Pregnancy after subzonal insemination with spermatozoa lacking outer dynein arms


1Laboratoire de Biologie de la Reproduction et du Développement — Histologie, Embryologie, Cytogénétique, Centre hospitalier, Université Paris XI, Bicêtre, 94275 Kremlin Bicêtre, France; and
2Service de Gynécologie Obstétrique, Hôpital A. Béclère, 157, rue de la Porte de Trivaux, 92141 Clamart, France

The absence of outer dynein arms in the sperm flagellum induces an abnormal movement pattern associated with male infertility. These spermatozoa can decondense in zona-free hamster oocytes but result in a very low fertilization rate in in vitro fertilization. We hypothesized that subzonal insemination could help achieve fertilization and pregnancy. A randomized prospective trial (five couples, five cycles) comparing subzonal insemination (n = 31 oocytes) and routine IVF (n = 23 oocytes) was carried out. Oocytes were microinjected with 8.5 ± 3.6 spermatozoa. In a second series (nine cycles), all the oocytes were microinjected with 10.5 ± 4.3 spermatozoa. In the randomized series, the fertilization rate was 16.1% without polyploidy, whereas no fertilization was obtained after control IVF insemination. In the second series involving nine couples, six of whom were included in the first series, the fertilization rate increased to 57.8% with a 27.8% polyspermic rate. Eighty-eight per cent of the zygotes cleaved normally (29 out of 33). A total of 11 embryo transfers resulted in three pregnancies, one of which terminated one month later, a second being ongoing and the third delivering a healthy girl. A 21.4% pregnancy rate per cycle, with a 37.5% pregnancy rate per couple, justifies the use of subzonal insemination to treat this particular flagellar dyskinesia.

Introduction

Various axonemal defects have been described in the spermatozoa of infertile men. Most defects are responsible for flagellar immotility (Aitzenius and Eliaison, 1979; Bacetti et al., 1979; Escalier and David, 1984; Escudier et al., 1990). In some cases, the absence of the outer dynein arms is the only detectable defect. The spermatozoa are motile, but there is a flagellar dyskinesia that is associated with male sterility: decreased sperm movement is observed. The diagnosis is based on finding less than three outer arms on all the electron micrograph axoneme cross-sections of the same sample. The movement pattern of these spermatozoa is characteristic. The shape of their principal flagellar curvature is similar to that of normal spermatozoa but their curvilinear velocity, straightline velocity and flagellar beat frequency are approximately half those of control values (Jouannet et al., 1983). These spermatozoa can migrate in cervical mucus but their penetration is partially or completely impaired (Jouannet et al., 1983). As a consequence, failure to ascend the female reproductive tract results in infertility as has been shown for other abnormal sperm movements that impede interaction between spermatozoa and cervical mucus (Aitken et al., 1986).

Microfertilization techniques may be a useful treatment in cases of male infertility (Ng et al., 1988; Garrisi et al., 1990; Fishel et al., 1990). Since the flagellar dyskinesia can impair the interaction between spermatozoa and zona pellucida, we hypothesized that subzonal insemination could help in achieving fertilization and pregnancy for patients whose infertility is related to this particular sperm pathology. The local Ethical Committee gave its approval. In a first series of trials, we ran a randomized clinical trial comparing a routine IVF procedure with subzonal insemination (n = 5 cycles for five different patients). In a second series of trials, three new patients were included in the programme, and six repeated attempts were performed for patients who were already unsuccessfully involved in the first series. In view of the encouraging results of the first series, all the oocytes of the second series were microinjected with a greater number of spermatozoa without any randomization.

Patients and Methods

Selection of patients

Eight couples with primary infertility for a mean duration of 7 years (range 3–12) were included in the study. The men

Received 6 May 1992.

© 1993 Journals of Reproduction and Fertility Ltd Downloaded from Bioscientifica.com at 03/22/2019 08:30:11PM via free access
were 31.5 ± 1.5 years old (range 29–34) and the women 30.7 ± 2.1 years old (range 27–34). Two couples had two previous IVF attempts each (38 oocytes) without any fertilization. Sperm counts and motility were determined by classical methods. The sperm analysis included a characterization of the sperm movement pattern using a computerized analysing system (M 2030; Hamilton Thorn, IMV France), an in vitro cervical mucus penetration and immunobead test. The sperm morphology was assessed on a smear stained with Schorr and haematoxilin according to the classification of David et al. (1975). The structural defect was suspected for these patients because their sperm exhibited a limited cervical mucus penetration test in spite of a normal or subnormal percentage of motile spermatozoon and an absence of anti-sperm antibodies. The study of their movement pattern confirmed the flagellar dyskinesia. The diagnosis was finally confirmed by examination of spermatozoon by electron microscopy, which revealed that spermatozoon from seven patients had a complete absence of outer dynein arms and 60% of spermatozoon from the last patient had this abnormality (A2) (Fig. 1). The sperm analysis was completed by a test of selection by centrifugation through a simplified Percoll gradient (95%, 47.5%) (Pharmacia, France) and a study of the percentage of motile forms in B2 Menezzo’s medium (Bio Mérieux, France) after incubation for 24 h at 20°C (Survival Test). The sperm fusiogenic function was evaluated by a zona-free hamster oocyte penetration assay before including the patients in the programme. This test was carried out by incubating thirty eggs in a sperm suspension containing 2.5 × 10^6 motile spermatozoon ml⁻¹ for 3 h. The spermatozoon had been collected the day before the test and the motile spermatozoon selected by centrifugation through a two-density Percoll gradient (95 and 47.5%) and then incubated for 18 h in BWW medium (Biggers et al., 1971) at 18°C.

Sperm preparation for oocyte insemination

The sperm specimen was obtained by masturbation on the day before oocyte retrieval (Mortimer et al., 1989). Motile spermatozoon were selected by centrifugation through a simplified Percoll gradient spun for 20 min at 300 g. Pellets were washed twice by centrifugation (5 min, 600 g), resuspended in B2 medium and kept for 16–18 h at 20°C before use.

Oocyte preparation

Follicular stimulation protocols were performed with a combination of a GnRH analogue and human menopausal gonadotrophin. Ovulation was induced with 5000 IU of hCG and oocytes were harvested 36 h later using a transvaginal ultrasound-guided procedure (Frydman et al., 1988). The cumuli were washed and individually placed in 40 μl of B2 medium under equilibrated mineral oil. The cumulus cells were removed with 0.1% hyaluronidase (Type III, Sigma, St Louis, MO) in B2 medium and the eggs checked for their nuclear status on the basis of the presence or absence of the first polar body or of the germinal vesicle (Veeck, 1986).

In the first series of experiments, the microinjection technique was tested in a trial during which oocytes were randomly divided into two groups. Oocytes from the first group were inseminated by a routine IVF technique (n = 23), and the others had subzonal insemination (n = 31).

In the second series (n = 90), all the oocytes were microinjected. For both series, the procedure took place 6–8 h after egg retrieval, regardless of nuclear maturity unless oocytes still presented a germinal vesicle. In this latter case they were matured in vitro before insemination or subzonal insemination (7 of 147 oocytes).

Sperm microinjection

Microinjection was performed using two Narishige micromanipulators and an inverted Olympus IMT-2 microscope. Differential interference contrast optics were suitable for carrying out the procedure. Micropipettes were pulled on a vertical Narishige PB-7 puller (8 μm diameter), and holding pipettes were prepared on a De Fonbrune microforge (Alcatel, France). Subzonal insemination was performed in 3 μl of Hepes-buffered B2 medium containing 0.1 mol sucrose 1⁻¹ at pH 7.4, under equilibrated mineral oil. The oocytes were transferred onto a glass depression slide, and a 3 μl of sperm suspension in B2 medium was placed on another slide. A few spermatozooa were aspirated into the microinjection pipette, flagellum first. The oocyte was then caught by the holding pipette and the zona pierced; spermatozooa were allowed to swim into the perivitelline space. Oocytes were then returned to the culture medium in an air:5% CO₂ incubator at 37°C. The total procedure took less than 5 min. The mean number of spermatozooa microinjected was 8.5 ± 3.6 in the first series and 10.5 ± 4.3 in the second (range 1–20).

Control IVF oocytes

Control oocytes were inseminated after the cumuli had been dispersed by 0.1% hyaluronidase in B2 medium. They were inseminated with 5000 motile spermatozooa in 40 μl B2 medium drops under mineral oil and kept in an air:5% CO₂ incubator at 37°C.

Embryos

The oocytes were checked 16–18 h after insemination or subzonal insemination for evidence of fertilization. Oocytes were examined for the presence of 2 pronuclei.
Table 1. Sperm parameters of patients included in the programme

<table>
<thead>
<tr>
<th>Patient</th>
<th>Volume of ejaculate (ml)</th>
<th>Number of spermatozoa × 10^6 ml⁻¹</th>
<th>Motile spermatozoa (%)</th>
<th>Total motile sperm count × 10⁶</th>
<th>Normal morphology (%)</th>
<th>Acrosome defect (%)</th>
<th>Hamster egg penetration test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>3.2</td>
<td>10</td>
<td>15</td>
<td>4.8</td>
<td>58</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>A2</td>
<td>4.6</td>
<td>23</td>
<td>28</td>
<td>29.6</td>
<td>45</td>
<td>20</td>
<td>51</td>
</tr>
<tr>
<td>A3</td>
<td>2.7</td>
<td>55</td>
<td>40</td>
<td>59.4</td>
<td>40</td>
<td>25</td>
<td>52</td>
</tr>
<tr>
<td>A4</td>
<td>2.8</td>
<td>80</td>
<td>20</td>
<td>44.8</td>
<td>34</td>
<td>25</td>
<td>71</td>
</tr>
<tr>
<td>A5</td>
<td>2.6</td>
<td>72</td>
<td>5</td>
<td>9.4</td>
<td>20</td>
<td>41</td>
<td>94</td>
</tr>
<tr>
<td>A6</td>
<td>1.9</td>
<td>65</td>
<td>40</td>
<td>49.4</td>
<td>30</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>A7</td>
<td>3.6</td>
<td>41</td>
<td>7</td>
<td>10.3</td>
<td>48</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td>A8</td>
<td>3.2</td>
<td>127</td>
<td>15</td>
<td>60.9</td>
<td>43</td>
<td>17</td>
<td>93</td>
</tr>
</tbody>
</table>

Mean: 3.0 ± 0.8 59.1 ± 36.4 21.2 ± 13.6 33.6 ± 23.1 39.7 ± 11.7 27.5 ± 9.2 49.8 ± 27.1

For each patient these values represent the mean of three analyses except for the zona-free hamster egg penetration test which was performed only once. *Percentage of oocytes with decondensed sperm heads.

Table 2. Fertilization rate in patients after subzonal and IVF insemination with spermatozoa lacking outer dynein arms

<table>
<thead>
<tr>
<th>Patient</th>
<th>Number of oocytes</th>
<th>Fertilized</th>
<th>Polyspermic</th>
<th>Number of oocytes</th>
<th>Fertilized</th>
<th>Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>yes</td>
</tr>
<tr>
<td>A2</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>5</td>
<td>0</td>
<td>23</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

exhibiting two pronuclei were considered normally fertilized. An oocyte was considered unfertilized or polyspermic when no pronucleus or more than two pronuclei, respectively, were found. Diploid zygotes were kept in culture for a further 24 h. Regularly cleaved embryos only were transferred.

Statistical analysis

Groups of oocytes were compared by Chi squared tests. Differences were considered significant at P < 0.05. Regression and correlation coefficients were studied using the STATWORKS package.

Results

Characteristics of individual patient’s spermatozoa

Except for patient A1, sperm counts were normal (Jouannet et al., 1988); the mean was 59.1 × 10⁶ spermatozoa ml⁻¹ (range: 10–127 × 10⁶). The percentage of motile spermatozoa was low for four patients (A1, A5, A7 and A8). The percentage of spermatozoa with normal morphology and the percentage with acrosome defects were comparable to those of a fertile population except for patients A5 and A7 who had an increased incidence of acrosome defects (41 and 40%, respectively). Patient A5 also presented a reduced percentage of oocytes with decondensed sperm heads in the zona-free hamster oocyte penetration test (9%), whereas the mean for all patients was 49.8 ± 27.1% (Table 1). All patients had positive survival tests and no anti-sperm antibodies. Analysis of sperm movement revealed the characteristic pattern of spermatozoa lacking outer dynein arms: reduced mean straight line velocity of 5.5 ± 14.9 µm s⁻¹ (range 7.8–21) and normal mean lateral head displacement of 2.6 ± 1.3 µm (range 0.8–3.7).

Results of subzonal insemination

A total of 147 oocytes were retrieved in 14 cycles. Sixty-eight per cent were at metaphase II at the moment of insemination or subzonal insemination. One hundred and twenty-four were microinjected. Cellular damage occurred in three cases (2.5%).

In the randomized series, fertilization was obtained for three of the five couples by subzonal insemination (Table 2). Five oocytes out of 31 were fertilized (16.1%). Four eggs cleaved normally and one remained blocked at the pronucleus stage.

Downloaded from Bioscientifica.com at 03/22/2019 06:50:17PM via free access
There was no polyploidy among these eggs and no fertilization was obtained in the control IVF group (23 oocytes). Three embryo transfers were performed, with one embryo in two cases (patients A1 and A2) and two embryos in the other (patient A3). One pregnancy resulted from the transfer of an embryo but miscarried one month later (patient A2).

In the second series (subzonal insemination only; Table 3), 90 oocytes were microinjected with a greater number of spermatozoa. Fifty-two fertilized (57.8%), of which 25 were polyspermic; the diploid fertilization was obtained for only 30.0% of the microinjected eggs. Of these diploid zygotes, 87.8% cleaved normally. Fertilization was obtained in every case in this second series, including patient A4 who had failed to fertilize during the first series. The number of normal embryos obtained for patients who had repeated attempts was generally improved. All the patients had an embryo transfer: four of them with three embryos, and two with one and two embryos, respectively. Two pregnancies were obtained. One gave birth to a healthy baby girl (patient A6), and the other pregnancy is still ongoing (patient A7).

The nuclear status at the time of subzonal insemination was recorded for 80 oocytes. Among metaphase II oocytes (n = 59), 45.8% fertilized after subzonal insemination. This percentage dropped to 28.5% for metaphase I oocytes (n = 21), but this difference was not statistically significant (P > 0.05). No fertilization occurred when less than five spermatozoa were placed in the perivitelline space (Fig. 2). Thirty-seven percent per cent of the oocytes microinjected with five to nine spermatozoa fertilized with 16.7% rate of polyspermy. When more than ten and 15 spermatozoa were microinjected, the fertilization rates increased to 46.7 and 47.3%, respectively, with a moderate augmentation of the polyspermy rate (21%). A correlation coefficient of 0.81 was found between the fertilization rate for the first attempt of five patients (A1, A2, A3, A5 and A6) whose oocytes were microinjected with the same range number of spermatozoa (8.5 ± 3.6) and the percentage of hamster oocytes with decondensed sperm heads (P = 0.09). Furthermore, the number of spermatozoa that allows a diploid fertilization was different from one couple to another, but was remarkably constant for individual couples, from one egg to another and between repeated attempts.

**Discussion**

It would be reasonable to query the ethics of assisting fertilization in patients with spermatozoa that lack outer dynein arms considering the potential risk of transmitted disease. However, no connection between the absence of outer dynein arms and genetically transmitted disease has been established, and neither the man who donated spermatozoa, nor male relatives, nor offspring born five years ago after IVF presented with respiratory disease (Spira et al., 1986). Furthermore, no defects in respiratory ciliary cells were found in patients whose spermatozoa lacked outer dynein arms (Escudier et al., 1990). In sea urchins, it has been shown that the alpha-heavy chains of sperm flagellar and ciliary outer arm dyneins are different (Ogawa et al., 1990). It is possible that such a difference also exists in other species. The potential risk of transmitting respiratory diseases, therefore, seems low. However, patients were informed of this possibility, and their acceptance of a long-term follow-up of the children has been obtained.

We report a series of 14 attempts of subzonal insemination with spermatozoa lacking outer dynein arms, including a comparison between subzonal and routine IVF insemination in a prospective randomized clinical trial, yielding three pregnancies and the birth of one baby. Zona-free hamster eggs decondensed human immotile spermatozoa lacking both dynein arms, suggesting that fertilizing ability may be, at least partially, dissociated from the sperm movement pattern (Aitken et al., 1983). These spermatozoa can undergo normal acrosome reaction as seen by transmission electron microscopy (Aitken et al., 1983). Fertilization of three human oocytes by subzonal insemination of a single immotile spermatozoon has also been achieved (Bongso et al., 1989). The embryos obtained cleaved normally but their transfer did not result in pregnancy. Spermatozoa lacking only outer dynein arms can also decondense in zona-free hamster eggs, although the mean percentage of oocytes with swollen sperm heads is lower than that with normal control spermatozoa (Courtot et al., 1985). These data...
demonstrate the ability of these spermatozoa to complete
capacitation, to undergo acrosome reaction and to attach
to the vitelline membrane and fuse with it (Courtot et al., 1985).
Pregnancy with spermatozoa lacking outer dynein arms has
been achieved. Spira et al. (1986) carried out an IVF programme
of eight cycles for five couples presenting this sperm defect.
Out of 36 oocytes, three were fertilized for one patient. The
embryo transfer resulted in a twin pregnancy. The two girls are
now five years old and appear normal. All these results demon¬
strate the low fertilization capability of spermatozoa lacking
dynein arms but that when fertilization occurs normal embryos
can be produced. We confirm the low fertilizing ability of
spermatozoa lacking outer dynein arms when placed in contact
with the zona pellucida of human oocytes; zygotes were not
obtained after the routine IVF insemination of 23 control
oocytes. The duration of the sterility of the couples, and the
very low fertilization rate obtained by classic IVF procedures
justify complementary techniques such as subzonal insemi¬
nation. Indeed, the overall fertilization rate was 47.1% after sub-
zonal insemination for 121 oocytes, with a 37.5% pregnancy
rate per patient.

Few characteristics of spermatozoa lacking outer dynein arms
seem to be important. In the first randomized series, the fer-
tilization rate was relatively low (16.1%) and the rate of
polyspermy was nil. In a similar study performed after IVF
failure with structurally normal spermatozoa, microinjection
of comparable numbers of spermatozoa under the zona pellucida
almost doubled the fertilization rate (29.4%) and the polyspermy
rate was very high (Wolf et al., 1992). This suggests that the
fusiogenic capability of spermatozoa lacking outer dynein arms
is decreased even when microinjected under the zona pellucida.
These findings confirm the observations previously made with
the hamster oocyte penetration test. This is why we decided to
increase the number of microinjected spermatozoa to more than
10 in the second series. This resulted in an improvement in the
fertilization rate (up to 57.8%) but with a polyspermy rate of
27.8%. However, the percentage of diploid zygotes was 30.0% of
microinjected oocytes, which is a better result than in the
first series. In every attempt of the second series, there was a
minimum of one egg fertilized per couple and one embryo
transfer, even for patient A4 who failed to achieve fertilization
in the first cycle. The difference in the rate of polyspermy
between the two series (nil for the first and 27.8% for the
second) is surprising since the increase of the number of
spermatozoa microinjected was relatively small. It suggests that
the fertilization rate and the rate of polyspermy are not linearly
related to the number of spermatozoa microinjected into the
perivitelline space. The fertilization rate reached a plateau
when more than ten spermatozoa were microinjected and data
collected from more than 1000 oocytes after subzonal insemination (not shown) suggest that a threshold might exist.

To reduce polyspermy, it would be useful to have criteria
that could predict the optimum number of spermatozoa to
microinject. The correlation between the hamster egg pen-
etration assays and the fertilization rate after zona drilling for
patients who had previous unsuccessful IVF cycles has been
studied but found not to be significant (Vazquez-Levin et al.,
1990). We also failed to find any correlation when we per-
formed subzonal insemination for patients with previous IVF
failure (Wolf et al., 1992). This correlation coefficient for theive patients who had the same number range of spermatozoa
microinjected almost reaches significance in spite of the small
number of cases. It could probably serve as a guideline to de-
terminie prospectively the number of spermatozoa to microinject.
This number would also probably depend upon the percentage
of motile and acrosome-reacted spermatozoa present in the
suspension after migration, as well as on oocyte quality. This
hypothesis is under evaluation.

In conclusion, subzonal insemination appears to be a reliable
technique for achieving fertilization and pregnancy in cases of
infertility related to flagellar dyskinesia owing to the lack of
outer dynein arms and should be preferred to IVF. The zona-
free hamster egg penetration assay appears to be a good indi-
cator of the number of spermatozoa to microinject, but this
needs to be confirmed by a study involving larger numbers of
patients.

This work has been supported by INSERM C/JF 8810 and Assistance
publique 'Contrat de Recherche Clinique no 910602'. Part of the results
were presented at the 7th World Congress on In Vitro Fertilization and

References

Afzelius BA and Eliasson R (1979) Flagellar mutants in man: on the hetero-
genosity of the immotile-cilia syndrome. Journal of Ultrastructure Research 69
43–52.

Afzelius RJ, Ross A and Lees MM (1983) Analysis of sperm function in
Kartagener’s syndrome Fertility and Sterility 40 696–698

Afzelius RJ, Warner P and Reid C (1986) Factors influencing the success of
sperm-cervical mucus interaction in patients exhibiting unexplained infertility
Journal of Andrology 7 3–10

Bacetti B, Burrini AG, Dallai R and Pallini V (1979) The dynein electrooeptic
bands in axoneeme lacking the inner or the outer arm Journal of Cell Biology 80
334–340

Bigger JS, Whitten WK and Whittingham DG (1971) The culture of mouse
Daniel. Freeman, San Francisco

Bongso TA, Sathananthan AH, Wong PC, Ratnam SS, Ng SC, Anandakumar C
and Ganatra S (1989) Human fertilization by microinjection of immotile
sperm Human Reproduction 4 175–179

Ability of human spermatozoa without dynein arms to penetrate zona-free
hamster oocytes. In Human in vitro fertilisation. INSERM Symposium No. 24,
Amsterdam

David G, Bisson JP, Czyglik F, Jouanett P, Gernigon C (1975) Anomalies morphe-
ologiques du spermatozoide humain: I (I) proposition pour un systeme de
classification Journal de Gynecologie Obstetrique et Biologie de la Reproduction 4
17–36

Escalier D and David G (1984) Pathology of the cytoskeleton of the human
sperm flagellum: axonemal and peri-axonemal anomalies Biology of the Cell 50
37–52

Escudier E, Escalier D, Pinchon MC, Boucharet M, Bernaudin JF and Fleury-Feith
J (1990) Dissimilar expression of axonemal anomalies in respiratory cilia and
sperm flagella in infertile men American Review of Respiratory Disease 142
674–679

insemination for the alleviation of infertility Fertility and Sterility 54 828–835

Frydman R, Forman RC, Belashch-Allart J, Hazout A, Rainhorn JD, Fries N and
Testart J (1988) Improvements in ovarian stimulation for in vitro fertilization
Annals New York Academy of Sciences 541 30–36

Clinical evaluation of three approaches to micromanipulation-assisted
fertilization Fertility and Sterility 54 671–677

Downloaded from Bioscientifica.com at 03/22/2019 08:30:11PM via free access


