Ooplastic injection of elongating spermatids for the treatment of non-obstructive azoospermia

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We applied the technique of ooplastic elongating spermatic injection to the treatment of non-obstructive azoospermia. Mature oocytes were injected with elongating spermatic nuclei isolated from testicular biopsy material obtained from 13 non-obstructed azoospermic men. Seventy-three oocytes were successfully injected with elongating spermatic nuclei and were then cultured for 36 h. At 13 h post-injection 68 oocytes were found to be activated and 52 of them were fertilized. Forty-one 2- to 4-cell stage embryos developed from normally fertilized oocytes were transferred. At least two embryos were transferred to each female partner. Two pregnancies were achieved. Elongating spermatic injection may have a role in the treatment of non-obstructive azoospermia.

Key words: azoospermia/infertility/spermatids/testis

Introduction

The male gamete that has just completed the second meiotic division in the testis is the round spermatid and therefore its nucleus contains a complete haploid set of chromosomes. The round spermatid is transformed to a spermatozoon via a complex series of processes known as spermiogenesis. The round spermatid is transformed into an elongating spermatid during the first stages of spermiogenesis. This is a metamorphic process accompanied by important nuclear biochemical alterations and acrosomal granule transformation. Coalescence of nuclear chromatin granules is followed by replacement of lysine-rich histones by arginine-rich molecules, and accumulation of lysine-rich proteins in the sphere chromatophile (De Kretser and Kerr, 1988). Proacrosomal granules appear in stage 2 round spermatids (Oakberg, 1956) and coalesce to form a single acrosomal granule rounded on the nuclear surface (stage 3 round spermatid). This granule is subsequently extended/flattened on the nuclear membrane (Russel et al., 1990).

Previous studies have shown that fertilization and delivery of healthy offspring can occur after transferring round spermatid nuclei into rabbit or mouse oocytes via microsurgical methods (Sofikitis et al., 1996a, b; Kimura and Yanagimachi, 1995a, b) Furthermore, Sofikitis et al. (1997a) have recently demonstrated that the reproductive potential of the rabbit early haploid male gamete, increases after stage 3 of spermiogenesis. If the results of that study could be transferred to the human, recovery of spermatids of stage ≥3 would be recommended in assisted reproduction programmes applying ooplastic injection of spermatids for the treatment of male infertility due to non-obstructive azoospermia. Edwards et al. (1994) and Sofikitis et al. (1994a) first suggested that ooplastic injections of spermatids may serve as a novel mode of therapy for non-obstructive azoospermia. The first human pregnancies following ooplastic injection of round spermatid nuclei were achieved in 1994 and reported in April 1995 (Sofikitis et al., 1995a; Hannay, 1995). However, these pregnancies resulted in abortions. A few months later, Tesarik et al. (1995) reported two human pregnancies achieved via ooplastic injection of round spermatids. Two healthy newborns were delivered. Additional human pregnancies or acceptable fertilization rates after ooplastic injection of spermatids were also reported by Fishel et al. (1995), Vanderzwalmen et al. (1995, 1997), Chen et al. (1996), Yamanaka et al. (1997), Antinori et al. (1997a, b), and Araki et al. (1997).

In the present study we report the results of ooplastic injection of elongating spermatids for the treatment of male infertility due to non-obstructive azoospermia.

Materials and methods

Participants

The participants of the present study were 13 men with non-obstructive azoospermia (karyotypes 46-XY; aged 26–41 years) who attended our infertility clinics. Their wives were 18–42 years old. Peripheral serum follicle-stimulating hormone (FSH) levels in the male patients were 18–41 IU/l. Normal levels of FSH for males of reproductive age in our facilities are considered to range from 3–11 IU/l. All the 13 men had previously undergone diagnostic testicular biopsy. Round spermatids and elongating spermatids were demonstrated in a few seminiferous tubules in all men. However, most of the tubules showed spermatogenic arrest at the primary spermatocyte stage.

Oocyte preparation

Details on ovarian stimulation and oocyte recovery in our facilities have been previously published (Sofikitis et al., 1994b; Yamanaka et al., 1996a, b). Details on ovarian stimulation and oocyte recovery in our facilities are considered to range from 3–11 IU/l. All the 13 men had previously undergone diagnostic testicular biopsy. Round spermatids and elongating spermatids were demonstrated in a few seminiferous tubules in all men. However, most of the tubules showed spermatogenic arrest at the primary spermatocyte stage.

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After treatment with 0.1% bovine testicular hyaluronidase (type VIII; Sigma Co., St Louis, MO, USA) for 1–2 min the oocytes were freed from cumulus and corona cells, transferred into human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 15% patients’ serum (modified HTF medium), and incubated at 37°C under 5% CO2. Oocytes that had already completed the first meiotic division were injected with elongating spermatids 3–5 h after oocyte recovery.

**Therapeutic testicular biopsy/isolation of elongating spermatids**

A small amount of tissue (213–318 mg) was obtained from the testis biopsies of patients (to which had undergone previous diagnostic biopsy). The testicular tissue was washed three times with normal saline. The seminiferous tubules were then washed in Dulbecco’s phosphate buffered saline (DPBS; Sigma Co.) containing 5.6 mM glucose and 5.8 mM sodium lactate (modified DPBS) and subsequently minced into small pieces. Samples were maintained at 5°C and observed via a dissecting microscope (Olympus SZ-STS®; Olympus, Tokyo, Japan) during the tissue mincing process. To minimize blood cell contamination, seminiferous tubules with blood vessels in their surfaces were not further cut. The overall mincing process lasted 1 h. Then the samples were centrifuged at 500 g for 20 min, sedimanted pieces of tissue and cells were suspended in modified DPBS, and the samples were passed through a filter paper of 20–30 µm pore size (Whatman Co., New York, NY, USA). The total volume of testicular cell suspension which passed through the filter was 2–5 ml. The filtration procedure was performed at room temperature and lasted 10–15 min. The filtrate was collected, centrifuged at 750 g for 30 min, and sedimented cells were resuspended in modified DPBS. Then, the cells were observed via an inverted microscope (IX-70®; Olympus; Normarski lens) computer-assisted system (Apple Computers Inc., Cupertino, CA, USA). Elongating spermatids were identified in all participants (Figure I) and isolated via a micropipette attached to a micromanipulator (ONO-125®; Olympus/Narishige, Tokyo, Japan)-microinjecctor (Stoelting Co., Wood Dale, IL, USA) system. The internal diameter of the micropipette was 25 µm. There were neither elongated spermatids nor testicular spermatozoa in the testicular samples. The ethical committee of our Center had requested exclusion of a high percentage of elongating spermatids and to distinguish them from damaged or degenerate round cells, eight fractions of elongating spermatids recovered from eight participants were processed for confocal scanning laser microscopy computer-assisted system, using standard procedures (Sofikitis et al., 1994b, 1996b).

Elongating spermatids selected for ooplasmic injections were transferred to SOF medium (Table I). Before use, 2.85 µg of bicarbonate was added per ml of SOF medium. This medium was developed by the first author to prolong the viability of spermatids and has been already used for maintenance of human and rabbit round spermatids (Yamanaka et al., 1997; Sofikitis et al., 1997a, b). It contains lactate and glucose as energy substrates. Previous studies have demonstrated that lactate is the preferred energy substrate for round spermatids (Nakamura et al., 1978). In addition, SOF medium contains amino acids. Cholesterol was included in a low concentration to stabilize the spermatid membrane and subsequently protect the cell during incubation. Iron-binding protein (transferrin) and vitamin-binding proteins are normally secreted by Sertoli cells (Sylvester, 1993). These proteins may regulate the concentrations of iron and vitamins within the seminiferous tubules. Iron and vitamins are important for the spermatogenic process and a receptor-mediated endocytosis of transferrin by spermatids has been proposed (Sylvester, 1993). We speculated that iron and vitamins might influence spermatid viability and reproductive capacity and hence included them in the SOF medium. Preliminary experiments in our facilities have shown a lower fertilization rate after ooplasmic injection of nuclei extracted from rabbit round spermatids preincubated in SOF medium without amino acids for 9 h compared with injection of nuclei recovered from round spermatids preincubated in SOF medium for the same period.

**Elongating spermatid injections into oocytes**

For manipulations of oocytes, HEPES-buffered modified HTF medium was used.

Prior to ooplasmic injections spermatids were washed in SOF medium without cholesterol. They were then transferred to droplets (10 µl) of SOF medium without cholesterol supplemented with 1 mM MgCl2 and 4% polyvinylpyrrolidone (K90; mol. wt. 360 000; ICN Biochemicals, Costa Mesa, CA, USA) (Yamanaka et al., 1997). The internal diameter of the injecting micropipettes was approximately 10 µm. Elongating spermatids were aspirated/expelled forcefully several times with an injecting micropipette prior to ooplasmic injections. They were then compressed 8–10 times by the injecting micropipette. These manoeuvres resulted in removal of a small amount of cytoplasm from the spermatid. The spermatids were finally aspirated easily into another injecting micropipette without exerting a significant force during the aspiration technique. One spermatid was placed at the tip of the injecting micropipette. This position ensured the injection of a minimal amount of medium within the oocyte. The computer service department of Tottori University calculated that the amount of medium injected into the oocyte together with the elongating spermatid under the above described conditions was smaller than 1.8 pl. Placing the spermatid at the tip of the injecting micropipette required skill at injection to avoid losing the spermatid during penetration of the oocyte coverings. Each oocyte was placed with the first polar body at 12 o’clock position. The injecting micropipette penetrated the zona pellucida and vitelline membrane, and the spermatid was gently expelled at the centre of the oocyte to a direction opposite to that of the first polar body (6 o’clock direction). Vigorous ooplasmic aspiration/expelling manipulations were not performed during elongating spermatid injections. However, a very small amount of ooplasm was aspirated within the injecting micropipette to confirm rupture of the vitelline membrane. This minor amount of ooplasm was expelled together with the spermatid within the oocyte. After each ooplasmic injection the placement of the spermatid within the ooplasm was confirmed. In a few non-successfully injected oocytes, the elongating spermatids either failed to enter the oocytes or were extruded.

Following injection all oocytes were transferred to modified HTF medium and cultured at 37°C under 5% CO2 for 36 h. Oocytes were checked for pronuclei and second polar body at 13 h post-injection. Medium was changed at that time. Oocytes were also observed at 36 h post-injection.

An activated oocyte was defined as one with a first and second polar body extruded into the perivitelline space and at least one (female) pronucleus (Ogura and Yanagimachi, 1993; Kimura and Yanagimachi, 1995a, b; Sofikitis et al., 1996a; Yamanaka et al., 1997).

**Embryo transfer**

At 36 h post-injection 2- to 4-cell stage embryos developed from normally fertilized oocytes were transferred to the female partners by standard techniques (Sofikitis et al., 1994b).
Figure 1. An elongating spermatid observed via inverted microscope (×600) computer-assisted system (X1.96). The average diameter of the ‘head’ of the elongating spermatid is 9.3 μm.

Figure 2. An embryo containing one pronucleus and second polar body generated by ooplasmic injection of an elongating spermatid.

Results

Confocal scanning laser microscopy of spermatids
Confocal scanning laser microscopy confirmed that 100% of the evaluated cells in each fraction were elongating spermatids.

Outcome of ooplasmic injection of elongating spermatids
The outcome of ooplasmic injection of elongating spermatids is shown in Table II. Forty-one embryos generated from normally fertilized oocytes completed the first cleavage and were transferred. At least two embryos were transferred to every woman. All of the transferred embryos were at the 2- to 4-cell stage. At least one normally fertilized oocyte developed to 4-cell stage in each cycle. Two, four, five, and six embryos were transferred in eight, two, one, and two female partners, respectively. The pregnancy test (measurement of β-subunit of human chorionic gonadotrophin in serum) was positive in two women 15 (325 and 343 IU/l), 18 (1500 and 1710 IU/l) and 25 (7000 and 16 600 IU/l) days post-transfer. At 8–9 weeks post-embryo transfer a gestational sac with one embryo of normal growth was demonstrated via ultrasonography in two women. Amniocentesis at 12–14 weeks post-transfer demonstrated one XY fetus in each pregnant woman.
Development was arrested at the first cleavage stage (injection). None of these oocytes developed further; or their pronucleus plus second polar body (Figure 2), 13 h post-pronuclei without a second polar body and 16 had one body weight of the newborns was 2780 g and 3100 g.

### Table II. Outcome of ooplasmic injection of elongating spermatids

<table>
<thead>
<tr>
<th>No. ( % )</th>
<th>Hours of Culture</th>
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<tbody>
<tr>
<td>Injected oocytes</td>
<td>79</td>
</tr>
<tr>
<td>Successfully injected oocytes</td>
<td>73 (92)</td>
</tr>
<tr>
<td>Activated oocytes</td>
<td>13 (68)</td>
</tr>
<tr>
<td>Two pronuclei plus second polar body</td>
<td>13 (66)</td>
</tr>
<tr>
<td>One pronucleus plus second polar body</td>
<td>13 (66)</td>
</tr>
<tr>
<td>Cleaved oocytes</td>
<td>36 (41)</td>
</tr>
<tr>
<td>Two pronuclei plus second polar body</td>
<td>36 (11)</td>
</tr>
<tr>
<td>One pronucleus plus second polar body</td>
<td>36 (13)</td>
</tr>
<tr>
<td>Arrest at the process of first cleavage</td>
<td>36 (3)</td>
</tr>
<tr>
<td>Pregnancies</td>
<td>2</td>
</tr>
</tbody>
</table>

Two healthy male newborns were normally delivered. The body weight of the newborns was 2780 g and 3100 g.

Of the 73 successfully injected oocytes, one had two pronuclei without a second polar body and 16 had one pronucleus plus second polar body (Figure 2), 13 h post-injection. None of these oocytes developed further; or their development was arrested at the first cleavage stage (n = 3). The single oocyte with two pronuclei without a second polar body did not further develop and was recorded as unactivated since it did not satisfy the criterion of the extrusion of the second polar body into the perivitelline space (see Materials and methods).

### Discussion

The results of the present study confirm previous reports demonstrating that ooplasmic injection of human spermatids can result in fertilization and pregnancies (Sofikitis et al., 1995a, b, 1997b; Hannay, 1995; Tesarik et al., 1995; Vanderzwalmen et al., 1995, 1997; Fishel et al., 1995; Antinori et al., 1997a, b; Yamanaka et al., 1997; Araki et al., 1997). It appears that the chromosomes of the human elongating spermatozoon can pair with the chromosomes of the oocyte to participate in syngamy and further embryonic and fetal development. Furthermore, our findings support the thesis that post-meiotic maturation of the human elongating spermatozoon may not be required for the final events of the fertilization process (i.e., induction of oocyte activation, male gamete nucleus decondensation, pronuclei formation, syngamy).

The transfer of the elongating spermatozoon into oocytes that had not received any electrical or major mechanical ooplasmic prestimulation resulted in activation of 86% of the injected oocytes. It has been suggested that activation of oocytes depends on a male gamete nucleus-associated oocyte-activating factor present both in mature (i.e., epididymal) or immature (i.e., testicular) spermatozoa (Kimura and Yanagimachi, 1995a, b). Oocyte-activating factor induces the development of a characteristic series of NaCa\(^{2+}\) oscillations in oocytes by releasing NaCa\(^{2+}\) from intracellular stores (Swann, 1990). A sperm protein displaying oocyte-activating activity has been identified (Parrington et al., 1996). The oocyte-activating factor has been considered to appear/to be activated in the mouse male gamete after the round spermatid stage (Kimura and Yanagimachi, 1995a, b).

The current study shows that the transfer of a human elongating spermatozoon into an oocyte results in oocyte activation in a large percentage of cases. In preliminary experiments, 18 mature oocytes collected from superovulated women were injected using a technique and an amount of medium equivalent (qualitatively and quantitatively) to that used for injections in the present study. No oocyte was activated. Both sham injections and elongating spermatozoon injections took 14–31 s/oocyte. Since neither the injection of medium nor the technique alone were able to activate human oocytes in the present study, it suggests that the elongating spermatozoon must be responsible. Yamanaka and co-workers (1997) sham injected human oocytes with medium of the same quantity and quality as that injected into oocytes in the present study, confirming that the volume of medium or concentration of calcium injected into the oocytes together with the elongating spermatozoon did not itself activate the oocytes. Furthermore, they showed a high oocyte activation rate after injection of round spermatozoon nuclei into human oocytes with minor ooplasmic stimulation. Sousa and co-workers (1996) have demonstrated that human round spermatozooz have the ability to induce a calcium response in oocytes that is similar to that induced by mature spermatozoa. It appears that the human oocyte-activating factor has already appeared or become activated during or before the first stages of spermiogenesis.

Whether the technique applied for human male gamete injection influences the oocyte activation process is controversial. Mansour and co-workers (1996) have demonstrated that cytoplasmic stimulation prior to sperm injection into oocytes is not essential for oocyte activation and does not improve the fertilization rate. In contrast, other studies have shown that...
vigorously aspiration of oocyte cytoplasm during sperm injection facilitates oocyte activation by increasing the $\text{Ca}^{2+}$ load of the oocytes (Tesarik and Sousa, 1994, 1995). This increased $\text{Ca}^{2+}$ load may support the capacity of the oocyte-activating factor of the male gamete to trigger oocyte activation. However, the mechanical stimulus alone is not sufficient to trigger human oocyte activation during microinjection. Fishel and co-workers (1996) and Tesarik and Sousa (1995) have suggested that a mechanical vigorous ooplasmic stimulation alone does not activate human oocytes. Dozortsev and co-workers (1995) and Meng and Wolf (1997) have emphasized that human or monkey mechanical ooplasmic stimulation and oocyte exposure to a low or a relatively high extracellular calcium concentration can alter intracellular $\text{Ca}^{2+}$ but do not, alone, cause oocyte activation. Similarly, injection of dead spermatozoa does not cause oocyte activation (Dozortsev et al., 1995). The minimal ooplasmic aspiration/expelling manoeuvres applied during elongating spermatid injection in the current study may have acted synergistically with the oocyte-activating factor of the elongating spermatid. The relatively high oocyte activation rate after ooplasmic injection of elongating spermatids is unlikely to be attributable to either the age of oocytes (i.e., aged oocytes are easily activated; Ahmadi et al., 1995) or to pH fluctuations of the medium chosen for oocyte manipulation (Ahmadi et al., 1995), since microinjections were performed 3–5 h after oocyte recovery and HEPES-buffered medium was used for oocyte manipulation. The high oocyte activation rate after ooplasmic injections of elongating spermatids is also not attributable to the treatment of oocytes with hyaluronidase. In additional preliminary experiments seven human oocytes were treated with 0.1% hyaluronidase for 2 min and subsequently cultured for 48 h. None became activated.

Oocytes were observed to assess fertilization 13 h post-injection. Previous studies have indicated that the peak of the curve for the appearance of two pronuclei in a group of human oocytes injected with round spermatids or mature spermatozoa is 9 h or 16 h post-injection, respectively (Nagy et al., 1994; Sofikitis et al., 1995b; Yamanaka et al., 1997). The elongating spermatid represents a stage of maturation of the male gamete between the round spermatid and the spermatozoon. Since the speed of pronuclei appearance and embryonic development depends on the maturation stage of the male gamete (Ogura and Yanagimachi, 1993; Kimura and Yanagimachi, 1995a,b; Sofikitis et al., 1996a), we assessed fertilization between 9 h and 16 h post-injection. We speculated that at 13 h post-injection most of the oocytes normally fertilized by elongating spermatids would reveal two pronuclei. Additional studies are necessary to define the appropriate time for assessment of fertilization after ooplasmic elongating spermatid injections.

Oocytes with one pronucleus and second polar body (1PN–2SB; $n = 13$) or two pronuclei without second polar body ($n = 1$) after ooplasmic injections of elongating spermatids did not further develop or their development was arrested at the first cleavage stage. Yamanaka and co-workers (1997) also reported that 1PN–2SB stage oocytes obtained following ooplasmic injection of human round spermatid nuclei did not further develop, consistent with the findings of Kimura and Yanagimachi (1995a, b), who also suggested that some of the zygotes which are identified as IPN–2SB may have a minute male pronucleus.

Transfer of more than three or four embryos may result in multiple pregnancy. In the present study all the cleaved oocytes generated from normally fertilized oocytes were transferred. Thus, in three cycles five or six embryos were transferred. Ooplasmic injection of elongating spermatids are currently offered at a limited number of IVF centres. The couples treated in the present study may not have the opportunity to participate in an additional cycle in the future and the highest probability for pregnancy was thus offered to them by transferring all the normally fertilized and subsequently cleaved oocytes. Cryopreservation of embryos was not attempted in the current study. All the couples requested transfer of all the embryos derived from normally fertilized oocytes.

Janny and Ménězo (1994) have shown that the mission of the male gamete is not only to activate and fertilize the oocyte but also to contribute to the zygote potential to undergo the first mitotic divisions. It appears that there is a paternal effect on early embryonic development. This thesis has been recently supported by Sofikitis and co-workers (1996b) and Ono and co-workers (1997). The latter studies showed a defect in the capacity for early development and implantation of embryos generated from fertilization of oocytes by round spermatids or spermatozoa recovered from animals with varicoceles. Thus, embryos derived from fertilization of oocytes by spermatids or spermatozoa recovered from men with primary testicular damage may have an impaired potential for further development and implantation. In addition, the elongating spermatid factors mediating the paternal influence on embryonic development may be deficient since the elongating spermatid represents an immature stage of the male gamete. Therefore, we recommend transfer of all embryos generated from the fertilization of oocytes by spermatids.

We suggest that ooplasmic injection of elongating spermatids has a role in the treatment of male infertility due to non-obstructive azoospermia. However, genetic risks inherent to ooplasmic spermatid injections should be considered (see for review Sofikitis et al., 1997b). More research is necessary to understand better the physiology of elongating spermatids and subsequently improve the outcome of elongating spermatid injection procedures.

References


