

SHORT COMMUNICATION

## Successful pregnancy after spermatid injection

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**We present nine cases of spermatid intracytoplasmic injection for the treatment of non-obstructive azoospermia. In eight cases, no elongated spermatids or spermatozoa were found in previous spermograms or testicular biopsies. In these patients, treatment was performed using ejaculated ( $n = 6$ ) and testicular ( $n = 2$ ) retrieved round spermatids (Sa type). In cases where ejaculated round spermatids were used, they were isolated on the day before oocyte retrieval and left in culture for 24 h before intracytoplasmic sperm injection (ICSI). No pregnancy was obtained in either group, although culturing seemed to increase the fertilization rate. In one other case, elongated spermatids were observed in the previous spermogram and thus a normal ICSI procedure was scheduled. However, on the day of oocyte retrieval, no spermatids could be recovered from fresh sequential ejaculates, and a testicular open biopsy was then performed. Both round and elongated spermatids were found in the testicular tissue, but only the more mature germinal cells (Sb2) were injected. From this case, a normal pregnancy was obtained which resulted in the birth by Caesarean section at 37 weeks of gestation of a normal healthy baby girl, weighing 2700 g.**

**Key words:** ICSI/non-obstructive azoospermia/pregnancy/spermatid injection

### Introduction

The successful introduction of intracytoplasmic injection of round (Tesarik *et al.*, 1995, 1996) and elongated (Fishel *et al.*, 1995, 1996) spermatids, has created many expectations for azoospermic men, who want to become genetic fathers. Analysis of the recent reported data suggests that the use of testicular spermatids may have an advantage over those retrieved from the ejaculate (Antinori *et al.*, 1997a,b; Araki *et al.*, 1997). It also seems to indicate that the complete absence of spermatozoa from the ejaculate or testicular biopsy in the previous history of a patient has an adverse effect on the clinical outcome (Amer *et al.*, 1997; Vanderzwalmen *et al.*,

1997). Taking together the data presented by those authors, for cases where spermatozoa were found at least once, the fertilization and pregnancy rates per cycle respectively were 45 and 18% when using spermatids taken from the ejaculate (11 cycles), and 36 and 26% when spermatids were retrieved from the testicle (53 cycles). By comparison, for those cases where spermatozoa were never previously detected, the fertilization and pregnancy rates per cycle respectively were 19 and 0% when using spermatids taken from the ejaculate (25 cycles), and 38 and 14% when spermatids were retrieved from the testicle (83 cycles). Thus, results suggest that a worse prognosis is to be expected when using early spermatids and spermatids taken from the ejaculate, and when treating patients in whom there has never been any previous history of advanced spermatogenesis. Our present results, although of a very small case series, seem to confirm those findings and suggest new strategies to improve the outcome of the early spermatid block.

### Materials and methods

Ovulation induction and oocyte retrieval were performed as described by Barros *et al.* (1997). For intracytoplasmic sperm injection (ICSI), oocytes were prepared and injected as reported by Tesarik and Sousa (1995), using micropipettes (SweMed, Frolunda, Sweden) with internal diameters of 5 and 9  $\mu\text{m}$  for elongated and round spermatids respectively. Fertilization was assessed 16–18 h after injection by the presence of two pronuclei (PN) and two polar bodies for elongated spermatids and also by the presence of one syngamic PN in the case of round spermatids (Tesarik and Mendoza, 1996). Embryo cleavage and quality, embryo transfer, luteal supplementation, and pregnancy assessment were carried out as described by Barros *et al.* (1997). All therapeutic procedures have been approved by the National Ethical Committees, and informed consent was obtained from all patients. Despite adequate counselling in relation to the experimental nature of the treatment, the couple in whom a successful pregnancy was established refused prenatal diagnosis.

Preparation of round spermatids from the ejaculate was as described by Tesarik and Mendoza (1996), except that we used a short Percoll (Pharmacia, Medicult, Copenhagen, Denmark) gradient (90–70–40% layers). Isolated cells were then left in culture for 24 h in a microdrop of sperm preparation medium (SPM, Medicult, Copenhagen, Denmark) under light mineral oil (Embryo tested, Medicult) at 37°C with 5% CO<sub>2</sub>. For isolation of testicular spermatids, tissue samples were collected in SPM, squeezed with surgical blades, and the resultant fluid was then washed in SPM by centrifuging for 5 min at 800 g. Testicular round spermatids were not cultured because the biopsy was performed on the same day as oocyte retrieval, whenever the search was negative the day before.

Spermatids were classified according to de Kretser and Kerr (1994):

Sa, round; Sb1, round with flagellum; Sb2, elongating; Sc, elongating, nucleus fully elongated; Sd1, elongated, head still not separated from mid-piece; Sd2, mature, large cytoplasmic sheath in mid-piece. Spermatids of Sa type were further tested by two sequential steps. First, they had to deform when aspirated (size and consistency selection), without sticking to the internal tip of the microneedle. Sticking appears characteristic of lymphocytes (data not shown). Second, they were washed in 10% polyvinyl pyrrolidone (PVP)-SPM (Medicult), and only those cells that did not shrink or became stuck in this medium were then put into culture and/or injected.

## Results

There were eight treatment cycles in which round spermatids (Sa type) were used from non-obstructive azoospermic men with documented testicular biopsies showing Sertoli cell-only syndrome and in whom spermatozoa or elongated spermatids had never been observed either in previous ejaculates or in testicular diagnostic biopsies. Female partners had a normal gynaecological examination and both members of the couples presented normal karyotypes. Six treatment cycles were made by using ejaculate-retrieved round spermatids, as male patients preferred this option as a first trial instead of directly performing a testicular open biopsy. Male and female mean ages were 33 (range 28–36) and 30 (27–32) years respectively. Of 52 injected oocytes, 44 survived injection (15% degeneration). One syngamic nucleus and two polar bodies were found in 20 zygotes (45%), whereas only six had two normal PN (14%). Embryo cleavage rate was 100% (including those from syngamic zygotes), with 42% grade A and 39% grade B embryos. No pregnancy was achieved. In two other treatment cycles, no round spermatids were found in the ejaculate and therefore testicular biopsy was needed. Only round spermatids were found and injected. Male ages were 29 and 32 years, and female ages were 27 and 32 years respectively. From 17 injected oocytes, two degenerated after ICSI (12%). Syngamy was found in four zygotes (27%), whereas only one showed two normal PN (7%). All these cleaved normally (grade B embryos) and were transferred, but no pregnancy resulted.

In one other treatment cycle, some elongated spermatids had been found in the previous diagnostic ejaculate, and therefore a normal ICSI was scheduled. The patient, 26 years old, had a normal karyotype and no diagnostic testicular biopsy. The female partner, 24 years old, had a normal gynaecological examination and karyotype. On the day of oocyte recovery, however, no spermatozoa or spermatids were found in two sequential ejaculates. A testicular biopsy was then performed, and both round (Sa) and elongating (Sb2) spermatids were found. Only Sb2 cells were used for injection. Of the seven oocytes that were microinjected, three fertilized normally (2PN) and cleaved (grade A). From this embryo transfer, a normal pregnancy was achieved which resulted in birth by Caesarean section at 37 weeks of gestation of a normal healthy baby girl weighing 2700 g. This represented the first successful spermatid conception obtained in Spain.

## Discussion

Spermatid conception was proposed by Edwards *et al.* (1994), and successfully achieved by microinjection of round sperm-

atids (ROSI) retrieved from the ejaculate (Tesarik *et al.*, 1995, 1996) and of elongated spermatids (ELSI) recovered from a testicular biopsy (Fishel *et al.*, 1995, 1996). In both cases, however, spermatozoa had been found in previous ejaculates or testicular biopsy respectively. Tesarik *et al.* (1995, 1996) reported seven cycles of ROSI (Sa type) and four cycles of ELSI (Sd1 type) with spermatids retrieved from the ejaculates. In ROSI cycles, the fertilization rate was 46% (with 10% of syngamic zygotes) and the pregnancy rate per cycle was 29% (two full term deliveries), with these having been obtained from 2PN zygotes. In ELSI cases, the fertilization rate was higher (74%, with 13% of syngamic zygotes), but no pregnancy was achieved. In the treatment cycle presented by Fishel *et al.* (1995, 1996), using testicular ELSI, the fertilization rate was only 10% (one 2PN zygote), but a full term pregnancy was obtained. These initial encouraging results stimulated further treatment cycles in an attempt to solve the conception problem of non-obstructive azoospermic patients.

However, with larger series, results became less optimistic. Antinori *et al.* (1997b) described 36 treatment cycles performed with spermatids retrieved from testicular biopsies. Those patients never presented spermatozoa or elongated spermatids in their ejaculates. In 19 testicular ROSI cycles (Sa type), the fertilization rate was comparatively higher in relation to ejaculated round spermatids (56% of 2PN zygotes), but the pregnancy rate was only 11% per cycle. In the 17 testicular ELSI cases (types of spermatids not specified), the fertilization rate was 58% (2PN zygotes), and the pregnancy rate was again only 12% per cycle. One further pregnancy was also obtained with frozen-thawed testicular ROSI (Antinori *et al.*, 1997a).

Vanderzwalmen *et al.* (1997) then showed the influence of testicular pathology and type of spermatids selected on clinical outcomes. They presented a large series of non-obstructive azoospermic patients treated with testicular spermatid injection. Patients were divided into groups according to whether testicular biopsies showed no complete spermatogenesis (11 cycles), or where biopsies showed some foci of complete spermatogenesis (26 cycles). In the former group, the fertilization rate (2PN zygotes) was 12% and no pregnancy was achieved, whereas in the latter, the fertilization rate (2PN zygotes) was 37% and the pregnancy rate 19% per cycle. In relation to the type of spermatids used, of the five pregnancies, one was obtained with round (Sa), three with elongating (two Sb2, one Sc) and one with elongated (Sd2) spermatids. These results clearly demonstrate that an early germinal block markedly worsens the prognosis.

Finally, Amer *et al.* (1997) confirmed the poor prognosis associated with round spermatid injection, at least when there is no previous finding of a focus of complete spermatogenesis. In this work, none of the patients had ever presented spermatozoa or late spermatids in their ejaculates or testicular biopsies. In 25 cycles, round (Sa) (23 cycles) and elongating (Sb, Sc) (two cycles) spermatids from the ejaculate were used. In 34 other cycles, round (Sa) (31 cycles) and elongated (Sd2) (three cycles) spermatids were recovered from testicular biopsies. Overall, the fertilization rate (2PN and syngamic zygotes) was rather low (24%), and only two pregnancies were established (3%), both with elongated testicular spermatids.

Our present case series, albeit comprising a small number of cycles ( $n = 9$ ), supports the above finding that round spermatid injection is associated with a very low pregnancy success (eight cycles, no pregnancy), at least when there is no previous finding of a focus of complete spermatogenesis. On the other hand, injection of more developed testicular spermatids (one cycle, one successful pregnancy) seems to have an excellent prognosis. Because previous results suggested that a crucial developmental step may occur at the round spermatid stage, we attempted to culture round spermatids before injection to ascertain if removal from the seminiferous environment might favour maturation progression. Although culturing for 1–3 days, without special supplementation, seemed not to influence cell morphology, culturing for 24 h elicited a much better fertilization rate (59 versus 18% as in Amer *et al.*, 1997). This kind of procedure should therefore be tested further, in order to determine if it is accompanied by an improvement of the clinical outcome. However, until further results and experimentation have been done, it seems more prudent to advise patients that the success rate when using spermatid injection is rather low when a complete block of spermatogenesis occurs up to the round spermatid stage.

## References

- Amer, M., Soliman, E., El-Sadek, M. *et al.* (1997) Is complete spermiogenesis failure a good indication for spermatid conception? *Lancet*, **350**, 116.
- Antinori, S., Versaci, C., Dani, G. *et al.* (1997a) Successful fertilization and pregnancy after injection of frozen–thawed round spermatids into human oocytes. *Hum. Reprod.*, **12**, 554–556.
- Antinori, S., Versaci, C., Dani, G. *et al.* (1997b) Fertilization with human testicular spermatids: four successful pregnancies. *Hum. Reprod.*, **12**, 286–291.
- Araki, Y., Motoyama, M., Yoshida, A. *et al.* (1997) Intracytoplasmic injection with late spermatids: a successful procedure in achieving childbirth for couples in which the male partner suffers from azoospermia due to deficient spermatogenesis. *Fertil. Steril.*, **67**, 559–561.
- Barros, A., Sousa, M., Oliveira, C. *et al.* (1997) Pregnancy and birth after intracytoplasmic sperm injection with totally immotile sperm recovered from the ejaculate. *Fertil. Steril.*, **67**, 1091–1094.
- Edwards, R.G., Tarin, J.J., Dean, N. *et al.* (1994) Are spermatid injections into human oocytes now mandatory? *Hum. Reprod.*, **9**, 2217–2219.
- de Kretser, D.M. and Kerr, J.B. (1994) The cytology of the testis. In Knobil, E. and Neill, J.D. (eds), *The Physiology of Reproduction*, Vol. 1. Raven Press, New York, pp. 1177–1290.
- Fishel, S., Green, S., Bishop, M. *et al.* (1995) Pregnancy after intracytoplasmic injection of spermatid. *Lancet*, **345**, 1641–1642.
- Fishel, S., Aslam, I. and Tesarik, J. (1996) Spermatid conception: a stage too early, or a time too soon? *Hum. Reprod.*, **11**, 1371–1375.
- Tesarik, J. and Mendoza, C. (1996) Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. *Hum. Reprod.*, **11**, 772–779.
- Tesarik, J. and Sousa, M. (1995) Key elements of a highly efficient intracytoplasmic sperm injection technique:  $Ca^{2+}$  fluxes and oocyte cytoplasmic dislocation. *Fertil. Steril.*, **64**, 770–776.
- Tesarik, J., Mendoza, C. and Testart, J. (1995) Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med.*, **333**, 525.
- Tesarik, J., Rolet, F., Brami, C. *et al.* (1996) Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum. Reprod.*, **11**, 780–783.
- Vanderzwalmen, P., Zech, H., Birkenfeld, A. *et al.* (1997) Intracytoplasmic injection of spermatids retrieved from testicular tissue: influence of testicular pathology, type of selected spermatids and oocyte activation. *Hum. Reprod.*, **12**, 1203–1213.

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