Successful testicular sperm extraction (TESE) in spite of high serum follicle stimulating hormone and azoospermia: correlation between testicular morphology, TESE results, semen analysis and serum hormone values in 103 infertile men

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Spermatozoa recovered from testicular biopsies can be used through intracytoplasmic sperm injection (ICSI) to achieve a pregnancy. To assess the likelihood of successful testicular sperm extraction (TESE) in men suffering from severe oligo- or azoospermia, bilateral biopsy specimens were obtained. Following semi-thin sectioning, the morphology of testicular samples was graded according to a modified Johnsen score. TESE was performed in parallel to this histological examination. The number of isolated spermatozoa was assessed in a semiquantitative way. From 103 patients investigated, 64 (62.1%) showed azoospermia in a preceding semen analysis and 29 (28.2%) patients had sperm concentrations between 0.1 and 1×10^6/ml. In 10 patients who had higher sperm counts, most spermatozoa were non-motile. Spermatozoa could be detected after TESE in the testicular tissue of 49 (77%) azoospermic men. When follicle stimulating hormone (FSH) concentration was normal, most patients had detectable spermatozoa after TESE. Nearly one-third of patients with mildly elevated FSH had no spermatozoa. Thirty-nine percent of patients in whom FSH was elevated to more than twice normal and 50% of patients with grossly elevated FSH had no detectable spermatozoa. In all, 82.8% of men with sperm concentrations between 0.1 and 1×10^6/ml in their ejaculate showed spermatozoa in the tissue sample after TESE. Our data demonstrate that, contrary to previous recommendations, infertile men with azoospermia and high FSH values should be reconsidered for testicular biopsy, provided that tissue samples can be cryopreserved for later TESE/ICSI treatment.
Key words: azoospermia/biopsy/male infertility/testis/testicular sperm extraction

Introduction

Advanced methods of assisted reproduction have revolutionized treatment of male factor infertility. In particular, the introduction of intracytoplasmic sperm injection (ICSI) in combination with in-vitro fertilization (IVF) has offered for the first time a therapy with a high chance of producing a pregnancy, even from severely oligoasthenoteratozoospermic and azoospermic patients (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993; Redgment *et al.*, 1994; Tsirigotis *et al.*, 1995). In addition, a number of novel techniques for spermatozoa retrieval from the epididymis and testis, including microsurgical epididymal sperm aspiration (MESA) (Silber and Asch, 1992; Silber *et al.*, 1994; Tsirigotis *et al.*, 1995), percutaneous epididymal sperm aspiration (PESA) (Craft *et al.*, 1995; Tsirigotis *et al.*, 1995) and testicular spermatozoa extraction (TESE) (Schoysman *et al.*, 1993a,b; Silber *et al.*, 1995), have been recently described, and may be successfully combined with ICSI. TESE has been used to treat cases of obstructive azoospermia resulting from the congenital absence of the vas deferens (CAVD), when retrieval of epididymal spermatozoa is not possible, and/or the epididymis itself (Silber *et al.*, 1995). However, several reports have indicated that TESE can also be applied in cases of serious testicular damage (including multifocal atrophy of seminiferous tubules) or disturbances of spermatid differentiation (Fishel *et al.*, 1995; Gil-Salom *et al.*, 1995a; Silber *et al.*, 1995; Yemini *et al.*, 1995; Tournaye *et al.*, 1996).

The majority of procedures described for TESE use a mechanical method of sample preparation, i.e. fine mincing of the testicular biopsy and isolation of a few, vital, spermatozoa capable of fertilization (Abuzeid *et al.*, 1995; Gil-Salom *et al.*, 1995a,b; Tucker *et al.*, 1995). Recently, a new TESE concept has been proposed (Salzbrunn *et al.*, 1996), based on enzymatic treatment of the testicular tissue with a mild concentration of collagenase, instead of mechanical force. This procedure combines histological examination with an immediate post-surgical test for the presence of spermatozoa in the biopsy (test-TESE). The simultaneous cryopreservation of additional testicular biopsy fragments permits subsequent TESE/ICSI cycles to be performed without the need for the male to undergo further surgery. The present study was carried out to assess the likelihood of successful sperm retrieval in men suffering from azoospermia or severe oligozoospermia.

Materials and methods

Patients

All patients referred for testicular biopsy to the Infertility Clinic of the Department of Andrology, University of Hamburg, between June 1995 and May 1996, were eligible. The study population comprised men in whom previous semen analyses had shown azoospermia or such low numbers of viable spermatozoa that there was a high risk of a futile IVF/ICSI procedure if relying on ejaculated spermatozoa only. Results from previous semen analyses and hormone measurements [luteinizing hormone (LH), follicle stimulating hormone (FSH),
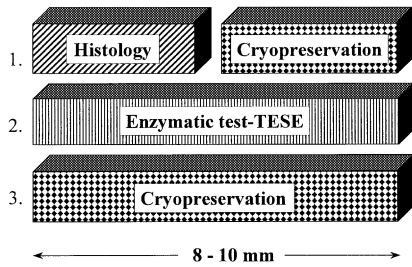
Figure 1. Work-up of tissue sample obtained by testicular biopsy prior to different investigations and cryopreservation. In order to obtain representative information of the tissue to be frozen, the whole biopsy was removed in three separate layers as indicated ('sandwich principle'). 1: upper layer, 2: middle layer, 3: lowest layer.

testosterone] were provided by the referring physicians. The procedure was explained in detail to the patients and written and informed consent was obtained. Biopsies were obtained from each testis under local anaesthesia.

Testicular biopsy

Tissue specimens for cryopreservation, histological analysis and trial TESE were obtained according to the method described by Holstein *et al.* (1994). An incision 8–10 mm in length into the tunica albuginea was made in order to reach at least four to five testicular lobules. The protruding tissue was removed by cutting with microsurgical scissors. In order to obtain representative information on the tissue to be frozen, the whole biopsy was removed in three separate layers. The upper layer was divided into two. One part was used for histological examination, while the remainder was cryopreserved. The total portion of material obtained from the middle layer was subjected to a TESE attempt. The tissue from the lowest layer was also cryopreserved. We refer to this work-up of the biopsy sample as the 'sandwich principle' (Figure 1).

Histological evaluation

The fragment selected for histological examination was immediately immersed in 5.5% glutaraldehyde at 4°C for 2 h and then postfixed in 1% OsO4 for a further 2 h. Afterwards, the tissue was dehydrated in a series of ascending alcohol concentrations and propylene oxide, and embedded in Epon 812 (Holstein and Wulfhekel, 1971). Tissue blocks were cut by a diamond knife (Diatome), using a Reichert ultramicrotome. Semithin sections (section thickness, d = 1 μm) were stained with Toluidine Blue/Pyronine (Ito and Winchester, 1963) and analysed under light microscopy (Olympus BL 91, Germany).

During histological analysis, the following variables were assessed: total number of tubules in the section; number of tubules containing germ cells and proportion of atrophic tubules ('tubular shadows'). When germinal epithelium was present, the state of spermatogenesis was evaluated using a modified Johnsen score of testicular biopsies (Table I, De Kretser and Holstein, 1976; Holstein *et al.*, 1994). In cases of impaired spermatogenesis where differences were present between individual tubules, the highest score found within the whole section was used to grade the biopsy.

Additionally, the tubular diameter and the thickness of the lamina propria were measured using an ocular micrometer. Morphology of interstitial tissue was also examined, with particular attention directed to changes in the Leydig cells, the presence of inflammatory and/or mast cells, the appearance of blood vessels, and interstitial fibrosis.

Cryopreservation

Tissue samples selected for cryopreservation were immersed in 0.5 ml Sperm-Freeze (Medicult, Hamburg, Germany) and subsequently

Table I. Modified Johnsen score of testicular biopsies

Score	Morphological base
1	Tubular sclerosis
2	Sertoli cells only
3	Spermatogonia only
4	Arrest at primary spermatocyte, no spermatids
5	Many spermatocytes, no spermatids
6	No late spermatids, arrest at spermatid stage
7	No late spermatids, but many early spermatids
8	Few late spermatids
9	Many late spermatids, disorganized tubular epithelium
10	Full spermatogenesis

frozen in two steps (cooling to –60°C during the first 5 min and then exponentially to –120°C in the following 55 min using a Nicoolbag 10 device (Air Liquide, Wiesbaden, Germany). They were then stored in liquid nitrogen until use (Salzbrunn *et al.*, 1996).

Testicular spermatozoa extraction (TESE)

The tissue from the middle layer of the biopsy was placed in 1 ml of prewarmed (37°C) Sperm-Prep Medium (Medicult) and incubated at 37°C for 2 h. At the end of this time, 1 ml sterile filtered prewarmed Sperm-Prep Medium supplemented with 0.8 mg collagenase, type AI (Sigma, Heidelberg, Germany) and 0.2 μg trypsin inhibitor (Sigma) was added. The sample was then incubated for a further 2 h and finally centrifuged for 10 min at 800 *g* and 37°C. After removal of the supernatant, the pellet was analysed [always by the same investigator (W.S.)] using a light microscope at ×400 magnification high power field (HPF). The result of the test-TESE was classified as follows: grade I: >10 spermatozoa/HPF; grade II: 1–10 spermatozoa/HPF; grade III: <1 spermatozoon/HPF. When very few spermatozoa could be detected, search time (< 5 min or >5 min) was used for further discrimination of sample quality. When no haploid gametes could be detected after at least 30 min of intensive exploration, the sample was considered sperm-free (grade IV).

Statistical analysis

Data were recorded on predesigned questionnaires and transcribed to computer files for further analysis by SPSS. Mean and SD are given where data are normally distributed; otherwise medians, ranges and percentiles are quoted. Means of related parametric samples were compared by paired *t*-test or, in the case of ordinal level measurements, by Wilcoxon matched pairs signed ranks test. To estimate correlations between nominal level data, contingency coefficients (CC) were used. Values of $P < 0.05$ were considered as statistically significant. To facilitate presentation of data and improve data reduction, serum FSH concentrations and sperm counts were allocated to discrete groups as given below.

Results

During the study period, a total of 132 patients underwent testicular biopsy for further diagnosis of male factor subfertility. Due to previous hemicastration, maldescensus or testicular atrophy in 24 cases, biopsies were obtained only unilaterally; thus results from bilateral samples were obtained for only 108 patients. In five of these men, data sets were not complete and were excluded prior to statistical analysis. Results from the remaining 103 cases are presented here.

Table II. Study population classified according to presurgical sperm concentration and TESE results

Sperm count	TESE results				
	No sperm	Sperm present in			
		Left testis	Right testis	Both testes	Total (%)
Azoospermia	15	6	6	37	64 (62.1)
0.1–1.0 × 10⁶/ml	5	1	6	17	29 (28.2)
1.1–5.0 × 10⁶/ml	1	0	1	7	9 (8.7)
> 5 × 10⁶/ml	0	0	0	1	1 (1.0)
Total (%)	21 (20.4)	7 (6.8)	13 (12.6)	62 (60.2)	103 (100)

TESE = testicular sperm extraction.

Semen parameters and serum FSH values

Semen analysis showed that 64 (62%) patients suffered from azoospermia. The remaining 39 (38 %) patients had severely reduced numbers of normally formed and motile spermatozoa (for details see Table II).

Serum FSH concentrations were normal (below 7.5 IU/l) in 41 (39.8%) patients, while 27 (26.2%) patients had concentrations between 7.6 and 15 IU/l. In 25 (24.3%) cases FSH concentrations ranged from 15.1 to 30 IU/l and the remaining patients ($n = 10$; 9.7%) had values between 30 and 62.3 IU/l. Of the 64 azoospermic men with azoospermia, 37 (58%) had FSH values >7.5 IU/l, while the remaining 27 (42%) had values within the normal range. Of the 39 men with severe oligozoospermia, 25 (64%) had elevated FSH levels, while the remaining 14 (36%) were normogonadotrophic.

Histological examination and biopsy scores

An average of 22.2 ± 7.9 tubules were evaluated per biopsy. Tubular diameters ranged from 80 to 240 µm (mean ± SD 195 ± 42.7 µm). The thickness of the lamina propria was 6.9 ± 2.8 µm (mean ± SD), ranging from 5 to 28 µm.

Histological examination of the biopsy material revealed a variety of pathological changes in seminiferous tubules and interstitial tissue, with scores ranging from 1 to 10. Thus, in ~2% of samples only atrophic tubules ('tubular shadows', score = 1) could be observed, while tubules lined with Sertoli cells exclusively (score = 2) were recorded in 20.4% of cases on the right side and 22.3% of cases on the left side. However, the majority could be classified as 'mixed atrophy' (Sigg and Hedinger, 1981), where seminiferous tubule showed different degrees of degeneration alternating with regions of almost intact spermatogenesis (score = 1–2–3–4–9). Full spermatogenesis (score = 10) was recorded in only ~5% of all samples. Further analysis revealed differences of up to six grades between the two sides in each direction. In only 53% of patients did biopsies from the two testes have an identical morphological aspect. Details are given in Table III.

Postsurgical testicular sperm extraction (test-TESE)

TESE was successful in 82 (79.6%) cases overall. In 16 (15.5%) samples from both the right and left testis, we were able to isolate more than 10 spermatozoa per microscopic field. One to 10 spermatozoa per microscopic field were visible in 25 (24.3%) and 24 (23.3%) samples taken from the right

Table III. Differences in scores of testicular biopsies between left and right testis

Difference in scores	Number of patients (n)	Relative proportion (%)
–6	3	2.9
–4	1	1.0
–3	1	1.0
–2	2	1.9
–1	12	11.7
0	55	53.4
1	18	17.5
2	3	2.9
4	4	3.9
5	2	1.9
6	2	1.9
Total	103	100

and the left side respectively. Less than one spermatozoon per HPF was seen in 34 (33%, right side) and 29 (28.2%, left side) preparations, while in the remaining cases [right side: 28 (27.2%); left side: 34 (33%)] no spermatozoa could be isolated. In 69 (67%) and 65 (63.1%) of all samples (right/left), spermatozoa could be detected following less than 5 min search time.

The individual distribution of TESE results and presurgical semen analysis are presented in Table III. This indicated that in 77% of azoospermic men ($n = 64$), spermatozoa could be detected by TESE. If the analysis is limited to azoospermic men with FSH serum concentrations <7.5 IU/l, which represent the majority of cases of obstructive azoospermia, 26 of 27 patients (96.2%) had positive TESE results on at least one side. However, even when FSH concentrations were increased in azoospermia, there was a 62% chance of isolating spermatozoa from testicular biopsies (Table IV).

Comparison of results obtained from histological examination of the biopsy and TESE showed a positive correlation between Johnsen scores and successful spermatozoa extraction (Table V), with highly significant contingency coefficients (CC right side: 0.58; CC left side: 0.60; approximate significance $P < 0.0001$).

The results suggest that the better the score, the better is the outcome of TESE. The best results were obtained following TESE where the histological analysis revealed mature spermatids within the seminiferous epithelium. However, if germ cells

Table IV. Presurgical serum FSH values and TESE results in individual patients with azoospermia

Serum FSH (IU/l)	No sperm	TESE results			
		Sperm present in			
		Left side	Right side	Both sides	Total (%)
<7.5	1	0	1	25	27 (42.1)
7.6–15	4	3	1	5	13 (20.3)
15.1–30	7	2	3	6	18 (28.2)
>30	3	1	1	1	6 (9.4)
Total (%)	15 (23.4)	6 (9.4)	6 (9.4)	37 (57.8)	64 (100)

TESE = testicular sperm extraction, FSH = follicle stimulating hormone.

were present only as primary spermatocytes or spermatogonia, or Sertoli cells only were present, the probability of a negative outcome for TESE (no spermatozoa isolated) was increased. The same situation applied to cases of tubular atrophy. Regular tubular diameter (220–280 μm) showed a positive correlation with spermatozoa isolation ($P < 0.01$). However, if tubular diameter was reduced (180–80 μm) or if lamina propria was thickened (>7 μm), the likelihood of a negative TESE result was increased ($P < 0.01$). A positive correlation was found between the score and the search time for spermatozoa in the TESE pellet.

Discussion

The correlation between the results obtained from histological analysis and from TESE has shown that the best outcome of TESE may be expected if the biopsy specimen contains tubules with mature spermatids, normal diameter and a regular thickness of the lamina propria. If the histological evaluation of the seminiferous epithelium shows the presence of only Sertoli cells, spermatogonia or primary spermatocytes, the probability of a negative outcome of TESE is significantly increased. The same is true for cases of tubular sclerosis or general reduction of tubular diameter and/or increased lamina propria thickness.

Histological analysis in our survey revealed a picture of 'mixed atrophy' in the vast majority of cases. Despite extensive morphological defects of the tubules, it was possible in an assessment frequently to detect tubular segments with spermatogenetic activity. Even in cases of hypergonadotropic azoospermia, such tubular segments may provide a source of spermatozoa for a successful TESE attempt. The worst prognosis is for those patients whose tubules are completely atrophic or are lined with Sertoli cells exclusively. However, positive TESE results obtained in some cases show that even these patients must have focal regions of spermatogenetic activity. This confirms previous data (Tournaye et al., 1996). In our experience, only the combination of histology and postsurgical test-TESE enables secure predictions to be made about the presence of spermatozoa in the cryopreserved testicular sample and the chances for a successful IVF/ICSI procedure in the female partner.

During the evaluation of the histological analyses and TESE, we noted differences between the right and left testes in almost

Table V. Correlation between testicular histology expressed as modified Johnsen score and TESE results expressed as frequency and quantity of sperm detection in samples from right and left testis

Score	>10/HPF	1–10/HPF	<1/HPF	No. sperm
1	0/0	0/0	0/0	2/2
2	0/0	0/0	7/4	14/19
3	0/0	0/0	1/0	0/1
4	0/0	0/0	0/3	4/4
5	0/0	0/0	0/0	1/1
6	0/0	1/0	6/5	4/2
7	0/2	3/1	7/8	2/5
8	3/3	9/8	8/6	1/0
9	8/7	12/14	5/3	0/0
10	5/4	0/1	0/0	0/0
CC	0.54*/0.46*	0.43*/0.51*	0.37/0.40*	0.58*/0.60*

Right testis/left testis; CC = contingency coefficient; * = estimated significance $P < 0.05$.
TESE = testicular sperm extraction, HPF = high power field.

half of all patients. Consequently, we recommend that a bilateral testicular biopsy should be performed whenever possible.

Earlier reports on TESE have pointed out that this method can be used in the case of obstructive azoospermia, which implies a relatively well preserved testicular structure (Silber et al., 1994, 1995; Craft et al., 1995; Tucker et al., 1995). Recently, Yemini et al. (1995) described a case of an azoospermic patient with tubular atrophy where, despite extensive tubular damage, a few spermatozoa were present and could be used for ICSI. Such studies are in good accord with our observations, which demonstrate on a much wider basis that 62% of our hypergonadotrophic patients with azoospermia still had enough intratesticular spermatozoa for ICSI treatment. Although there is a negative correlation between TESE result and serum FSH concentration, there appears to be no upper limit for FSH concentrations above which no spermatogenetic activity can be expected. This was confirmed in our series by the patient with the highest FSH concentration (62.3 IU/l) where viable spermatozoa could be retrieved in sufficient numbers for an ICSI treatment. This also agrees with results of other studies (Gil-Salom et al., 1995b; Kim et al. 1997).

In the treatment of severe oligoasthenozoospermic patients or in cases of azoospermia, microsurgical techniques, including MESA (Silber and Asch, 1992; Silber et al., 1994; Tsirigotis et al., 1995) or PESA (Craft et al., 1995; Tsirigotis et al., 1995), have been used. Despite successful retrieval of

spermatozoa from the epididymides of such azoospermic patients, in some cases a testicular biopsy nevertheless had to be performed in order to yield spermatozoa for ICSI (Silber *et al.*, 1994; Craft *et al.*, 1995; Tsirigotis *et al.*, 1995). In contrast to MESA and PESA, our method offers a histological evaluation of the testicular biopsy in parallel with TESE. Moreover, the biopsied material can be used for several TESE procedures, without further trauma and exploration of testicular tissue (Salzbrunn *et al.*, 1996). In addition, whenever it is possible to retrieve spermatozoa by MESA, TESE will also be successful, whereas the reverse is not true. The TESE procedure presented here has the additional advantage that it is less invasive, more economical, easier and faster to perform and without the need for general anaesthesia. High pregnancy rates after IVF/ICSI treatment demonstrate that the fertilizing capacity of spermatozoa retrieved by TESE is unsurpassed even when sperm numbers are low (Fischer *et al.*, 1996).

In contrast to others (Abuzeid *et al.*, 1995; Gil-Salom *et al.*, 1995a,b; Tucker *et al.*, 1995), we use an enzymatic approach to isolate spermatozoa from testicular biopsies (Salzbrunn *et al.*, 1996). Our experience with this method indicates a higher yield of viable spermatozoa in comparison to mechanical 'shredding'. This is especially important for the cases of 'mixed atrophy', where often only focally preserved spermatogenesis is evident.

In conclusion, we have demonstrated a correlation between the results of histological analysis and TESE outcome. Histological evaluation in combination with a postsurgical test-TESE is an optimal tool in the diagnosis and treatment of male-factor infertility, and furthermore provides reliable information about the chances of success in the subsequent treatment of the infertile couple.

Acknowledgements

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