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Multiple testicular sampling in non-obstructive azoospermia—is it necessary?

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Spermatogenesis may be focal in non-obstructive azoospermia. The present study was conducted to determine whether the performance of multiple, rather than a single testicular sample contributes to obtaining spermatozoa in amounts sufficient for fertilization and cryopreservation in non-obstructive, azoospermic patients. Furthermore, the aim was to clarify the significance of location for retrieval from the testis in such cases. Three biopsies were taken from identical locations in 55 testes of 29 men with non-obstructive azoospermia: (i) the rete testis region, ii) the midline, and (iii) the proximal region of the testis. When sperm cells were detected, they were used for intracytoplasmic sperm injection (ICSI), and the remainder were then cryopreserved in as many aliquots as possible (adjusted for ICSI procedure). Spermatozoa were found in 28 testes (50.9%) of 18 men (62.1%). In the testes from which spermatozoa were obtained, they were present in three, two or one locations in 15 (53.6%), five (17.9%) and eight (28.6%) cases respectively. The possibility of finding spermatozoa was not influenced by the location in the testis. Multiple testicular sperm extraction is recommended in cases of non-obstructive azoospermia, since it may enhance diagnostic accuracy of absolute testicular failure and increase the number of sperm cells retrieved.

Key words: ICSI/non-obstructive azoospermia/spermatogenesis/testis

Introduction

It is well established that mature testicular spermatozoa could be found in cases of non-obstructive, azoospermic men (Hauser *et al.*, 1994; Tournaye *et al.*, 1995). Moreover, it was also proved that such sperm cells have fertilizing ability once the intracytoplasmic sperm injection (ICSI) procedure is used (Devroey *et al.*, 1995; Gil-Shalom *et al.*, 1995; Silber *et al.*, 1995; Kahraman *et al.*, 1996; Mansour *et al.*, 1996; Silber *et al.*, 1996). These findings make the achievement of genetic offspring possible for a population of men who were previously advised to use donor spermatozoa. As a result, testicular sperm retrieval procedures in non-obstructive, azoospermic men are becoming increasingly popular.

With accumulated data and increased experience, it has been shown that mature spermatozoa can be found in only part of the testes of non-obstructive, azoospermic men. At present, there are no means of predicting the presence of such spermatogenesis (Hauser *et al.*, 1994; Tournaye *et al.*, 1995). The only diagnostic method available is the performance of surgical extraction of testicular tissue (Oates *et al.*, 1997).

In fertile men, sperm cells are produced throughout the testis. Roosen-Runge (1956) and Steinberger and Tjioe (1968) stated that a single, large sample is representative of the entire testis. When the testes of infertile men were examined, Levin (1979) found mixed histological patterns of germinal cell aplasia and focal spermatogenesis in 6%. A similar histology of side-by-side presence of different patterns (such as Sertoli-cell-only syndrome and normal spermatogenesis) was observed in non-obstructive azoospermic men (Giwercman and Skakkebaek, 1993; Devroey *et al.*, 1995; Gil-Shalom *et al.*, 1995). The incidence of such histology in cases of non-obstructive azoospermia has not, as yet, been determined.

Attitudes vary concerning the number of testicular samples that should be taken for sperm retrieval in men with non-obstructive azoospermia. Some perform multiple biopsies, while others perform a single testicular biopsy (Silber *et al.*, 1995; Verheyen *et al.*, 1995) based on the assumption that multi-focal distribution of spermatogenesis throughout the entire testis is present in non-obstructive azoospermia (Silber *et al.*, 1997).

Even if the multiple sampling approach is preferred, it evokes new dilemmas, i.e. the optimal number of biopsies that should be performed, and whether there is a site in the testis where mature spermatogenesis is more likely to be found. The aim of the present study was to try to answer these questions based on our experience.

Materials and methods

Patients

The study group included 29 consecutive, non-obstructive, azoospermic men who underwent multiple testicular biopsies. The age of patients ranged between 21 and 47 years. All patients suffered from primary infertility ranging between 1 and 13 years and had repeated documentation of semen analysis. Cases suspected of having congenital bilateral agenesis of vas deferens (CBAVD) on the basis of physical examination (vas deferens was not palpable), low ejaculate volume and low fructose concentration in seminal fluid, were not included in this study. In all cases, azoospermia was reconfirmed

prior to the operation using centrifugation of 600 *g* for 10 min and thorough examination of the pellet under the microscope (×600). The upper fluid was then recentrifuged (8000 *g*) for an additional 10 min and examined. All samples were Papanicolaou stained and re-examined under the microscope. In seven men, the serum follicle stimulating hormone (FSH) concentrations were normal (<9 mIU/ml) while in the remaining 22, serum FSH levels were elevated, ranging from 10 to 46 mIU/ml.

The procedure involved the extraction of multiple testicular tissue samples (multiple testicular sperm extraction). In most cases the extraction was performed in combination with an ovulation induction for in-vitro fertilization (IVF) in the spouse. Only in cases where the couple refused preoperative preparation of donor semen backup was the multiple TESE procedure performed on an elective basis, in order to avoid the situation of having retrieved oocytes with no spermatozoa available.

Operative technique

The procedures were performed under general anaesthesia. In all cases, the scrotum was opened via a median raphe incision and all layers were cut with meticulous homeostasis until full exposure of the testis was accomplished. The tunica albuginea was incised transversely for ~4 mm in three identical locations in each testis: (i) in the upper pole near the head of the epididymis and as close as possible to the rete region; (ii) in the midline of the testis; and (iii) in the proximal testicular pole opposite to the rete testis. The testis was then gently squeezed and the protruding tissues of ~50 mg were excised. Care was taken to ensure that all samples would be approximately the same size. The extracted testicular tissue was then immediately placed in HIF medium (Irvine Scientific, Irvine, CA, USA) and taken to the laboratory for sperm search and isolation. The tunica albuginea was closed using 6/0 nylon monofibre and the scrotum layers were closed separately.

All procedures were performed in our day surgery clinic and lasted between 30 and 45 min. After recovery from anaesthesia (about 2 h), the patients were released from the clinic, but were advised to rest at home for 2 days. All patients received prophylactic antibiotic treatment and were rechecked 1 week later.

Isolation and handling of spermatozoa

In the laboratory, all testicular tissue was cut into small pieces and then, with the use of an inverted microscope and a micromanipulator, sperm cells were identified either floating in the medium, or still attached to Sertoli cells. After release from their attachment, they were aspirated into an intracytoplasmic micropipe (Humagen Fertility Diagnostics, Inc., Charlottesville, VA, USA). In all cases smears were performed for all biopsy sites. The slides were examined after Papanicolaou staining. The isolated spermatozoa were injected into the oocytes (the ICSI procedure) that were retrieved from the spouse. Excess sperm cells and all spermatozoa found in the cases of elective multiple TESE were incubated for 3–4 h for motility acquisition. They were then cryopreserved in freezing medium (Irvine Scientific), according to a protocol published elsewhere (Yavetz *et al.*, 1991), in as many small aliquots as possible containing between a few and several hundreds of sperm cells for future ICSI procedures. In all cases, testicular tissue was also taken for histopathological evaluation to exclude a possible malignancy.

Evaluation of sperm quality

Each testicular sample was treated and recorded separately. A biopsy was considered positive for sperm presence even when a single mature sperm cell was found in the biopsy material. Since precise quantitation of sperm cells could not be done from testicular biopsy material, we adopted an estimation method based on a scale of four degrees of sperm numbers: 1 = a single spermatozoon was identified occasionally after reviewing at least several microscopic fields (×200); 2 = a single spermatozoon was identified in each microscopic field; 3 = a few sperm cells in each field; 4 = many sperm cells in each field. The presence of motile spermatozoa at the time of isolation was recorded for each sample.

Statistical analysis

Data are expressed as mean ± SD for continuous variables or proportions for discrete variables. The χ^2 test for independence was used to evaluate the difference in frequency of sperm detection between the right and left testes. The paired *t*-test was used for comparison of the total quantity of spermatozoa between the right and left testes. The χ^2 test for goodness of fit was used for evaluating the uniform distribution of locations positive for sperm presence. Analysis of variance (ANOVA) with repeated measures was used for comparison of the average sperm quantity in each individual location.

Results

A total of 165 testicular samples was extracted from 55 testes of 29 men. In three men, only a unilateral procedure was possible due to an undescending contralateral testis ($n = 2$) and orchidectomy post-torsion ($n = 1$). Malignancy was not detected in any of the samples evaluated histologically.

Presence of mature spermatozoa

Mature spermatozoa were found in 18 men (62.1%) and in 28 testes (50.9%). In five patients, sperm cells were found in only one testis, while no spermatozoa were found in the contralateral testis. No difference in the frequency of sperm detection was found between the right or left testis (15/27 = 55.6% for the right testis and 13/28 = 46.4% for the left; $P > 0.05$, χ^2 test of independence).

Comparison of the total quantity of spermatozoa obtained from the right or the left testis (the numbers represent the sum of sperm quantity in all the locations in each testis, based on the grading scale described above) also showed no significant difference. In the right and left testes, the mean ± SD was 4.6 ± 3.7 and 3.9 ± 3.8 respectively (paired sample *t*-test).

In 15 of the 28 testes with proven presence of sperm cells, spermatozoa were found in all three locations. In the other 13 testes, spermatozoa were found in only some of the locations sampled. In eight testes, spermatozoa were found in one location, and in five testes they were found in two locations only (Table I).

The distribution of the locations with sperm presence in the 13 testes with partial positive locations is shown in Table II. No difference in the frequency of any location was found using the χ^2 test for goodness of fit.

Estimation of sperm cell quantity in different testicular locations

The method used for estimation of sperm quantity in each individual sample has been described. The results of the average degree of sperm quantity in the three locations of testis (rete area, midline or proximal pole) are presented in

Table I. The testes with different number of locations positive for sperm presence

No. of locations positive for sperm cells	No. of testes	% of all testes	% of testes from which spermatozoa were obtained
0	27	49.1	–
1	8	14.5	28.6
2	5	9.1	17.9
3	15	27.3	53.6

Table II. Distribution of the locations with sperm presence in the 13 testes with partial positive locations

No. of locations	No. of testes	Rete area	Midline	Proximal pole opposite to the rete
1	8	3	1	4
2	5	3	3	4

Table III. Comparison of the average sperm quantity [degrees 1 (low) to 4 (high)] in each individual location (only testes with spermatozoa are included). Analysis of variance with repeated measures

	Testis location (n = 28)		
	Rete area	Midline	Opposite pole
Quantity of spermatozoa per location (average degrees of quantity ± SD)	1.65 ± 1.29	1.75 ± 1.43	1.75 ± 1.48

Table III. All the testes with spermatozoa present in at least one location are included. No significant difference was found in sperm quantity among the different locations.

The motility of sperm cells was evaluated and recorded immediately after isolation. In seven of 20 testes with two or three locations positive for sperm presence, motile spermatozoa were detected in some locations, while only non-motile spermatozoa were found in the others.

Operative and postoperative complications

No operative or major postoperative complications were recorded in any of the cases. In no instance was there a need for re-hospitalization, no haematoma developed, and no abscess was formed. In two cases, mild infections of the scrotum occurred with fever up to 38°C, moderate tenderness and swelling. All symptoms resolved after a few days of outpatient antibiotic treatment.

Fertility outcome

Operative procedures were performed in combination with ovulation induction and IVF treatment cycles in 21 couples. Of these, testicular spermatozoa were obtained from 18 patients and inseminations were performed using the ICSI technique. Donor spermatozoa were prepared preoperatively and were used whenever the testicular sampling was negative. A total number of 165 oocytes was inseminated with spermatozoa from the husband and 90 (54.5%) fertilized. Pregnancy was achieved in six cases (33.3% per embryo transfer). Extra embryos were frozen in eight cases. The remaining testicular sperm cells from the husband were frozen in vials containing from a few to several hundreds of sperm cells for future ICSI. Between one and 33 sperm vials from 16 couples were frozen.

Discussion

The introduction of ICSI has significantly changed our ability to assist couples with severe male factor infertility to achieve pregnancies. In fact, all definitions of male infertility, and even of male sterility, have changed. The finding of only a few sperm cells in testicular tissue of an azoospermic male can make him potentially fertile in the ICSI era. Therefore, it is of utmost importance to use the most reliable method available in order to detect and retrieve testicular spermatozoa, since a negative result implies sterility.

There is inconsistency in the literature concerning the pattern of testicular histology in non-obstructive azoospermic men. Silber *et al.* (1997) claimed that it is homogeneous, and suggested that a single testicular biopsy is sufficient to diagnose the presence of spermatozoa. Others (Devroey *et al.*, 1995; Gil-Shalom *et al.*, 1995) demonstrated the side-by-side different histological patterns, such as Sertoli-cell-only and normal spermatogenesis.

In the present study, in order to find mature sperm cells, we tried to evaluate the effectiveness of the performance of one, two or three open testicular biopsies in specific locations in the testis. The results suggest that the performance of multiple testicular biopsies increase the probability of finding of spermatozoa in cases of non-obstructive azoospermia. In almost half of the testes (46.5%) from which sperm cells were retrieved, spermatozoa were found in only some of the biopsies: in eight testes, they were recovered from one biopsy only, and in another five testes from two of three locations (Table I).

Based on our data (Table II), if, theoretically, one biopsy only had been taken from the centre of the testis, nine of the testes with proven sperm presence (after three biopsies) would have been missed. This accounts for 32.1% of the testes with proven sperm presence once three biopsies were performed. If one biopsy had been performed in the rete area, seven cases (25%) would have been missed, and likewise five cases (17.9%) if only the proximal pole (opposite the rete area) had been sampled in all testes. If two samples had been taken from two locations in each testis, between one and five of the testes in our study group would have been erroneously considered negative, which accounts for 3.6–17.9% of the testes from which sperm cells were obtained.

The performance of multiple biopsies may also contribute to the retrieval of motile testicular spermatozoa. In seven testes, motile spermatozoa were identified in one location, while only non-motile spermatozoa were identified in another,

which accounts for 35% of all testes with at least two positive locations per sperm presence.

Silber *et al.* (1997) showed that diagnostic biopsy is predictive for the finding of spermatozoa in subsequent TESE. Despite this, in five of 45 cases spermatogenesis was present in only one of the two procedures, implying that in 11% of cases the testes were not homogeneous after all.

Our results also showed that the finding of spermatozoa in one testis is not indicative of sperm presence in the contralateral testis, since in five cases spermatozoa were found in one testis only, and were absent from all three samples taken from the contralateral testis. The same is true for the number of positive locations in the contralateral testis.

No single location of the three tested showed any advantage compared to the others in terms of sperm presence (Table II) or sperm quantity (Table III). In some cases, spermatozoa were present in a proximal location and absent from the rete testis area. These results differ from those of Witt *et al.* (1997), who also questioned the benefit of additional testicular biopsies in non-obstructive azoospermic men. The results suggest that the entire testis is not homogeneous, but unlike our results, when spermatozoa were obtained from a testis, they were always present in the midline and medulla. We believe that in severe cases of testicular failure, when spermatozoa are being produced in low numbers and in focal regions in the periphery of the testis only, it is reasonable to find them in these areas only and they may be absent from the rete testis region. In a normal testis, sperm cells are produced in all the seminiferous tubules and then drain to the rete area prior to entering the epididymis. In severe cases of non-obstructive azoospermia, Silber *et al.* (1997) proved that spermatozoa are not found in the ejaculate, although they are being produced inside the testis. Since obstruction of the tubuli is unlikely (as implied from the term non-obstructive azoospermia), other factors are responsible for their 'disappearance' during their course to the rete testis. When the initial number of spermatozoa being produced is low, they do not reach more distal regions, or even the rete testis. This may explain our findings indicating the presence of sperm cells in proximal areas of the testis only.

There is no doubt that performing multiple biopsies lengthens operation time compared to performing a single biopsy and may exert an increased complication rate. Schlegel and Su (1997) elegantly described the consequences of multiple testicular sperm extraction procedures, which may even cause complete devascularization of a testis. However, the technique of an open testicular sampling, which, with careful observation, facilitates the avoidance of cutting through blood vessels of the tunica albuginea and the use of meticulous homeostasis, may contribute to a reduced complication rate. The use of such a technique, when performed in a population of mainly young and healthy patients, possibly contributed to the low complication rate in our series. The fact that some of the operations were performed on an elective basis probably also contributed to this low morbidity rate. No doubt, such complications mandate any available way to detect sperm cells in the ejaculate, such as extended sperm preparation (Ron-El *et al.*, 1997) in order to avoid an unnecessary operative procedure.

Another alternative to consider is the performance of multiple percutaneous needle aspirations, as suggested by Craft *et al.* (1995). One large biopsy was also suggested, but comparison of the chance of finding spermatozoa using this technique, to the multiple sampling technique, could not be made based on our results.

Based on the results in the present study, we suggest at least three open testicular biopsies of each testis in order to reach the most accurate diagnosis of sperm presence in cases of non-obstructive azoospermia. The theoretical performance of a single biopsy would have cost us up to 32% of missed diagnoses per testis, and up to 28% per patient. It is possible that the performance of more biopsies may discover more cases with sperm cells, but determination thereof is beyond the scope of the present study.

References

Craft, I., Tsirigotis, M., Bennett, V. *et al.* (1995) Percutaneous epididymal sperm aspiration and intracytoplasmic sperm injection in the management of infertility due to obstructive azoospermia. *Fertil. Steril.*, **5,** 1038–1042.

Devroey, P., Liu, J., Nagy, P. *et al.* (1995) Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum. Reprod.*, **6,** 1457–1460.

Gil-Shalom, M., Minguez, Y., Rubio, C. *et al.* (1995) Efficacy of intracytoplasmic sperm injection using testicular spermatozoa. *Hum. Reprod.*, **10,** 3166–3170.

Giwercman A. and Skakkebaek NE (1993) The role of testicular biopsy in the evaluation of male infertility. In Burger, H.G. and Oshima, H. (eds), *An Approach to Clinical Andrology.* Serono Symposia Review, Rome, pp. 67–75.

Hauser, R., Temple-Smith, P.D., Southwick, G.J. *et al.* (1994) Fertility in cases of hypergonadotropic azoospermia. *Fertil. Steril.*, **63,** 631–636.

Kahraman, S., Ozgur, S., Alatas, C. *et al.* (1996) High implantation and pregnancy rates with testicular sperm extraction and intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia. *Hum. Reprod.*, **11,** 673–676.

Levin, H.S. (1979) Testicular biopsy in the study of male infertility. *Hum. Pathol.*, **10,** 569–584.

Mansour, R.T., Aboulghar, M.A., Serour, G.I. *et al.* (1996) Intracytoplasmic sperm injection using microsurgically retrieved epididymal and testicular sperm. *Fertil. Steril.*, **3,** 566–572.

Oates, R., Mulhall, J., Burgess, C. *et al.* (1997) Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for intracytoplasmic sperm injection in 10 men with non-obstructive azoospermia. *Hum. Reprod.*, **12,** 734–739.

Ron-El, R., Strassburger, D., Friedler, S. *et al.* (1997) Extended sperm preparation: an alternative to testicular sperm extraction in non-obstructive azoospermia. *Hum. Reprod.*, **12,** 1222–1226.

Roosen-Runge, E.C. (1956) Quantitative investigations on human testicular biopsies. I. Normal testis. *Fertil. Steril.*, **7,** 251–259.

Schlegel, P.N. and Su, L. (1997) Physiological consequences of testicular sperm extraction. *Hum. Reprod.*, **12,** 1688–1692.

Silber, S.J., Van Steirteghem, A.C., Liu, J. *et al. (*1995) High fertilization and pregnancy rates after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum. Reprod.*, **10,** 148–152.

Silber, S., Van Steirteghem, A.C., Nagy, P.Z. *et al.* (1996) Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. *Fertil. Steril.*, **1,** 110–117.

Silber, S.J., Nagy, Z., Devroey, P. *et al.* (1997) Distribution of spermatogenesis in the testicles of azoospermic men: the presence or absence of spermatids in the testes of men with germinal failure. *Hum. Reprod*., **12,** 2422–2428.

Steinberger, E. and Tjioe, D.Y. (1968) A method for quantitative analysis of human seminiferous epithelium. *Fertil. Steril.*, **19,** 960–967.

Tournaye, H., Camus, M., Goossens, A. *et al.* (1995) Recent concepts in the management of infertility because of non-obstructive azoospermia. *Hum. Reprod.*, **10,** 115–119.

Tournaye, H., Liu, J., Nagy, P.Z. *et al.* (1996) Correlation between testicular histology and outcome after intracytoplasmic sperm injection using testicular spermatozoa. *Hum. Reprod.*, **1,** 127–132.

Verheyen, G., De Croo, I., Tournaye, H. *et al.* (1995) Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Hum. Reprod.*, **10,** 2956–2959.

Witt, M.A., Richard, J.R., Smith, S.E. *et al.* (1997) The benefit of additional biopsy sites when performing testicular sperm extraction in non-obstructive azoospermia. *53rd Annual Meeting of the American Society for Reproductive Medicine*, Cincinnati, Ohio, October 1997 (Abstract).

Yavetz, H., Yogev, L., Homonnai, Z.T. *et al.* (1991) Prerequisites for successful human sperm cryobanking: sperm quality and prefreezing holding time. *Fertil. Steril.*, **55,** 812–816.

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