Intracytoplasmic injection of round and elongated spermatids from azoospermic patients: results and review

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Microinjection is established as the method of choice in the treatment of severe male factor infertility as well as in azoospermic patients. Recent studies have shown that fertilization and cleavage can be achieved by injection of ejaculated as well as testicular elongated spermatids into oocytes. Here we report on the two first pregnancies worldwide resulting from elongated spermatid injection from frozen–thawed testicular tissue. Four patients with complete Sertoli cell-only syndrome (SCOS) and two with spermatogenetic maturation arrest were included in our microinjection programme. Tissues from open testicular biopsies were cryopreserved until the time of follicle puncture. A total of 67 oocytes were harvested. In the two patients with maturation arrest, cryopreserved elongated spermatids were successfully injected, while in two of the other four SCOS patients only cryopreserved round spermatids were available to be injected into the oocytes. Out of 18 injected oocytes, 10 were fertilized in the first group, while nine out of 49 injected oocytes showed fertilization and cleavage in the second group. Two clinical pregnancies were achieved with elongated spermatids from frozen–thawed testicular tissue, while no pregnancy was established in the case of round spermatids. This study confirms that fertilization, cleavage and pregnancy can be successfully achieved in cases with spermatogenetic maturation arrest by injecting cryopreserved elongated spermatids into oocytes. The literature on pregnancies following spermatid injection, as well as the problems using this technique and possible risks, are discussed.

Key words: cryopreservation/maturation arrest/Sertoli cell-only syndrome/spermatid injection/TESE
Spermatozoa may not be available in some azoospermic men with histologically confirmed maturation arrest at the spermatid stage. For this reason an alternative approach involving injection of spermatids extracted from semen or from testicular tissue was studied (Fishel et al., 1995; Tesarik et al., 1995).

The ability of spermatid nuclei to form male pronuclei and to participate in syngamy when injected into the cytoplasm of hamster oocytes was first demonstrated in 1993 (Ogura and Yanagimachi, 1993). Successive experiments on animals reported successful pregnancies after injection of round spermatids (Ogura et al., 1994; Sofikitis et al., 1994; Kimura and Yanagimachi, 1995).

Spermatid injection in assisted reproduction was first proposed in 1994 (Edwards et al., 1994). Edwards et al. estimated the number of cases in which this procedure would be necessary to be in the range of 1–2% of all cases of infertility. They also saw the possibility of culturing spermatids to retrieve more mature stages, and the theoretical possibility of cryopreserving those cells in testicular tissue.

Subsequent studies were performed on human oocytes and showed that the spermatids have potential capacity to fertilize oocytes. The fertilization rate, however, is low (25–50%) and to date (excluding this study) there have been 29 clinical pregnancies reported, seven with round spermatids (ROS) and 22 with elongated spermatids (ELS) (Table I).

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testicular tissue from two patients with spermatogenic maturation arrest. The first pregnancy established using frozen–thawed ROS was achieved by Antinori et al. (1997b).

Materials and methods

Patients

Six patients were treated, four with SCOS and two with a focal functional spermatogenesis arrested at the stage of spermatid elongation. All treated cases had pure male factor infertility, and no additional factors were present in the female partner. The women’s mean age was 29 years, that of the men was 34 years, with a mean duration of infertility of 4 years. All patients and their partners had normal karyotypes. The histology of testicular samples showed maturation arrest in the ELS injection (ELSI) cases and SCOS in the ROS injection (ROSI) cases. Embryos were at the 4-cell-stage or higher at the time of transfer, with >50% high quality embryos.

Testicular biopsy preparation

The biopsies were divided into portions in Ham’s F10 medium (Biochrom Corp., Berlin, Germany) in a Petri dish using sterile scissors; the rice grain-sized specimens were placed in 0.5 ml of HEPES-buffered medium (Sperm Freeze, Medicult, Hamburg, Germany). The samples were frozen in screw-top tubes immediately, using a Planer Kryo 10 III apparatus (Messer Griesheim, Krefeld, Germany). After 60 min the samples were stored in liquid nitrogen. The cooling procedure was performed in liquid nitrogen vapour, down to −30°C within the first 5 min and exponentially to −150°C in the following 55 min. The tissues were cryopreserved until the time of follicle puncture.

For thawing, the tissue samples were placed in a 37°C water bath for 3–5 min. Then they were washed twice in Ham’s F10 medium supplemented with 15% human umbilical cord serum (retrieved from a new-born baby within 30 min postpartum in the Department of Obstetrics). Next, they were placed into a Petri dish containing Ham’s F10 medium, where the tissue was minced in Falcon centre well dishes containing Ham’s F10. Culture tubes were incubated for 3–5 h in Ham’s F10 medium. The supernatant was transferred into 2 ml Eppendorf tubes and centrifuged at 500 g for 1 min. The pellet was resuspended using 3 μl Ham’s F10 medium.

Ovarian stimulation and oocyte preparation

Ovarian stimulation was performed according to the long protocol using gonadotrophin-releasing hormone (GnRH) analogues and human menopausal gonado-
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The spermatid injection procedure was similar to the conventional method with mature spermatozoa (Van Steirteghem et al., 1993; Al-Hasani et al., 1995). Because of the larger size of ROS, the injection needle had to be 8–10 μm in diameter, but in every other respect it was the same as that used for the conventional method.

In case of ELSI, 3–4 μm pipettes were used and the tails of the spermatids were immobilized by crushing with the injection pipette.

Oocytes were examined 16–18 h after injection to determine whether they were fertilized. Embryos were transferred into the uterine cavity 24 h later. Luteal support was performed with the administration of either human chorionic gonadotrophin (HCG) i.m. or natural micronized progesterone vaginally daily (Utrogest, 600 mg; Dr. Kade Pharmazeutische Fabrik GmbH, Berlin, Germany) as described previously (Ludwig et al., 1997).

Results

A total of 67 oocytes at metaphase II were recovered from six patients (Table II). Eighteen oocytes were injected with ELS and 16 with ROS. The fertilization rate observed 18 h after injection was 56% in cases of ELSI (10 out of 18 oocytes) and 18% in cases of ROSI (9 out of 49 oocytes). In ELSI cycles, five embryos were transferred in two patients and two pregnancies were achieved; in ROSI cycles, nine embryos were transferred in four patients but no pregnancy was established.

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**Table II. Results of microinjection of round and elongated spermatids**

<table>
<thead>
<tr>
<th></th>
<th>Round spermatids</th>
<th>Elongated spermatids</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cycles</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Total no. of oocytes</td>
<td>53</td>
<td>27</td>
</tr>
<tr>
<td>No. of injected oocytes</td>
<td>49</td>
<td>18</td>
</tr>
<tr>
<td>No. of fertilized oocytes (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 PN + 2 PB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 PN + 2 PB</td>
<td>9 (18)</td>
<td>10 (56)</td>
</tr>
<tr>
<td>No. of embryos transferred (mean per transfer)</td>
<td>9 (2.3)</td>
<td>5 (2.5)</td>
</tr>
<tr>
<td>CES* (mean)</td>
<td>21.0</td>
<td>21.5</td>
</tr>
<tr>
<td>No. of transfers</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>No. of ongoing pregnancies</td>
<td>–</td>
<td>2</td>
</tr>
</tbody>
</table>

*CES = cumulative embryo score according to Steer et al. (1992). A modification was made, since not four but three embryo quality gradings were used.*

...trophins. Oocyte preparation was carried out as described previously (Al-Hasani et al., 1995).
Discussion

Here we have reported the first pregnancies achieved using spermatid injection in Germany, and the first two pregnancies worldwide using frozen-thawed testicular ELS. Despite the limited number of cases, we confirmed that the success rate using ELS is better than that using ROS, as observed by others (Table I).

Several problems have been encountered since the introduction of spermatid injection into the techniques of assisted reproduction. These are especially (i) the injection technique for ELS and ROS, (ii) the identification of ROS, (iii) possible genetic problems arising from using undifferentiated or partially differentiated cells.

The injection procedure using ROS or ELS is similar to that used in conventional intracytoplasmic sperm injection (ICSI) programmes (Van Steirteghem et al., 1993; Al-Hasani et al., 1995). However, since ROS have a larger diameter than mature spermatozoa or ELS, larger pipettes with an inner diameter of 8 µm must be used.

The main problem when using ROS is their identification and differentiation from other cells. The gold standard for identifying these cells is transmission electron microscopy (Sofikitis et al., 1998b). However, ROS can also be identified by light microscopy. They can be distinguished from lymphocytes that can have the same diameter by their larger nuclear/cytoplasmic ratio. In addition, their nuclei appear kidney-shaped (Holstein and Rosen-Runge, 1981), or round and acentric, and possess a marbled chromatin structure. In ROS a developing acrosome can sometimes been seen as a small bright protrusion adjacent to the nucleus. ROS are more easily distinguished from other white blood cells and from other sperm precursors (spermatocytes and spermatogonia) by their smaller size; they are distinguished from red blood cells by the presence of a nucleus and by their slightly larger size (Tesarik and Mendoza, 1996b; Angelopoulos et al., 1997; Vanderzwalmen et al., 1997; Sousa et al., 1998).

Mendoza et al. (1996) reported a method to distinguish ROS by combining the immunocytochemical visualisation of proacrosin with autosomal DNA fluorescence in-situ hybridization (FISH) to assess ejaculated germ cells from patients with a spermiogenesis defect. All the cells of ROS size possessing proacrosin immunoreactivity were found to be haploid by FISH. Extending their study, they found that cell size is the main criterion for the identification of ejaculated ROS, whereas the presence of the developing acrosome represents only an auxiliary criterion. The scoring of acrosomal development in ejaculated spermatids may be useful as part of a pre-treatment diagnosis before the inclusion of infertile couples in a spermatid conception programme.

However, this classification was based on observations of normal spermiogenesis; abnormal sperm development may not produce such definite subdivisions. In addition, it is sometimes very difficult to recognize ROS solely by morphological characteristics, especially as there is no supravital staining to identify viable from non-viable cells, which is important for any improvement of the
fertilization rate. Yamanaka et al. (1997) reported a method to identify ROS by using computer-assisted image analysis which is unfortunately not available in the majority of IVF laboratories. A simplified method to recognize ROS has been reported (Mendoza and Tesarik, 1996) using three different staining techniques: Papanicolaou, fluorescein-labelled *Pisum sativum* agglutinin binding and antiacrosin antiserum immunolabelling, but all these methods do not involve vital stainings.

ELS are more easy to identify than ROS cells, since they have a completely different head shape but are morphologically distinct from mature sperm cells; thus it would be preferable to use them. Fertilization and pregnancy rates are higher with ELS than with ROS (see Table I). A lower fertilization rate has been reported following injection of spermatids from the ejaculate (24%) compared with testicular spermatozoa (79%) from the same patients into sibling oocytes (Fishel et al., 1997). Some authors have suggested that, in cases where no further matured spermatids (i.e. ELS) can be found, there are in fact no ROS present (Silber and Johnson, 1998), whereas others found ROS even in testicular biopsy specimens in which no ELS were present (Tesarik et al., 1998a).

It is now well known that fertilization, implantation and pregnancy rates are different after ELSI and ROSI. The difference may be due to the fact that spermiogenesis failure is incomplete where ELS are present. In fact, occasionally more biochemical pregnancies are reported after ROSI than after ELSI (e.g. in the study of Amer et al., 1997; Table I). Thus it has been proposed to use the terms ‘complete spermiogenesis failure’ if only ROS are present, and ‘incomplete spermiogenesis failure’ if ELS can also be identified.

It is possible that certain factors which are important for the implantation process are not yet fully developed in immature spermatids, leading to the outcome of improved implantation and ongoing pregnancy rates with increasing spermatid maturity, e.g. in ELSI cycles (Tesarik et al., 1998b). It seems, however, that even in ROS the genetic material is able to pair with that in the oocyte and participate in syngamy, fertilization and subsequent development (Sofikitis et al., 1998b). The oocyte-activating substance that is present in spermatozoa must also be present in spermatids, otherwise the fertilization process would not be induced. Recent studies (Tesarik and Sousa, 1995; Vanderzwalmen et al., 1995; Sousa et al., 1996) have reported that spermatids do contain this factor but that the oocyte requires a further boost for activation; this could be the vigorous aspiration of the cytoplasm during the injection procedure or the addition of Ca²⁺ ionophore to the medium 30 min after injection. This is not true for all species, however, since in mice no fertilization occurs after spermatid injection (Sofikitis et al., 1998b).

After the identification of ROS, the main problem with ELSI and ROSI is the inability to recognize the viability of spermatids without staining, making it impossible to know if a viable or a non-viable cell is injected (Tesarik and Mendoza, 1996; Tesarik et al., 1998a; Vanderzwalmen et al., 1998). The advantage of using a fluorescent-activated cell sorter (FACS) to isolate large homogeneous populations of viable spermatids within a short time period has been demonstrated (Aslam et al., 1998a). However, Aslam et al. also discussed possible disadvant-
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ages, including the high cost of the equipment and the exposure to a laser and its possible detrimental effect on DNA integrity. Further experience is required before these techniques can be introduced into clinical practice.

Another problem, especially for the comparison of the results of different workgroups on this topic, is the definition of ELS. Thus, the same intervention may be called ELSI by some workers and ICSI with testicular spermatozoa by others (Sousa et al., 1998). This is particularly problematic since in 60% of testicular specimens from men with germinal failure and azoospermia, occasional spermatozoa can be found.

Other problems relate to the protein content of immature spermatids compared to spermatozoa (Sousa et al., 1998) and to cell cycle asynchrony (Edwards et al., 1994). The experiments of Vanderzwalmen et al. (1997) using an ionophore to increase the fertilization rate show that there really might be a problem of oocyte activation. Since the centriole is essential for further development of the zygote and embryo, this may also be a factor which might explain the better fertilization rate following ELSI compared to ROSI: in ELS cytoskeletal maturation has progressed further (Fishel et al., 1995, 1996; Tesarik et al., 1995; Sousa et al., 1998). However, Sofikitis et al. (1998a) recently discussed the possibility of reorganization of centrosomes in mammalian oocytes when centrosomes are absent in the fertilizing spermatozoa or spermatids. This theory has still to be proven.

To overcome the lower fertilization capacity of ROS, it was recently proposed to culture these cells in vitro (Aslam and Fishel, 1998). Aslam and Fishel were able to show a progressive development of ROS towards more advanced stages of spermatogenesis.

Finally, the use of spermatids could imply some risk of genetic disorders because genomic imprinting, which also may occur during late gametogenesis, plays an important role in the regulation of embryo development. During development, specific modification of genes control the differential expression of genes on homologous chromosomes. Differential expression of the alleles of imprinted genes is related to the DNA methylation process (Fishel et al., 1996). This fundamental genetic process in sperm cells is completed in the testes before the second meiotic division in mouse cells or within the cytoplasm of the mature oocyte after spermatid injection in human cells (Kimura and Yanagimachi, 1995a,b). However, the problem of genomic imprinting has been discussed extensively (Tesarik et al., 1998a), since it has been shown that the imprinting process has not even been finished in male gametes engaged in the fertilization process (Latham et al., 1995). On the other hand, Bonduelle et al. (1998), in studies with nearly 2000 children born after ICSI, did not find an increased risk of malformations compared with the general population. However, these authors described an increased risk of XY chromosomal abnormalities in prenatal diagnosis, as also reported by others (Tesarik, 1995). All prenatal karyotyping done so far in pregnancies following spermatid injection has not shown any abnormalities in these children (Table I). Therefore, the pregnancies and children
born following spermatid injection should be carefully evaluated, as already proposed (Tesarik, 1996).

ROSI is a delicate issue and patients who undergo this procedure must be advised about the possible increased risk of genetic abnormalities resulting from immature sperm cell injection. An abnormality possibly associated with genomic imprinting is Prader–Willi syndrome (PWS). In fact PWS and Angelman syndrome (AS) are due to the lack of imprinted gene expression within chromosome 15q11-q13: the differential phenotype results from a paternal (PWS) or maternal (AS) genetic deficiency. The 15q11-q13 chromosome contains genes which are differentially expressed in the maternally and paternally inherited allele, because of the genomic imprinting which produces different modifications during male and female gametogenesis (Glenn et al., 1997). The loss of expression of only paternally expressed genes within 15q11-q13 causes PWS, and so it is obvious that genomic imprinting has to occur correctly. The problem of genomic imprinting has also been extensively discussed (Tesarik and Mendoza, 1996a; Aslam et al., 1998b; Tesarik et al., 1998b). Sofikitis et al. (1998b) proposed that even if the DNA methylation process, important for genomic imprinting, has not been completed at the ROS stage, it still may be completed after injection. This theory is supported by observations of waves of DNA methylation occurring during early embryonic development (Fishel et al., 1996).

Nevertheless, a potential genetic risk, which is also possible for offspring resulting from ICSI, should not be the reason to cancel treatment of male infertility by ELSI or ROSI. However, it should force us to study extensively the health and development of children born by these techniques. Aslam et al. (1998b) have proposed the application of spermatid injection in defined clinical trials, but restricted to specialized centres. Otherwise, there may be no possibility of collecting more data on the safety of this technique.

Patients must also be informed about the possible clinical outcome when using ROS or ELS. Tesarik et al. (1998b) estimated the take-home-baby rate for a spermatid conception attempt to be in the range of 5–10% in cases with past sperm production and in which the wife’s age is <35 years. They proposed an even lower chance of 1–5% for those couples with complete spermiogenesis failure and when the wife’s age approaches 40 years. Because of this low probability of conception, Sofikitis et al. (1998b) counsel their patients to have all available embryos transferred in a single treatment cycle under these circumstances.

To conclude, even when the diagnosis is SCOS or maturation arrest, multiple treatment biopsies are worthwhile in men suffering from non-obstructive azoospermia because a focus of complete spermatogenesis or tissue containing ROS may be found. Several authors have suggested using an operation microscope to identify such foci more easily and to avoid removal of too much testicular tissue (Balet et al., 1998; Silber, 1998). In men in whom spermatozoa or elongating/elongated spermatids have never previously been found in semen or testicular biopsies, there is to date no record of a pregnancy after ROSI that resulted in the birth of a normal baby (Tesarik et al., 1995, 1996, 1998b; Tesarik and
Mendoza, 1996a; Antinori et al., 1997a,b; Barak et al., 1998; Barros et al., 1998; Bernabeu et al., 1998; Kahraman et al., 1998; Sofikitis et al., 1998a,b; Sousa et al., 1998). Therefore, in these cases the probability of conception must be even lower than the 5% suggested by Tesarik et al. (1998b). Using ELS, however, the clinical outcome may be as good as using spermatozoa (Vanderzwalmen et al., 1997; Barros et al., 1998), but it is too soon to draw final conclusions. To summarize, the pregnancy rate using ROS is much lower than with ELS, which may be due to problems of oocyte activation, centrosome maturation or genomic imprinting. The overall experience using spermatids in human conception has developed far enough to propose their use in further cycles. However, since the problems of genomic imprinting and genetic abnormalities in the offspring are still under debate, a strong follow-up of the children must be guaranteed. Couples who undergo spermatid injection for the treatment of their infertility should be counselled about the possible as yet undefined genetic risks. Ultimately, as with other genetic risks, it should be up to the individuals to decide whether to proceed or not.

References


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