Male subfertility and assisted reproduction: the quest for the ultimate treatment strategy
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Success rates of ICSI with ejaculated sperm in men with near azoospermia

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Submitted
Abstract

Men with near azoospermia have only a few motile spermatozoa in their ejaculate and on occasion no spermatozoa at all. The use of ICSI with ejaculated spermatozoa in couples with near azoospermia is controversial. Here, we report on the risk of cancellation of these cycles due to complete absence of injectable spermatozoa, as well as on pregnancy rates.

We performed a cohort study involving all couples undergoing ICSI in the Center for Reproductive Medicine in the Academic Medical Centre from 1999-2009. We compared three groups.

In group A (men with near azoospermia) there were no motile spermatozoa after conventional semen analysis and subsequent semen preparation at the time of follicle aspiration; in these men an extended sperm preparation was performed in which droplets of ejaculate sediment were extensively investigated for the presence of spermatozoa.

In group B there were also no motile spermatozoa after conventional semen analysis at the time of follicle aspiration but they were found at subsequent semen preparation.

In group C motile spermatozoa were found after conventional semen analysis at the time of follicle aspiration. Outcome measures in the three groups were number of cycles without injectable spermatozoa, number of oocytes injected, fertilisation rate, rate of total fertilisation failure, number of embryos transferred and ongoing pregnancy rates. A total of 6499 ICSI cycles were started, of which 6412 cycles resulted in follicle aspiration: 102 cycles were in group A, 145 cycles in group B and 6170 cycles in group C.

In group A seven cycles (7%) were cancelled due to the complete absence of injectable spermatozoa. This did not happen in groups B and C. The mean number of oocytes injected and the overall fertilisation rate were statistically significantly lower in group A, 7.1 and 40% respectively, compared to 8.5 and 60% for group B and 9.5 and 60% for group C (p=0.03 and p<0.001). There were 19 ongoing pregnancies (19% per cycle) in group A versus 28 and 1253 for groups B and C (20% per cycle for both groups) (p=0.43 and p = 0.38, respectively). In conclusion, although 7% of ICSI cycles in couples with near azoospermia had to be cancelled due to complete absence of spermatozoa in the ejaculate, ongoing pregnancy rates were comparable to overall ICSI pregnancy rates.

Key words: ICSI, male subfertility, semen parameters, near azoospermia.
Success rates of ICSI in near azoospermia

Introduction

The choice for the most effective assisted reproductive technique (ART) in male subfertility has proven to be a challenge. Possible options are intrauterine inseminations (IUI), in vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI) with ejaculated spermatozoa, and ICSI with surgically retrieved spermatozoa. The choice of treatment depends mainly on female factors and semen quality.

On the outer spectrum of male subfertility, where there are just a few or no motile sperm in the ejaculate, i.e. virtual azoospermia or near azoospermia, the question arises if ICSI with ejaculated sperm is a feasible treatment option or whether spermatozoa should be retrieved surgically using either microsurgical epididymal sperm aspiration (MESA) or testicular sperm extraction (TESE). This later option is of course more invasive, more time and resource consuming and is often difficult to conduct ad hoc.

The major risk in men with near azoospermia is the complete absence of injectable spermatozoa on the day of follicle aspiration. In this situation oocyte vitrification could be applied, which generates additional cost but has comparable pregnancy rates to cycles with fresh oocytes in women younger than 40.

Thus far, only two studies have reported on the effectiveness of ICSI in couples with near azoospermia on the day of follicle aspiration.

In the first study, a cohort of 49 men scheduled for TESE/ICSI underwent extended sperm preparation (ESP). ESP consists of conducting a thorough and extensive microscopic search through many droplets of ejaculate sediment when no spermatozoa have been found after regular semen preparation. In 17 men (35%) injectable spermatozoa were found in their ejaculate after ESP. The ICSI results in these 17 cycles were compared to 32 cycles of ICSI/TESE where no (motile) spermatozoa had been found after ESP. Clinical pregnancy rates were 23% per cycle in the ICSI group and 12.5% per cycle in the TESE/ICSI group.

The second study reported on 57 ICSI cycles of men with near azoospermia and compared these to a control group of 43 ICSI cycles of men with moderate semen impairments with a total sperm count between $1 \times 10^6$ and $5 \times 10^6$ spermatozoa. The ongoing pregnancy rate was 17% per cycle in the group with near azoospermia and 25.6% per cycle in the control group, a difference that was not statistically significant.
These data do not answer the question whether ICSI with ejaculated spermatozoa can achieve acceptable pregnancy rates in couples with near azoospermia at the time of follicle aspiration without the cost of too many cancelled cycles due to complete absence of injectable spermatozoa after semen preparation or whether ICSI/TESE is a better option for these couples.

To gain more insight into the success rate of ICSI in near azoospermia, we performed a large cohort study on ICSI with ejaculated spermatozoa in couples with near azoospermia on the day of follicle aspiration to determine the risk of not finding injectable spermatozoa and to report on success rates compared to all other couples undergoing ICSI in the same period.

**Material and methods**

We included cycles from all couples that underwent ICSI treatment between January 1998 to December 2009 at the Center for Reproductive Medicine (CRM) in the Academic Medical Center, Amsterdam, the Netherlands. All steps leading to the ICSI procedure are listed in Figure 1.

Inclusion criteria for our ICSI program were a post-wash total motile sperm count (TMC) of less than 1 million motile spermatozoa during the fertility workup or fertilization failure, i.e. poor fertilisation or total fertilisation failure, in a previous IVF cycle. When these inclusion criteria were met, women underwent controlled ovarian hyperstimulation using the same regime as mentioned in earlier publications, followed by follicle aspiration.

**Semen analysis and preparation**

Immediately after the follicle aspiration, semen analysis was performed according to WHO guidelines. Men had a minimal sexual abstinence of two days and analysis of the semen was performed after liquefaction and within 1 hour after ejaculation. After the semen analysis a pre-wash TMC was calculated. In case no motile spermatozoa were found, ie the pre-wash TMC was zero, the sample was washed once with culture medium (180x g, 10 minutes), examined again for the presence of spermatozoa and a post-wash TMC was calculated.

In case no motile spermatozoa were found, ie the post-wash TMC was zero, couples were classified as group A. In case motile spermatozoa were present, i.e. the post-wash TMC was above zero, couples were classified as group B. In group A an extended sperm preparation (ESP) was performed, for which a resuspension of the specimen in 0.3 ml of culture medium was made.
The resuspension was pipetted in a culture dish and flattened into a thin drop and covered with oil. This drop was then scanned microscopically for motile spermatozoa. Oocytes were only injected with motile spermatozoa as in the Netherlands it is prohibited to use non-motile ejaculated spermatozoa for ICSI. If no motile spermatozoa were found after ESP, ICSI could not be performed.

In all other cases where motile spermatozoa were found upon direct visualization of the ejaculate immediately after the follicle aspiration, i.e. the pre-wash TMC was above zero, semen preparation was performed as follows: Semen was diluted 1:1 with culture medium (Ham’s-F10, Gibco BRL, Life Technologies Ltd, Paisley, UK or Human Tubal Fluid, Cambrex, Verviers, Belgium supplemented with 15% pasteurized plasma protein solution) and subjected to density gradient centrifugation using 70% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) or 70% PureSperm (Nidacon, Gothenburg, Sweden) (650x g for 10 min.). Then the pellet was washed once with culture medium (180x g for 10 min.) and, depending on the sperm concentration, resuspended in 1-2 ml of culture medium. The sample was then incubated for 1h at 37 °C / 5% CO₂ during which the motile spermatozoa were allowed to swim to the bottom of the tube (“swim-down”). Finally, the pellet was washed again (180x g for 10 min.) and the post-wash TMC was calculated. All measurements were performed with the Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). These couples were classified as group C.

ICSI procedure

In all groups, all meta-phase II oocytes were then injected with one motile and morphologically normal spermatozoon approximately 40 hours after hCG. Oocytes were inspected for fertilization, which was defined as the presence of two or more pronuclei approximately 18 hours after injection. At this time, all embryos were transferred individually to a fresh volume of 75 μl culture medium for further culture. Embryos were cultured under oil at 37°C in 5% CO₂ in air. Embryo transfer was performed on day 3 or in some instances on day 4. Clinical pregnancy was determined by measuring serum hCG 12 and 18 days after follicle aspiration. Ongoing pregnancy was defined as positive fetal cardiac activity of at least one fetus at 12 weeks gestation.

Data analysis

We compared the baseline characteristics between groups A, B and C, including female and male age and the pre-and post-wash TMC obtained during
the fertility work-up, preceding the ICSI procedure. To assess the reproducibility of the sperm characteristics from the fertility work-up as compared to the sperm characteristics at the time of follicle aspiration, we calculated which couples from group A at the time of follicle aspiration would have been assigned to group A according to the pre-and post-wash TMC during the fertility work-up, and which couples to groups B and C. For this purpose a 3x3 table was constructed with the actual group classification according to the pre- and post-wash TMC at the time of follicle aspiration, and the predicted group classification according to the pre-and post-wash TMC from the fertility work-up.

We next compared data obtained during the ICSI cycles, like number of oocytes, number of oocytes injected, fertilisation rates, total fertilisation failure rates, and number of embryos transferred between the three groups. Finally, we compared clinical and ongoing pregnancy rates. Statistical analysis for fertilization rates was performed on the percentage values of the variables within each group. The mean number of oocytes, oocytes injected, number of embryos transferred, female age, male age and the post-wash TMC during the fertility work-up were also expressed as the mean of the values of these variables within each cycle. Global significance was tested by paired comparison of t-test.

The comparison of the pregnancies and the occurrence of total fertilization failure in the different groups were compared by means of the chi²-test. Pregnancy rates were compared using relative risks (RR). When applicable Fisher’s exact test was used. A p-value < 5% was considered statistically significant for all tests.

Results

A total of 6,499 ICSI cycles were started, of which 87 cycles were cancelled because of hyper- or hyporespons, personal reasons or the absence of oocytes on the day of follicle aspiration. In the remaining 6412 cycles semen was obtained and the cycles were allocated to the different study groups: 102 cycles to group A (57 couples), 140 cycles to group B (61 couples) and 6170 to group A (3122 couples) (Figure 1).
Success rates of ICSI in near azoospermia

Figure 1, Flow diagram

Post-wash TMC <1 beyond during the fertility work-up or fertilization failure during a previous IVF (n=6499)

Started ICSI cycles (n=6499)

Cancelled cycles (n=87)

Semen analysis at FA (n=6412)

Pre-wash TMC at FA (n=6412)

=0 (n=242)

> 0 (n=6170)

Semen preparation (wash) at FA

Semen preparation (density gradient and wash) at FA

Post-wash TMC at FA (n=242)

>0 (n=140) group B

>0 (n=6170) group C

Post-wash TMC at FA (n=6170)

=0 (n=102) group A

Extended sperm preparation (ESP)

=0 (n=7)

>0 (n=95)

No treatment

ICSI

FA=follicle aspiration; TMC= total motile count
Baseline characteristics are summarized in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age</td>
<td>33</td>
<td>33.6</td>
<td>35.4</td>
<td>(^1) p=0.72; (^2) p&lt;0.001</td>
</tr>
<tr>
<td>Male age</td>
<td>37</td>
<td>37</td>
<td>38</td>
<td>(^1) p=0.95; (^2) p=0.86</td>
</tr>
<tr>
<td>Pre-wash TMC</td>
<td>1.5</td>
<td>17</td>
<td>21.5</td>
<td>(^1) p&lt;0.001; (^2) p&lt;0.001</td>
</tr>
<tr>
<td>Post-wash TMC</td>
<td>1.3</td>
<td>9.3</td>
<td>11.8</td>
<td>(^1) p=0.04; (^2) p&lt;0.001</td>
</tr>
<tr>
<td>(fertility work-up)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 statistical difference between group A and B
2 statistical difference between group A and C

Female age was 33.0 years for group A versus 33.6 and 35.4 years in group B and C, respectively (p<0.001). The mean pre- and post-wash TMC during the fertility work-up were significantly lower in group A as compared to group B and group C (p<0.05).

The relation between the findings at the pre-and post-wash TMC from the fertility work-up and final group assignment according to the pre- and post-wash TMC at the time of follicle aspiration is shown in Table 2.

<table>
<thead>
<tr>
<th>Couples</th>
<th>Fertility work-up (pre-wash TMC=0 and post-wash TMC=0)</th>
<th>Fertility work-up (pre-wash TMC=0 and post-wash TMC&gt;0)</th>
<th>Fertility work-up (pre-wash TMC&gt;0 and post-wash TMC&gt;0)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>A: Follicle aspiration (pre-wash TMC=0 and post-wash TMC=0)</td>
<td>14 (25%)</td>
<td>9 (16%)</td>
<td>34 (59%)</td>
</tr>
<tr>
<td></td>
<td>B: Follicle aspiration (pre-wash TMC=0 and post-wash TMC&gt;0)</td>
<td>10 (16%)</td>
<td>9 (15%)</td>
<td>42 (69%)</td>
</tr>
<tr>
<td></td>
<td>C: Follicle aspiration (pre-wash TMC&gt;0)</td>
<td>18 (0.6%)</td>
<td>10 (0.3%)</td>
<td>3094 (99%)</td>
</tr>
</tbody>
</table>

In 23 out of 57 couples in group A (41%), the pre-wash TMC during the fertility work-up showed no motile spermatozoa. Only 14 couples (25%) would have been allocated to group A according to the pre-and post-wash TMC during the fertility work-up. In 19 out of 61 couples (31%) in group B, the pre-wash TMC showed no motile spermatozoa during the fertility work-up. Only 9 couples (15%) would have been allocated to group B according to the pre-and post-wash TMC during the fertility work-up. In group C, 28 out 3122 couples (0.9%) had no motile spermatozoa pre-wash during the fertility work-up.
Success rates of ICSI in near azoospermia

The majority of cases (99%) in group C would have been allocated to group C according to the pre-and post-wash TMC during the fertility work-up.

Data obtained during the ICSI cycles are summarized in Table 3.

<table>
<thead>
<tr>
<th>Table 3 ICSI data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>ICSI cycles</td>
</tr>
<tr>
<td>No injectable sperm</td>
</tr>
<tr>
<td>Oocytes</td>
</tr>
<tr>
<td>Oocytes inseminated</td>
</tr>
<tr>
<td>Oocytes fertilized</td>
</tr>
<tr>
<td>TFF</td>
</tr>
<tr>
<td>Embryos transferred</td>
</tr>
<tr>
<td>Fertilisation grade</td>
</tr>
<tr>
<td>Clinical pregnancies</td>
</tr>
<tr>
<td>Ongoing pregnancies</td>
</tr>
</tbody>
</table>

¹ statistical difference between group A and B
² statistical difference between group A and C
³ per started cycle
⁴ per ET

In group A, in seven out of 102 cycles (7%) no injectable spermatozoa were found, even after ESP. By definition, injectable spermatozoa were found in all cycles in group B or C. The overall chance of the absence of injectable spermatozoa on the day of follicle aspiration, if no motile spermatozoa were found in the initial semen sample, was 3% (7 out of 242 cycles).

The number of oocytes per cycle did not differ between the three groups, but the number of injected oocytes did differ between group A, compared to groups B and C. The number of oocytes fertilized, fertilization grade and the number of embryos transferred were all statistically significant lower in group A as compared to groups B and C. The risk of total fertilization failure (TFF) in the cycles with injectable spermatozoa differed slightly between group A compared to B and C, but this was not statistically significant (6/102= 6% compared to 11/140=8% and 645/6170=10%, respectively, p=0.52 and p=0.11).

The number of ongoing pregnancies per ICSI cycle in the total cohort was 1300 in 6412 cycles (20% per cycle). The number of ongoing pregnancies in group A was 19 pregnancies in 102 cycles (19% per cycle), which was not significantly different from the ongoing pregnancy rate per cycle in group B with 28 ongoing pregnancies in 140 cycles (20% per cycle (RR 0.91(95%CI 0.54-
1.5). When comparing group A with group C, (1253 ongoing pregnancies in 6170 cycles), we found a RR of 0.91 (95%CI 0.60-1.4). The ongoing pregnancy rates per embryo transfer were 19 per 89 transfers (21% per ET) in group A, 28 in 129 transfers (22% per ET) in group B and 1253 in 5525 cycles (23% per ET) in group C, respectively (RR 0.98 (95%CI 0.58-1.7) for group A versus group B and RR 0.95 (95%CI 0.63-1.4) for group A versus group B).

**Discussion**

The introduction of ICSI in 1992 was a tremendous breakthrough in the reproductive arsenal for couples with severe male subfertility. Shortly thereafter, it also became possible to obtain spermatozoa from the epididymis and the testis in obstructive and non-obstructive azoospermia and to perform ICSI with these surgically retrieved spermatozoa. In 1998 a moratorium on the use of surgically retrieved spermatozoa for ICSI became operational in the Netherlands because of concerns about the health of offspring. Around 2001 the use of microsurgical epididymal sperm aspiration (MESA) was allowed under a research protocol. Similarly, in 2008 the use of testicular sperm extraction (TESE) was allowed under a research protocol. Given these circumstances, ICSI with ejaculated spermatozoa was performed in the Netherlands even if the risk of azoospermia was high, simply because it was the only available option for couples with near azoospermia.

In our ICSI cohort with almost 6,500 cycles, more than 200 cycles showed no motile spermatozoa before preparation (near azoospermia) at the day of follicle aspiration. Only seven out of 242 cycles (3%) had to be cancelled because no spermatozoa could be found at the day of follicle aspiration. Although the number of fertilized oocytes and the number of embryos were lower in the near azoospermia group, the ongoing pregnancy rate was comparable to the overall ICSI group.

More than 60% of the male partners were not expected to have near azoospermia on the day of follicle aspiration, as their semen samples in the fertility work-up contained motile spermatozoa both pre- and post-wash. This means that the semenparameters from the fertility work-up do not always help to predict semenparameters at follicle aspiration. So, in an IVF/ICSI program near azoospermia can occur at any random day of follicle aspiration and an ESP should then be performed.

The strength of our cohort study lies in the large number of cycles analyzed. Furthermore, we have established a large experience with ESP in those
years that we could not perform TESE/ICSI in our clinic because of regulatory restrictions. This has led to detailed information on semen preparation and ESP performance. This detailed information is mostly lacking in the few other published studies on this subject.

One could say that the population with near azoospermia is comparable to a TESE population with non-obstructive azoospermia. In the early days of TESE/ICSI, one study made a comparison between ICSI with ejaculated spermatozoa in a moderately severe male subfertility population (mean TMC 8x10^6) and testicular or epididymal spermatozoa in azoospermic men. The fertilization rates were significantly lower for surgically retrieved spermatozoa than after injection with ejaculated sperm with comparable ongoing pregnancy rates of around 22% per cycle. Our findings confirm these data, although the study population that underwent ICSI with ejaculated spermatozoa did not suffer from near azoospermia. Recently, another study was published comparing TESE/ICSI and ICSI with ejaculated spermatozoa in men with near azoospermia. This study reports on 13 couples, where TESE/ICSI was performed with fresh and frozen/thawed spermatozoa and ICSI with spermatozoa from the ejaculate, depending on the presence of vital spermatozoa in the ejaculate in consecutive cycles. They found better fertilisation grades and implantation rates for the cycles with fresh testicular spermatozoa compared to frozen/thawed testicular spermatozoa or ejaculated spermatozoa, but the sample size of this study was very small.

To determine if we should perform ICSI with ejaculated spermatozoa in all cases of near azoospermia there are three important issues to consider.

Firstly, in many cycles the event of near azoospermia pre-wash is an unexpected finding and TESE will therefore never be considered in the first place. Secondly, the risk of ICSI with ejaculated sperm in near azoospermia, namely the absence of injectable spermatozoa after preparation, is low (7%). Finally, the logistic problems with ad hoc TESE, and the burden for the male partner can be avoided by ICSI.

Overall, we conclude that ICSI with ejaculated spermatozoa in couples with near azoospermia should be preferred above TESE-ICSI as the chances of not finding injectable spermatozoa are very small and success rates are acceptable. If there are no injectable spermatozoa on the day of follicle aspiration in men with near azoospermia, all oocytes can be vitrified and then thawed again in a natural cycle after spermatozoa are found in the ejaculate or after TESE.
References