

## Successful fertilization and pregnancy after injection of frozen–thawed round spermatids into human oocytes

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**The effect of cryopreservation on the integrity and fertilizing capacity of round spermatids was studied in two azoospermic patients. In December 1995 the patients, both with maturation arrest of spermatogenesis, were submitted to testicular sperm extraction (TESE) after an extensive examination of their ejaculate. Only round spermatids were found after testicular biopsy. Some of the spermatids were isolated and used for a first injection, while the remainder of the preparation was cryopreserved for successive cycles. Because of the failure of the first attempt, 3 months later, the same two patients were submitted to a second one. The frozen preparation was thawed and examined to evaluate the integrity and the viability of surviving round spermatids. More than 70% of the thawed spermatids were viable for injection. Fifteen oocytes at metaphase II, retrieved from the patients' wives, were microinjected with thawed round spermatids. Eighteen hours after the injection, seven out of 15 oocytes showed normal fertilization, with the presence of two pronuclei. The zygotes were cultured to observe embryonic development. After 48 h, six cleaving embryos had developed to at least the two-cell stage, while one had arrested at the pronuclear stage. At 72 h, the cleaving embryos showed further development to the four- to six-cell stage. They were then transferred into the uterus. After 3 weeks a clinical pregnancy was established in one patient [ $\beta$ -human chorionic gonadotrophin ( $\beta$ HCG) concentration was 2100 UI]. At 16 weeks of gestation, chromosomal analysis was performed, which confirmed the presence of a fetus with normal karyotype. The pregnancy is ongoing.**

*Key words:* cryopreservation/fertilization/frozen–thawed spermatid/pregnancy

### Introduction

Before the introduction of recent techniques, many cases of non-obstructive azoospermia, due to an arrest of spermatogenic maturation, were excluded from assisted reproduction. Recent studies have reported fertilization and subsequent embryo development in hamster and mouse following the fusion of round spermatid nuclei with oocytes (Ogura and Yanagimachi, 1993; Ogura *et al.*, 1993). Delivery of normal young mice has been obtained after microinjection of round spermatids into

mouse oocytes (Ogura *et al.*, 1994; Kimura and Yanagimachi, 1995a,b). The reproductive capacity of round spermatids injected into rabbit oocytes has also been demonstrated by Sofikitis *et al.* (1994). These findings suggest that the use of spermatids might be applicable to the treatment of non-obstructive azoospermia in humans (Edwards *et al.*, 1994). The injection of spermatids into human oocytes has led in some cases to successful fertilization, embryo development and pregnancy (Fishel *et al.*, 1995; Tesarik *et al.*, 1995, 1996; Vanderzwalmen *et al.*, 1995). Since the treatment of azoospermic patients with maturation arrest of spermatogenesis requires surgical operation to retrieve sperm cells from the testis, we have attempted to freeze the sperm cells recovered from the first attempt in order to avoid a second surgical treatment. In the present work we report the first successful fertilization and pregnancy with frozen–thawed spermatids.

### Materials and methods

#### *Source and preparation of frozen–thawed spermatids*

Two patients with azoospermia of secretory origin underwent our spermatid injection programme after counselling about the risk of this procedure. The preliminary histological biopsy diagnosed a maturation arrest of the spermatogenesis. The two patients had a high concentration of follicle stimulating hormone (FSH) (24 and 26 IU/l) and small testicular volume (<15 ml). An extensive examination of the ejaculates was performed, which revealed the complete absence of sperm cells. The two patients underwent testicular sperm extraction (TESE) to retrieve sperm cells.

The testicular samples were collected in a large volume of in-vitro fertilization (IVF) culture medium (Earle's Balanced Salt Solution) and rinsed twice. Subsequently the samples were minced into small pieces with a pair of scissors, and the suspension was collected in a 14 ml Falcon tube (Cat. N. 2095; Becton Dickinson, USA) and vibrated for 5 min at 2200 g with a Vortex (MS1 minishaker, IKA-WORKS, Inch, USA). A small amount of the suspension was placed immediately in 2 drops of IVF culture medium (Medi-cult) under paraffin oil in a Petri dish. The drops were examined carefully under an inverted microscope (Diaphot 300; Nikon Corporation, Tokyo, Japan) for the presence of sperm cells. Only round spermatids were found in the patients. Half of the fresh sample suspension was used for the first IVF attempt. The other half was cryopreserved using sperm freezing cryoprotectant [HEPES + 15% glycerol + 0.4% human serum albumin (HSA)] in an equal volume (1:1) to the sample suspension. After incubation in a 37°C water bath for 10 min, the mixture was put into 0.5 ml freezing straws. These were then initially exposed to liquid nitrogen vapour for 10 min, from room temperature to –80°C (rate: –10°C/min), and then plunged into liquid nitrogen (–196°C). For the thawing process, the straws were removed from the liquid nitrogen and immediately warmed at 37°C in a 5% CO<sub>2</sub> chamber for 30 min. To remove the cryoprotectant, the thawed

suspension was washed in Earle's medium and centrifuged at 1600 g for 5 min. The pellet was then diluted in 1.5 ml of Medi-cult medium and a sample was examined under an inverted microscope at  $\times 400$  magnification to determine survival and integrity of spermatids after freezing. Normal shaped spermatids, showing an intact membrane and a central nucleus with an adjacent acrosomic granule, were isolated and used for the injection.

#### Source and preparation of oocytes

Seventeen oocytes retrieved from the patients' wives were used for a second IVF attempt. The corona-cumulus complex was removed using hyaluronidase enzyme 80 IU/ml and the oocytes were then washed in Dulbecco basal salt solution (BSS) twice. Fifteen oocytes were at metaphase II stage with extruded polar body.

#### Microinjection procedure

The round spermatids were isolated with the use of a fine pipette and placed in a drop of Medi-cult culture medium without PVP in a Petri dish. Microinjection was performed by the usual procedure, using an injection pipette of 7 mm inner and 9 mm outer diameter. The polar body of the oocyte was at 12 or 6 o'clock and the injection point was at 3 o'clock. During the injection the cytoplasm was aspirated vigorously to stimulate the oocytes. The injected oocytes were then placed in drops of Medi-cult medium and incubated at 37°C with 5% CO<sub>2</sub> in air.

#### Results

Of the 15 oocytes injected with frozen-thawed round spermatids, seven became fertilized, as shown by the presence of two pronuclei 18 h after injection. Two days later, six zygotes had a normal cleaved embryo, with at least two cells, while one did not develop beyond the pronuclear stage. After a further 24 h, the cleaving embryos had further developed to the four- to six-cell stage. The embryo transfer (three embryos per patient) took place by the usual procedure, 72 h after injection. A resulting clinical pregnancy was established in one patient. At 16 weeks of gestation, chromosomal analysis confirmed the presence of a fetus with a normal karyotype.

#### Discussion

One of the major problems in the treatment of azoospermic patients is that they must undergo a microsurgical operation to retrieve the sperm cells from the epididymis, or from the testis, each time they attempt an ICSI cycle. This has meant many micro-operations before a pregnancy could be finally achieved. Recently, several approaches have been successfully used to avoid this inconvenience, including percutaneous epididymal sperm aspiration (PESA) (Craft *et al.*, 1995) and fine needle testicular sperm aspiration (TESA) (Craft and Tsirigotis, 1995). Both methods may have a traumatic effect on the epididymal or the testicular tissues after treatment. Recent studies reported the successful use of frozen-thawed epididymal (Devroey *et al.*, 1995) and testicular spermatozoa (Gil-Salom *et al.*, 1996; Romero *et al.*, 1996), in conjunction with ICSI, in azoospermic cases with normal spermatogenesis maturation.

In azoospermic cases with primary testicular failure, or maturation arrest at different stages of spermiogenesis, the

injection of spermatids into animal (Ogura *et al.*, 1993, 1994; Sofikitis *et al.*, 1995) or human oocytes (Edwards *et al.*, 1994; Fishel *et al.*, 1995) has proved their fertilization capacity. Moreover, successful pregnancies and delivery using round spermatids isolated from ejaculates have already been achieved by Tesarik *et al.* (1995, 1996). This may be of great value for the treatment of azoospermic patients because it avoids surgical operation, but in cases where spermatids cannot be found in the ejaculate the surgical retrieval of sperm cells remains the only available method.

The use of spermatids, however, may imply some genetic alteration related to immaturity and genomic imprinting, and this should be emphasized during patient counselling (Antinori *et al.*, 1996). The concern regarding gamete imprinting and DNA maturity (review in Fishel *et al.*, 1996) seems to be considerably reduced following the birth of normal offspring in animals and humans. Moreover, our results and previous clinical data (Fishel *et al.*, 1995; Antinori *et al.*, 1996; Tesarik *et al.*, 1996) support the biological studies reported by Ogura *et al.*, 1994, and by Kimura and Yanagimachi (1995a,b), and may indicate that essential imprinting occurs at the secondary spermatocyte stage in mice and human round spermatids.

However, genetic aberration related to genomic imprinting and DNA methylation must be studied in depth. The couples included in a spermatid injection programme must be assessed thoroughly in order to detect the possible presence of genetic abnormalities responsible for defects in sperm maturation and should also be monitored before and after conception.

The current result may indicate that frozen-thawed round spermatids maintain their integrity, viability and the potential capacity to achieve fertilization and embryo development. Before we can consider the above result as a positive treatment of azoospermic patients, careful investigations should be carried out.

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**S.Antinori et al.**

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