

Article

Factors affecting outcome after ICSI with spermatozoa retrieved from cryopreserved testicular tissue in non-obstructive azoospermia



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Abstract

There is a lack of data regarding variables affecting the treatment outcome for non-obstructive azoospermia when spermatozoa from cryopreserved testicular specimens are utilized for ICSI. The objective of the present retrospective analysis was to investigate the effect of various parameters on treatment outcome in such cases. One hundred and sixty five couples with non-obstructive azoospermic males undergoing a total of 297 cycles were included. In all cases the testicular tissue retrieved by multiple open-biopsy testicular sperm extraction was stored in liquid nitrogen and, after thawing, only mature spermatozoa were used for ICSI. When no motile spermatozoa were recovered, immotile spermatozoa were used. In 159 cycles, motile spermatozoa were utilized for ICSI, while in 138 cycles immotile spermatozoa were utilized. Higher normal fertilization rate (60.4 ± 3.1 versus $51.3 \pm 1.6\%$, $P < 0.05$), number of embryos transferred (2.8 ± 0.06 versus 2.6 ± 0.04 , $P < 0.05$), modified cumulative embryo score (31.2 ± 1.6 versus 23.9 ± 0.8 , $P < 0.001$), and proportion of motile spermatozoa injected (67.8 versus 49.8% , $P < 0.05$) were observed in cycles that resulted in clinical pregnancies. Binary logistic regression analysis showed that sperm motility (odds ratio 2.06, 95% CI 1.1–3.9, $P < 0.05$), but not maternal age, number of treatment cycle, type of GnRH-analogue used for pituitary suppression, number of oocytes retrieved or number of embryos transferred was a significant determinant of the likelihood of clinical pregnancy. In conclusion, sperm motility after freeze/thawing of testicular tissue is the major determinant of the success of ICSI in non-obstructive azoospermia.

Keywords: TESE, ICSI, non-obstructive azoospermia, sperm motility

Introduction

Non-obstructive azoospermia (defective spermatogenesis mainly) accounts for 60% of azoospermic patients (Matsumiya *et al.*, 1994). After the first reports of using spermatozoa from testicular tissue derived by testicular sperm extraction (TESE) for intracytoplasmic sperm injection (ICSI) (Schoysman *et al.*, 1993; Devroey *et al.*, 1994), this method has become an established technique for assisted reproduction in cases of non-obstructive azoospermia. The recovered spermatozoa may be used freshly for ICSI or the tissue may be cryopreserved for future ICSI.

There is a lack of data regarding variables affecting the treatment outcome for non-obstructive azoospermia when spermatozoa from cryopreserved testicular specimens are utilized for ICSI. The aim of the present retrospective analysis was to investigate the effect of sperm motility and other parameters on treatment outcome in such cases.

Materials and methods

Sample size calculation

The number of cycles needed to detect a difference in clinical

pregnancy rate of 10% between the two groups was calculated. Assuming that a clinical pregnancy rate of 25% would be feasible and higher in the group in which motile spermatozoa were utilized, a one-sided z-test with continuity correction was used. Sample sizes of 206 cycles in each group would be needed to achieve 80% power at a significance level (α) of 0.05. The significance level and power were calculated for 10 sequential steps (looks) using the O'Brien–Fleming spending function.

Patients

In the present retrospective study, 165 non-obstructive azoospermic males pursuing assisted conception in the Department of Obstetrics and Gynaecology at the University of Schleswig-Holstein, Germany from December 1995 to December 2002, were included. In all cases the testicular tissue retrieved by open multiple-biopsy TESE was cryopreserved and only mature spermatozoa (no early spermatids) were used for ICSI after thawing. The age (mean \pm SEM) of the male and female partners was 34.2 ± 0.6 and 32.2 ± 0.2 , respectively. Histologic diagnoses of the testicular biopsies showed some form of spermatogenesis defect in all cases, such as germ cell aplasia, maturation arrest and tubular sclerosis, and in the majority of cases mixed forms were diagnosed. All patients had a normal karyotype. Clinical pregnancy was defined as fetal heart activity in transvaginal ultrasound at 5–6 weeks after embryo transfer, although this definition is somewhat early and might overestimate the clinical pregnancy rate.

Retrieval of testicular tissue

Testicular tissue was obtained under local or general anaesthesia using an open multiple-biopsy technique (one cranial and one caudal tissue section in each testis). From each part a sample was fixed for the histopathological examination. Testicular specimens in Ham's F10 medium were examined under 40 \times objective phase-contrast microscopy to confirm the presence of spermatozoa, and were subsequently frozen in up to 10 fractions.

Freezing method of testicular tissue

Spermatozoa were not extracted before freezing, and tissue the size of a rice grain was frozen, although there are reports about successfully freezing few spermatozoa in empty zona pellucida (Cohen *et al.*, 1997; Montag *et al.*, 1999). From the present authors' experience, the difficult isolation of a very small number of spermatozoa in non-obstructive azoospermia cases and the action of testicular tissue as a cryoprotectant, reducing the freezing shock to spermatozoa, advocates freezing of testicular tissue. The testicular tissue suspension samples were placed in 0.5 ml HEPES-buffered medium, consisting of modified Earle's balanced salt solution with 0.4% human serum albumin and 15% glycerol as a cryoprotectant (Sperm Freeze, Medicult, Hamburg, Germany). The samples in 2 ml vials (Greiner, Frickenhausen, Germany) were placed on the top of a styrofoam box filled with liquid nitrogen and left there for 20–30 min. Afterwards they were immersed in liquid nitrogen and stored until required for ICSI.

Ovarian stimulation

Ovarian stimulation was performed using urinary or recombinant gonadotrophins [Menogon (Ferring Arzneimittel GmbH, Kiel,

Germany) or Gonal-F (Serono International S.A., Geneva, Switzerland); Puregon (Organon, Oss, The Netherlands) respectively] and either gonadotrophin-releasing hormone (GnRH) agonists [Enantone Gyn (Takeda Pharma GmbH, Aachen, Germany) or Decapeptyl-Gyn Depot (Ferring Arzneimittel GmbH)] (205 cycles) or antagonists [(Cetrotide (Serono International S.A.) or Orgalutran (Organon)] (90 cycles) for pituitary suppression in various protocols, while in two cycles there was no pituitary suppression. Ovulation was induced by administration of either urinary or recombinant human chorionic gonadotrophin (HCG) [Choragon (Ferring Arzneimittel GmbH) or Ovitrelle (Serono International S.A.) respectively]. Oocyte retrieval was performed by transvaginal ultrasound guided puncture of follicles 36 h after the HCG injection with or without general anaesthesia. For luteal-phase support, progesterone [Utrogest (Dr Kade, Berlin, Germany) or Crinone (Serono International S.A.)] with or without additional HCG was utilized.

Thawing procedure

After oocyte retrieval, testicular tissue samples containing spermatozoa were thawed in a 37°C water bath for 3–5 min and prepared by using the mechanical method (Baukloh, 2002) for ICSI. The minced tissue was incubated in Ham's F-10 medium up to 5 h before being used for the ICSI procedure.

ICSI

ICSI of spermatozoa was performed as previously described (Al-Hasani *et al.*, 1995). The supernatant medium was centrifuged for 1–2 min in an Eppendorf tube. One μ l from the pellet was added to one or two drops of medium under oil to be used for injection. In cases where no motile spermatozoa could be recovered, immotile spermatozoa with otherwise normal morphology were injected. If at least slight movements of the head or tail were observed, spermatozoa were considered motile, which is suggestive of viability. Retrieved oocytes were incubated for 3–4 h before ICSI, and this practice has been shown to be associated with improved maturation of oocytes, fertilization and cleavage rates and embryo quality (Isiklar *et al.*, 2004).

Statistical analysis

Statistical analysis was performed using Student's t-test, chi-squared test, Pearson correlation and binary logistic regression analysis. Numeric data were normally distributed (one sample Kolmogorov–Smirnov test). Descriptive statistics are presented as mean \pm SEM. The statistical software package used was SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) for Windows version 11.0 and NCSS (Number Cruncher Statistical Systems, Karysville, UT, USA) 2001.

Results

The present study ended once the significance level and power boundaries of the chi-squared test, in terms of difference in clinical pregnancy rate between the two groups (motile and immotile sperm), were fulfilled. In particular, for 288 cycles in both groups a significance level of 0.024 and a power of 52.2% would suffice to establish a statistically significant difference and interrupt the study. The 297 cycles analysed showed clinical pregnancy rates of 26.4 and 14.5% in the motile and immotile

sperm groups, respectively, accounting for a 0.017 significance level and 66.4% power.

A total of 297 cycles in 165 couples were performed, accounting therefore, for 1.8 ± 0.08 cycles performed for each couple (range 1–6). However, the inclusion of other cycles as well as first treatment cycles might constitute a methodological drawback of the study and bias the results. The total number of oocytes retrieved was 3669, and 12.3 ± 0.3 (range 1–30) were retrieved per cycle. The overall normal fertilization rate was 0.53 ± 0.01 , and 2.6 ± 0.04 embryos were transferred (range 1–3) with a modified cumulative embryo score (Al-Hasani *et al.*, 1999) of 25.5 ± 0.7 . The clinical pregnancy rate per cycle was 20.9% (62/297) and the multiple pregnancy rate (twins) per cycle was 2% (6/297). The clinical pregnancy rate per couple was 37.6% (62/165). Fifty-three (32.1%) of the 165 couples achieved at least one clinical pregnancy. In these 53 couples, each couple had an average of 1.17 ± 0.07 clinical pregnancies (range 1–4).

In 159 (53.5%) cycles motile spermatozoa were injected, while in the other 138 cycles immotile spermatozoa were used for ICSI. The age of the women and men did not differ significantly between these two groups. A lower number of oocytes retrieved (11.7 versus 13.1 , $P < 0.05$), a higher normal fertilization rate (65.2 versus 39.4% , $P < 0.001$), a higher number of embryos transferred (2.8 versus 2.4 , $P < 0.001$), a higher modified cumulative embryo score (28.0

versus 22.5 , $P < 0.001$), and a higher clinical pregnancy rate (26.4 versus 14.5% , $P < 0.05$) were observed in cycles in which motile spermatozoa were injected (**Table 1**).

Table 2 shows the comparison of various parameters between cycles that did, and those that did not, result in clinical pregnancy. A higher normal fertilization rate (60.4 versus 51.3% , $P < 0.05$), a higher number of embryos transferred (2.8 versus 2.6 , $P < 0.05$), a higher modified cumulative embryo score (31.2 versus 23.9 , $P < 0.001$), and a higher proportion of motile spermatozoa injected (67.8 versus 49.8% , $P < 0.05$) were observed in the cycles that resulted in clinical pregnancies.

Binary logistic regression analysis was performed to evaluate the relative strength of dependence of clinical pregnancy likelihood from relevant independent (predictive) factors. It was found that sperm motility (odds ratio 2.08, 95% CI 1.1–3.92, $P < 0.05$), but not maternal age, number of treatment cycle, type of GnRH-analogue used for pituitary suppression, number of oocytes retrieved or number of embryos transferred, was a significant determinant of the likelihood of clinical pregnancy (**Table 3**). This was the case whether block entry of variables was used in the model or any of the following stepwise methods: forward conditional, forward likelihood ratio, forward Wald, backward conditional, backward likelihood ratio or backward Wald.

Table 1. Comparison of various parameters between cycles with motile or immotile sperm utilization for ICSI.

	Type of sperm injected		P-value
	Motile	Immotile	
Maternal age (years)	32.1 ± 0.3	32.3 ± 0.3	NS
No. of oocytes retrieved	11.7 ± 0.4	13.1 ± 0.5	<0.05
Normal fertilization rate (%)	65.2 ± 1.8	39.4 ± 1.7	<0.001
No. of embryos transferred	2.8 ± 0.04	2.4 ± 0.06	<0.001
Modified cumulative embryo score	28.0 ± 1.0	22.5 ± 1.0	<0.001
Clinical pregnancy rate (%)	26.4	14.5	<0.05

NS = not significant.

Table 2. Comparison of various parameters between cycles that did and did not result in clinical pregnancy.

	Clinical pregnancy	No clinical pregnancy	P-value
Maternal age (years)	32.3 ± 0.5	32.2 ± 0.3	NS
No. of oocytes retrieved	12.8 ± 0.7	12.2 ± 0.4	NS
Normal fertilization rate (%)	60.4 ± 3.1	51.3 ± 1.6	<0.05
No. of embryos transferred	2.8 ± 0.06	2.6 ± 0.04	<0.05
Modified cumulative embryo score	31.2 ± 1.6	23.9 ± 0.8	<0.001
Percentage of motile spermatozoa injected (%)	67.8	49.8	<0.05

NS = not significant.

Table 3. Relative dependence of clinical pregnancy likelihood from various independent (predictive) factors (model using block entry of variables). The indicator contrast method was used in the logistic regression procedure.

<i>Independent factors</i>	<i>Odds ratio</i>	<i>95% confidence limits</i>	<i>P-value</i>
Maternal age	1.02	0.95–1.1	NS
No. of treatment cycles ^a	–	–	NS
GnRH analogue ^b	(i) 11.9	0.6–235.4	NS
	(ii) 0.9	0.46–1.73	NS
No. oocytes retrieved	1.01	0.96–1.07	NS
Sperm motility	2.06	1.1–3.9	<0.05
No. embryos transferred ^c	(i) 0.32	0.06–1.75	NS
	(ii) 0.59	0.26–1.3	NS

NS = not significant.

^aOdds ratios with 95% confidence limits are not presented for each of the six treatment cycles, due to non-significant values and simplicity of the table.

^bOdds ratios with 95% confidence limits are presented for no analogue (i) and GnRH agonist (ii) with reference to the GnRH-antagonist utilization.

^cOdds ratios with 95% confidence limits are presented for one (i) and two (ii) embryos transferred with reference to the transfer of three embryos.

Discussion

The successful outcomes after ICSI (Palermo *et al.*, 1992) established the potential of treatment in cases of non-obstructive azoospermia, where spermatozoa may be recovered by TESE. These spermatozoa may be used fresh, or can be cryopreserved for future ICSI cycles with similar treatment success (Friedler *et al.*, 1997; Ben-Yosef *et al.*, 1999). However, cryopreservation of testicular tissue may provide more advantages, such as excluding the possibility of unfruitful commencement of ovarian hyperstimulation and minimizing the repeats of the TESE procedure (Oates *et al.*, 1997) and its potential adverse local effect on the testis (Tash and Schlegel, 2001).

In ICSI cycles, sperm motility is an important parameter for fertilization and pregnancy and indicates sperm viability, although immotility does not preclude viability. The hypo-osmotic swelling test and variants (Casper *et al.*, 1996; Liu *et al.*, 1997; Salam *et al.*, 2001), and more recently others such as the phenomenon of sperm tail curling and the mechanical touch technique to observe tail flexibility (Ma *et al.*, 2000; de Oliveira *et al.*, 2004), have been suggested for selecting viable immotile spermatozoa before ICSI. However, due to low sperm count it is technically difficult to perform the hypo-osmotic swelling test in TESE tissue and it was not applied in the series reported here. Although pentoxifyllin treatment of the fresh or frozen sperm suspension activates sperm motility even in totally immotile spermatozoa, contributing to selection of viable spermatozoa (Terriou *et al.*, 2000), in the present cases this method has not been used. Furthermore, another method, the hyaluronic acid-coated slide sperm binding assay has been shown to facilitate the selection of single mature spermatozoa with high DNA integrity and low frequencies of chromosomal aneuploidies for ICSI (Cayli *et al.*, 2003).

Recently, a microfluidic device has been developed for isolation of motile spermatozoa from oligozoospermic samples without the need for centrifugation, even with high numbers of immotile spermatozoa and contamination of other cell types, with promising results (Schuster *et al.*, 2003). This may have an application in motile sperm retrieval from testicular tissue after properly designed future studies.

Sperm motility is quite uncommon in men with severe sperm axonemal defects, and it is known that the centrosome is responsible for motility of spermatozoa and the microtubule-organizing centre of the oocyte and/or zygote. However, although such axonemal defects might preclude normal fertilization after ICSI, when detected in a very high proportion of spermatozoa, they are not indicative of a global centrosomal defect (Alosilla Fontis *et al.*, 2002).

Sperm motility has been reported to be the single most important factor associated with successful outcome of ICSI with freshly ejaculated spermatozoa (Nagy *et al.*, 1995). Similarly, in cases of azoospermia (obstructive and non-obstructive), the normal fertilization rate was significantly lower (45.8 versus 64.9%) when fresh immotile versus motile testicular spermatozoa were used, but the pregnancy rates (positive HCG) were similar (30.8 versus 26.7%) (Nagy *et al.*, 1998). When the authors performed a separate analysis for non-obstructive azoospermic cases (60 cycles), the respective mean values for fertilization rates were 31.2 versus 60.8% ($P < 0.05$). Another retrospective study (Shulman *et al.*, 1999) with a small number of azoospermic cases (53 cycles in 50 couples) treated by ICSI with fresh testicular spermatozoa, has also shown lower fertilization rates (51 versus 62%), but not significantly different pregnancy rates (15.8 versus 23.5%) when immotile versus motile spermatozoa were used. The reason for the pregnancy rate being unaffected by sperm

motility in these two retrospective studies may be due to the small numbers of cycles with utilization of immotile spermatozoa (14 cycles) and total cases, respectively. Park *et al.* (2003) analysed retrospectively 261 TESE-ICSI cycles in couples with obstructive azoospermia. In 177 cycles, cryopreserved testicular tissue was utilized for ICSI and significantly lower fertilization and pregnancy rates (determined by serum β -HCG) (50.9 versus 70% and 27.3 versus 33.9%, respectively) were found when thawed immotile versus motile spermatozoa were used. However, ongoing pregnancy rates were similar (23.6 versus 30.3%).

Therefore, motility constitutes an important factor in selecting spermatozoa for ICSI. However, the influence that this parameter, among others, has on the treatment outcome has not yet been evaluated. In the present study, the relative effect that sperm motility has, among other factors, on the clinical pregnancy likelihood was investigated. These independent factors were selected on the basis of biological relevance and being at the same time widely accepted as important factors affecting assisted reproduction treatment outcome.

In the present study, a higher number of cycles than in previous studies (Nagy *et al.*, 1995, 1998; Shulman *et al.*, 1999; Park *et al.*, 2003) was included, and a different type of analysis was performed in order to clarify the importance of sperm motility in treatment outcome in cases of ICSI with cryopreserved testicular spermatozoa in non-azoospermic cases. To the present authors' knowledge this is the first study addressing this issue.

These results, in accordance with previous studies (Nagy *et al.*, 1995, 1998; Shulman *et al.*, 1999; Park *et al.*, 2003), showed higher normal fertilization rates in cycles in which motile spermatozoa were injected. The chance of clinical pregnancy seemed to be twofold when motile spermatozoa were used for ICSI compared with immotile sperm. Furthermore, binary logistic regression analysis showed that sperm motility was the only significant determinant of this treatment outcome. This finding is consistent with the results of a previous study, although in that study ejaculated spermatozoa were used (Nagy *et al.*, 1995).

In 53.5% of the cases reported here, motile spermatozoa were found after thawing of the cryopreserved testicular tissue. As suggested previously (Nagy *et al.*, 1998), a high percentage of motile spermatozoa (a range of 54 to 79% according to histologic diagnosis) may be found in fresh testicular tissue in cases of non-obstructive azoospermia. The relatively lower yield of motile spermatozoa in the cases reported here may be due to the cryopreservation/thawing procedure, and different distribution of testicular pathologies. The high percentage (90.5%) of motile sperm motility recovery presented in the study by Gil-Salom *et al.* (2000) could be due to sperm extraction before freezing, increasing the probability of cryoprotectant penetration into the sperm cells.

The diminution of maternal age in the list of significant determinants of clinical pregnancy, as was found by comparison between successful and unsuccessful cycles (the power of the t-test was 0.94) and binary logistic regression analysis, seems to agree with the results of a previous study (Silber *et al.*, 1997). In this study, although in 249 couples with

obstructive azoospermia maternal age had a negative impact on delivery rates, in 63 non-obstructive azoospermic couples a non-significant difference of the delivery rates between various age groups was found; however, the authors underlined the higher (but not significant) delivery rates in the younger women. In the present study, further comparisons (data not shown) of the clinical pregnancy rates between various maternal age subgroups also showed that maternal age did not affect clinical pregnancy rate. However, the age distribution in the women of the present study might account for this result, because only 11.4 and 2% of the women were older than 37 and 40 years, respectively. This was also the case for the effect of the number of oocytes retrieved on clinical pregnancy. Maternal age and number of oocytes retrieved were significantly and inversely correlated ($r = -0.21$, $P < 0.001$) as was expected, but none of these major factors, known to affect assisted reproduction treatment outcome in general, were significant determinants of clinical pregnancy in cases of cryopreservation-TESE cycles. This finding underlines the major importance of sperm motility in this severe form of male infertility. Furthermore, in the majority of the cases reported here no evidence of a female contribution to infertility has been recorded, although the female partners have not been uniformly assessed.

Another important issue that has not been clarified so far is the value of performing transfers of frozen/thawed embryos after ICSI in non-obstructive azoospermia cases. A recent study (Osmanagaoglou *et al.*, 2004) has shown the advantage of multiple frozen/thawed embryo transfers after ICSI but has not investigated the subgroup of non-obstructive azoospermia, although the authors reported that there was no significant difference in the crude delivery rate achieved by either non-surgically or surgically retrieved spermatozoa groups (8.6 versus 5% respectively).

To conclude, in this retrospective analysis of non-obstructive azoospermia cases, the major determinant of the success of ICSI was sperm motility after freeze/thawing of testicular tissue.

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