SHORT COMMUNICATION

BOLOGNA, ITALY

Ongoing Pregnancy After Intracytoplasmic Sperm Injection of Epididymal Spermatozoa into Cryopreserved Human Oocytes

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INTRODUCTION

Human embryo cryopreservation presents several ethical, legal, and religious problems and is a matter of discussion in several countries. The possibility of storing human oocytes would virtually eliminate these problems and, in addition, could give a reproductive chance to young patients at risk of losing ovarian function. However, only sporadic clinical success has been reported in the past decade (1). The recent technical improvement (2) and clinical success (3) have renewed interest in oocyte cryopreservation, whose possible applications are expanding. The present report describes the first pregnancy achieved with intracytoplasmic injection of epididymal sperm into frozen-thawed eggs.

MATERIALS AND METHODS

A 29-year-old woman whose partner suffered from bilateral obstruction of the vas deferens for inflammatory disease was enrolled in our assisted reproduction program. Multifollicular development was induced as described previously (4).

Transvaginal ultrasound-guided oocyte pickup was performed 34 hr after human chorionic gonadotropin

(hCG) and 54 oocytes were retrieved. Of the 54 oocytes retrieved, 33 good-quality metaphase II eggs were cryopreserved.

Since the patient showed severe ovarian hyperstimulation syndrome (OHSS), and she did not accept the alternative to freeze the embryos for personal ethical reasons, we decided to include the patient in our oocyte freezing program and postpone the transfer to avoid further worsening of the OHSS. After 4 months, the patient underwent hormonal replacement therapy to improve endometrial growth as described previously (3). When the endometrial thickness reached acceptable levels, percutaneous epididymal sperm aspiration was performed. Epididymal fluid was collected with a syringe filled with 1 ml of buffered human tubal fluid (HTF) supplemented with 0.5% human albumin. The syringe was connected to a 21-gauge butterfly, which was inserted directly through the skin and the epididymal tubule. The sperm was aspirated and examined under a microscope. Two aspiration from both right and left epididymis were performed and only a few sperms were retrieved. The isolated sperm were washed (300g, 10 min) and then resuspended in HTF supplemented with 0.5% human albumin. The sperm were incubated in a CO₂ incubator until use.

Before oocyte freezing the cumulus–corona complex of each oocyte was removed by briefly exposing it for 30–40 sec to a buffered culture medium containing 40 IU/ml hyaluronidase enzyme (Type VIII; Sigma, Aldrich S. r. l., Milano, Italy) and aspirating it through hand-drawn glass pipettes. Afterward, the oocytes were examined under an Olympus IMT-2 inverted microscope at $\times 400$ magnification and assessed for their nuclear maturity. The oocytes were cryopreserved using a slow freeze–rapid thaw protocol with 1,2-propanediol as cryoprotectant. They were equilibrated for 10 min in phosphate-buffered saline (PBS) supplemented with 1.5 M 1,2-propanediol (PROH) and 30% Plasmanate (Diasint, Firenze, Italy). After equilibration, the oocytes were transferred in PBS supplemented with 1.5 M PROH, 0.2 M sucrose, and 30% Plasmanate, loaded into plastic straws, and

placed in an automated Kryo II 10/17 biological vertical freezer (Planer Product Ltd., Datamed Milano, Italy), with the chamber temperature set at 23°C. The temperature was slowly reduced from 20 to -8°C at a rate of -2°C/min.

Ice nucleation was induced manually by seeding. Then the temperature was gradually reduced to -30°C at a rate of -0.3°C/min and rapidly lowered to -150°C at a rate of -50°C/min. After 10 min of temperature equilibration, the straws were transferred to liquid nitrogen tanks.

The straws were removed from liquid nitrogen, held at room temperature for 30 sec, and put in a 30°C water bath for 40 sec. The cryoprotectants were removed by stepwise dilution. The oocytes were equilibrated in 1.0, 0.5 M PROH solution for 5 min, then in 0.2 M sucrose and PBS for 10 min, and, finally, transferred to a fresh culture medium at 37°C in an atmosphere of 5% CO₂ for 3 hr before intracytoplasmic sperm injection (ICSI).

After thawing, the oocytes were incubated at 37°C in an atmosphere of 5% CO₂ until ICSI. The sperm suspension was kept in the 37°C incubator until the intracytoplasmic injection of the oocytes.

The ICSI technique was performed as reported previously (3).

RESULTS

Ten cryopreserved oocytes were thawed. Seven oocytes survived (70%) and were inseminated, leading to five normal fertilizations. Three fertilized oocytes cleaved, and on day 3 one good-quality 10-cell embryo and two 8-cell embryos were transferred. On day 15 the β -hCG level was 832 IU/L. One week later the β -hCG levels were 9300 IU/L. A twin healthy gestation was confirmed by ultrasound examination at the seventh week with the presence of embryonic cardiac activity.

DISCUSSION

For many years, oocyte storage has been considered an attractive but hardly feasible alternative in assisted reproduction, because of technical difficulties leading to low survival and fertilization rates.

Several cell structures can be damaged during the entire process of freezing/thawing and by the cryoprotectants, including the zona pellucida, the cytoskeleton, the meiotic spindle, and the cortical granules. This

damage can affect the survival and the fertilization rate and determine, at least in theory, aneuploidia. However, reassuring results have been achieved by Gook *et al.* (5), who demonstrated normal karyotypes and the absence of stray chromosomes in basic investigations.

A substantial improvement has been achieved by inseminating thawed oocytes by ICSI (2). The association of two novel techniques, such as egg freezing and ICSI, might raise some concern about the safety of the procedure. However, in our experience, the introduction of ICSI has improved the results in terms of fertilization and embryo development (6), and the birth of a healthy female using this technique was announced by our team (3).

The present report documents for the first time that epididymal sperm can also be used successfully to fertilize thawed oocytes and that a viable pregnancy can be achieved. Oocyte cryopreservation is thus likely destined to become a reliable technique offering several advantages. Among others, it could provide a solution to the ethical and legal problems of embryo cryopreservation. This paper describes an example of the ethical application of oocyte storage which gave an alternative to cycle cancellation.

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