three follicles were >2.0 cm on the ultrasound scan. At oocyte pick-up 36 h after the trigger injection, 18 cumulus masses of good quality (graded 2–3 on a scale of 0–

3, 3 being the best quality) were collected. However, following hyaluronidase (80 IU/ml) treatment it was found that two masses contained only zonae, eight contained metaphase II, five contained metaphase I and three contained GV stage oocytes. All oocytes were cultured in human tubal fluid medium (HTF) containing 10% human serum until ICSI.

The husband's sperm concentration on the day was 40×10^6 total spermatozoa/ml and 25×10^6 motile spermatozoa/ml with >50% progressive motility (WHO criteria, 1992). The semen was prepared using Percoll density gradient (95%:47.5%) separation and the motile sperm pellet was washed twice in culture medium. The ICSI procedure was performed 5 h after oocyte recovery. The micromanipulation set-up used was a Nikon Diaphot inverted microscope (Tokyo, Japan) with Narishige course and joystick hydraulic micromanipulators (Model MN-108 and MO-108). The microtools were prepared in-house and the ICSI procedure performed was similar to that described by Van Steirteghem *et al.* (1993). However, the spermatozoa were immobilized without placing them in polyvinylpyrrolidone (PVP). This was performed with the injection needle, which was pointed on top of the sperm tail at right angles and with a quick movement touched and stroked the sperm tail gently against the bottom of the dish until no movement was seen. By the time of ICSI three of the metaphase I oocytes had undergone maturation to metaphase II and thus ICSI could be performed on 11 oocytes. Fertilization (appearance of two pronuclei) was confirmed in six oocytes and one oocyte contained a single pronucleus. On the next day, three 2-cell embryos were transferred and the remaining three embryos were disposed of due to poor quality.

The five immature oocytes (two metaphase I, three GV oocytes) were examined after 24 h in culture and the first polar body was observed in all oocytes. Second day ICSI was performed on all five oocytes using sperm prepared the previous day. After 20 h, four of these oocytes (two metaphase I and two GV oocytes) contained two pronuclei and the other one showed fragmentation. The four pronuclear oocytes were pooled and cryopreserved in propanediol using the rapid freezing protocol described by Lassalle *et al.* (1985). Since the patient failed to conceive in the ICSI-IVF treatment cycle, the transfer of frozen embryos was planned for a stimulated cycle a few months later. The patient received 1 ampoule of human menopausal gonadotrophin (HMG, Pergonal; Serono) for 7 days but, due to the poor response, the dosage was increased to 3 ampoules. When the oestradiol concentration reached 1400 pmol/l, an ultrasound scan indicated three follicles of 1.9, 1.7 and 1.5 cm and an endometrial thickness of 12 mm. Ovulation was then initiated with 10 000 IU of HCG and frozen embryo transfer was arranged on the third day following the HCG administration. The couple refrained from sexual intercourse during this time. All four pronuclear oocytes were thawed 1 day prior to the transfer day using the rapid thaw technique (Lassalle *et al.*, 1985). Two embryos survived the freeze-thaw technique and developed to 4-cell stage embryos of average quality (score of 2/4) during overnight culture. The embryos were transferred to the uterus and the patient had a positive pregnancy test 15 days later (β -HCG of

375 mIU/l). The patient delivered a healthy baby boy following an uneventful pregnancy.

Discussion

To our knowledge this is the first baby born from cryopreserved embryos generated from ICSI carried out on in-vitro-matured metaphase I and GV stage oocytes the day following oocyte recovery. Nagy *et al.* (1996) achieved a birth from the transfer of fresh embryos generated by ICSI performed on GV stage oocytes. A similar pregnancy, but achieved using frozen-thawed embryos, ended in an early abortion.

Bonada *et al.* (1996) reported reduced fertilization rates (42.9%) with metaphase I oocytes which had been matured for 8 h, compared to mature oocytes (67.4%) following ICSI. Good fertilization rates were obtained for both metaphase I and GV oocytes in the present report. Due to pooling of embryos at cryopreservation, it is not known whether the pregnancy originated from metaphase I or GV stage oocytes. Furthermore, again because of pooling, data could not be obtained on the effects of freeze-thaw procedures on the survival of embryos originated from either metaphase I or GV stage oocytes. Thus properly planned research is required to study various aspects of immature oocytes, in particular the cytoplasmic and nuclear development of metaphase I and GV stage oocytes during in-vitro culture in order to assess the possible risks involved. However, it is clear from the available data that a certain proportion of these oocytes can achieve complete maturity *in vitro* and give rise to healthy embryos and pregnancies.

Since, for this patient, only 50% of oocytes were mature at the time of oocyte aspiration, an inherent meiotic maturation problem may have been present. This is supported by the fact that the follicles were of adequate size at the time of HCG trigger and the oocyte-corpora-cumulus complexes were graded mature at oocyte aspiration. Thus, immaturity of oocytes may have contributed, in part, to the poor fertilization rates obtained in previous IVF attempts.

Normally, germinal vesicle breakdown and resumption of meiotic maturation occur in response to the preovulatory gonadotrophin surge or to the injection of HCG. In ~37 h the majority of oocytes will complete meiosis I, extrude the first polar body and progress to metaphase II. In this case, the metaphase I and GV oocytes completed maturation after 24 h of in-vitro culture, which indicates that the meiotic maturation had commenced following HCG administration but did not continue, probably due to some disturbance in the removal of cyclic adenosine monophosphate (cAMP)-mediated inhibitory mechanism of GV breakdown (Downs, 1995). Furthermore, possible deleterious follicular factors may have been removed by in-vitro culture of immature oocytes, enabling them to complete maturation and produce good quality metaphase II oocytes and thus viable embryos following ICSI. The above mechanisms may explain the possible reasons for the failure of fresh embryo transfer to establish a pregnancy, while the frozen-thawed embryos generated from immature oocytes were able to implant and yield a live birth. In view of these factors it is unlikely that the patient would have achieved a

pregnancy in a natural or in an ovulation-induced cycle, even though some fertilization did occur with the husband's spermatozoa. This is a unique case where an inherent oocyte maturation problem, in addition to the possible male factor, may have been the cause of the couple's infertility.

Fertilization and pregnancies have been achieved from ICSI carried out on oocytes which had failed to fertilize in the regular IVF programme (Tsirigotis *et al.*, 1994; Sjogren *et al.*, 1995). The majority of the unfertilized oocytes in IVF are considered to be mature (metaphase II) at the time of oocyte retrieval and these have been maintained in culture for a further 24 h before being subjected to ICSI. Under these conditions oocytes may undergo ageing, increasing the risk of chromosomal aberrations. Furthermore, the work of Asch *et al.* (1995) indicated the presence of spermatozoa in a majority of unfertilized IVF oocytes, and this may further question the normality of the embryos resulting from ICSI on these oocytes. As suggested by Tsirigotis *et al.* (1994), it is advisable to use ICSI on unfertilized IVF oocytes only for diagnostic rather than therapeutic purposes. However, carrying out ICSI on in-vitro-matured metaphase I and GV is a different situation. Thus the performance of second day ICSI on 24 h in-vitro-matured oocytes can provide a valuable source of embryos for the future use of an infertile couple.

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