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Enzymatic digestion of testicular tissue may rescue the intracytoplasmic sperm injection cycle in some patients with non-obstructive azoospermia

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Recovery of testicular spermatozoa from azoospermic patients with testicular failure, followed by intracytoplasmic sperm injection (ICSI) is a recent advance in the treatment of male infertility. In most cases, free spermatozoa are recovered from testicular tissue after mechanical mincing of multiple biopsies. Testicular sperm retrieval, however, remains unsuccessful in 30–50% of male patients suffering from Sertoli cell-only syndrome and maturation arrest. In this study, a strategy was developed in order to maximize the chance of sperm retrieval in difficult cases of testicular failure. The ultimate step was the use of enzymatic procedures (collagenase type IV) to dissociate the testicular tissue completely. Testicular tissue of 41 patients for whom no spermatozoa were found after mechanical mincing of the testicular tissue was investigated. In 14 out of the 41 cases (34%), enough spermatozoa for ICSI were found after fine mincing of multiple biopsies and several hours' search in the cell suspension treated with the erythrocyte-lysing buffer (ELB). In 27 out of the 41 patients, no spermatozoa were found even after the use of ELB. In seven out of these 27 failures (26%), spermatozoa for ICSI were retrieved after enzymatic dissociation of the residual minced tissue pieces, thus making ICSI possible despite failure to find spermatozoa with conventional mincing. From this study, we may conclude that enzymatic digestion of testicular tissue is easy to perform, is not time-consuming and constitutes a successful method in reducing the sperm recovery failures in patients with non-obstructive azoospermia.

Key words: enzymatic treatment/intracytoplasmic sperm injection/non-obstructive azoospermia/testicular biopsy

Introduction

Recovery of testicular spermatozoa followed by intracytoplasmic sperm injection (ICSI) has now become a common treatment for azoospermic patients (Schoysman *et al*., 1993; Devroey *et al*., 1995; Silber *et al*., 1995). In patients with obstructive azoospermia and normal spermatogenesis, spermatozoa can easily be retrieved for ICSI in sufficient numbers by various methods (Devroey *et al*., 1994; Craft *et al*., 1995). In cases of testicular biopsy retrieval, the spermatozoa for ICSI are obtained from testicular tissue mostly after mechanical treatment of the biopsies (Verheyen *et al*., 1995; Ron-El *et al*., 1997).

Although repeated testicular surgery may risk destroying parts of the testes (Friedler *et al*., 1997; Schlegel and Li-Ming Su, 1997**)**, a single biopsy taken at random may not reveal spermatozoa in non-obstructive cases, while using multiple samples taken for wet preparation has been successful in a high number of cases (Tournaye *et al*., 1995). However, even after multiple biopsies, testicular sperm retrieval remains difficult and unsuccessful in some patients suffering from testicular failure (Mulhall *et al*., 1996; Silber *et al*., 1996; Tournaye *et al*., 1996). Spermatozoa have not been recovered after mincing of multiple biopsies in 49% of patients with germ cell aplasia (Sertoli cell-only syndrome) and in 51% of patients with maturation arrest on the basis of histological findings (Tournaye *et al*., 1997).

Isolated human testicular germ cells have been obtained in the past after enzymatic digestion of testicular tissue using trypsin/DNase or collagenase type I (Blanchard *et al*., 1991; Rivarola *et al*., 1995; Salzbrunn *et al*., 1996). We have used collagenase type IV, a specific protease which degrades collagen type IV (Lance *et al*., 1981) which is present in the basement membrane and the extracellular matrix (Hadley *et al*., 1987), to retrieve spermatozoa from testicular tissue. Collagenase type IV provided a higher yield of intact cells than collagenase type IA and therefore its use had been suggested in order to reduce the number of sperm recovery failures (Crabbé *et al*., 1997).

In this study, the enzymatic treatment of testicular biopsies with collagenase type IV was applied in clinical ICSI cases where no spermatozoa had been found after mechanical mincing of the biopsies, in order to see whether spermatozoa might be recovered from the residual tissue pieces. In this way, the chance of sperm retrieval may probably be maximized and the proportion of failed cases may be reduced.

Materials and methods

Patients

In this study, 41 azoospermic patients were included whose mean age was 34 years (range 25–46 years). Testicular sperm extraction (TESE) was performed to recover spermatozoa for ICSI. In all cases, multiple biopsies were excised (range 2–9 pieces). Only patients where no spermatozoa were found after shredding of each of the biopsies with microscope slides, followed by at least a 1-h search

(depending on the number of biopsies) for spermatozoa under the inverted microscope, were allocated to the study. The mean age of the female partners was 31 years (range 21–43 years).

Stimulation protocol

Female partners underwent ovarian stimulation using a gonadotrophin-releasing hormone analogue suppression protocol with buserelin (Suprefact nasal spray; Hoechst, Frankfurt, Germany) combined with human menopausal gonadotrophins (Humegon; Organon, Oss, The Netherlands). Oocyte–cumulus complexes were recovered by vaginal ultrasound-guided retrieval, 36 h after administration of 10 000 IU of human chorionic gonadotrophin (HCG). After removal of the surrounding cumulus and corona cells (Van de Velde *et al*., 1997), the nuclear maturity of the oocytes was assessed under an inverted microscope.

Testicular sperm recovery

TESE was performed under general anaesthesia. After unilateral hemiscrototomy, a small testicular incision was made and a specimen was sampled using a pair of curved scissors. This specimen was then transferred into a Petri dish (Falcon Plastics, Becton-Dickinson, Aalst, Belgium), filled with 3 ml modified HEPES-buffered Earle's medium supplemented with 2.25% human serum albumin (HSA) (Belgian Red Cross, Brussels, Belgium). One small tissue specimen per testis was sent for histology. The testicular biopsy specimen was shredded using two glass slides in a Petri dish (3002; Becton Dickinson, Aalst, Belgium). Microscopic examination of the wet preparation was carried out at ×400 magnification under an inverted microscope with Hoffman modulation optics (Modulation Optics Inc., Greenvale, New York, USA). If spermatozoa were observed, no further testicular incision was made. If initial microscopic assessment did not show any sperm cells, more tissue specimens were retrieved from the same side, and also from the contralateral side, wherever necessary. If after at least 1 h of initial searching no spermatozoa were observed in multiple biopsies, the patient was allocated to this study.

Biopsy fractions were further minced with two fine forceps (Lawton, Tuttlingan, Switzerland) in the Petri dish until tissue pieces of ~1 mm^3 or free tubuli pieces of a few millimetres in length were obtained (Verheyen *et al*., 1995). Spermatozoa were recovered directly from the pellet after centrifuging the supernatant of the shredded tissue at 300 *g* for 5 min (Nagy *et al*., 1995). In all cases, an erythrocyte-lysing buffer (ELB) was used in order to increase the chance of visualizing any spermatozoa present (Verheyen *et al*., 1995; Nagy *et al*., 1997). In some cases, extensive searching for hours was needed to find spermatozoa or elongated spermatids, in others, sperm retrieval failed even after ELB. To avoid misunderstanding, it is important to mention that elongated spermatids and immature spermatozoa were both called 'spermatozoa' throughout the study. In a wet cell preparation, it is impossible to make a reliable distinction between both types on the basis of morphological characteristics. In the meantime, all the residual tissue pieces from the shredded biopsies were further dissociated by the use of enzymatic procedures (Crabbé *et al*., 1997) in order to check whether spermatozoa might still be found, even where mechanical mincing and ELB had been unsuccessful.

Enzymatic treatment

The residual tissue pieces of each of the separate biopsies were put into 1 ml of prewarmed HEPES-buffered Earle's medium supplemented with 5% HSA, 1.6 mM $CaCl_2$ (Merck, Darmstadt, Germany), 25 μg/ml DNase (Sigma Chemical Co., St Louis, MO, USA, Crude DN25), and 1000 IU/ml collagenase Type IV (Sigma C5138) was added. DNase was added to the incubation medium to prevent clotting of the resulting cell suspension due to the release of free DNA from dead cells (Crabbé *et al*., 1997). The tissue samples were digested in an incubator at 37°C for 1 h. To facilitate complete enzymatic digestion, the samples were shaken every 10–15 min during this incubation period. The digested tissue solution was gently centrifuged for 5 min at 50 *g* to remove any residual pieces and/or debris not dissolved by the enzymes. The remaining cell suspension (supernatant) containing the loose cells was then washed twice with Earle's medium and centrifuged for 5 min at 1000 *g*. The supernatant was removed and the pellet resuspended in 50–100 μl. A drop of 5 μl from each of the suspensions representing one biopsy was taken for examination on a glass slide with coverslip under an upright phase-contrast microscope. When spermatozoa were found in one of the suspensions, multiple small droplets under oil in a Petri dish were examined in order to retrieve spermatozoa for ICSI.

ICSI procedure

Metaphase-II oocytes were each injected with a single spermatozoon. These procedures have been described in detail elsewhere (Liu *et al*., 1994; Van Steirteghem *et al*., 1995). Fertilization was confirmed if after 16–18 h two distinct pronuclei and two polar bodies were observed under an inverted microscope. Cleavage was assessed 24 h later and embryos were classified according to their morphological appearance. Embryos without fragments were classified as type A; when ⩽20% fragmentation was present, the embryos were classified as type B. Type C embryos had >20 but ⩽50% fragments and type D embryos had >50% fragments. Cleaving embryos were replaced into the uterine cavity about 48 h after the ICSI procedure. A rise in serum HCG on two consecutive occasions from 11 days on after transfer indicated pregnancy. Clinical pregnancy was defined by the presence of a gestational sac at ultrasonography after approximately 7 weeks of pregnancy.

Results

Enzymatic digestion of testicular tissue remnants was carried out in 41 patients undergoing TESE. The patients were allocated to the study because no spermatozoa had been found after at least a 1-h search of the shredded biopsy suspensions. The results of histopathology and the corresponding cytology as observed at the moment of searching for spermatozoa in the wet preparation after enzymatic treatment are summarized in Table I.

In 20 patients, Sertoli cell-only syndrome (SCOS) was diagnosed by histopathology. At the wet preparation on the day of ICSI, nine of them (45%) showed SCOS in all biopsies, three cases (15%) showed maturation arrest (MA) in some of the examined biopsies and in eight cases (40%) spermatozoa were found after cytological examination of some of the biopsies. The histological analysis diagnosed maturation arrest (MA) in 11 cases, whereas from the wet preparation on the day of ICSI, complete MA was observed in only three cases (27%), while in seven cases (64%) spermatozoa were observed in some biopsies. One case (9%) diagnosed as MA only showed Sertoli cells in the wet preparation. In four cases, spermatogenesis was found in some tubuli on histology and this was confirmed cytologically. In six cases, no histological diagnosis was available.

Spermatozoa were found for ICSI after fine mechanical mincing of multiple biopsies and several hours of searching in the cell suspension treated with ELB in 14/41 (34%) cases.

Table I. Summary of histopathology and cytology results from wet preparation after enzymatic treatment

Patient no.	Histological diagnosis of one diagnostic biopsy	No. biopsies taken	Observations in the wet preparation at the day of ICSI		
			Spermatozoa present after:		
			Mincing + ELB	Mincing + enzymes	Conclusion at cytological findings
1	SCOS	5	+	+	SCOS/spermatogenesis
2	SCOS	6	+	+	SCOS/spermatogenesis
3	SCOS	8	+	−	SCOS/MA
4	Not available	3	+	+	Spermatogenesis
5	SCOS + 1% MA	7	+	+	SCOS/spermatogenesis
6	MA	5	−	+	MA/spermatogenesis
7	MA	5	+	+	Spermatogenesis
8	SCOS + 5% spermatogenesis	3	+	+	Spermatogenesis
9	SCOS	8	−	+	Spermatogenesis
10	SCOS	6	−	+	MA/SCOS/spermatogenesis
11	SCOS + 10% spermatogenesis	5	+	+	Spermatogenesis
12	SCOS + 10% MA	3	+	+	Spermatogenesis
13	SCOS	6	+	+	Spermatogenesis
14	Not available	6	+	+	MA/spermatogenesis
15	Not available	7	−	−	SCOS
16	MA	5	−	−	SCOS
17	SCOS	5	−	−	SCOS
18	SCOS	5	−	−	SCOS
19	SCOS	3	+	−	SCOS/spermatogenesis
20	SCOS	4	−	−	SCOS
21	Not available	5	−	−	SCOS
22	SCOS	8	−	−	SCOS
23	MA	6	−	−	MA
24	SCOS	6	−	−	SCOS
25	MA	4	−	−	MA
26	SCOS	4	−	−	SCOS
27	SCOS	5	−	−	SCOS
28	MA	4	−	−	MA
29	SCOS	4	−	−	MA
30	Not available	3	−	−	MA
31	Not available	5	−	−	SCOS
32	SCOS	9	−	+	SCOS/spermatogenesis
33	MA	6	−	+	SCOS/spermatogenesis
34	SCOS	7	+	−	SCOS/spermatogenesis
35	SCOS	7	−	−	SCOS/MA
36	SCOS	7	−	−	SCOS
37	SCOS	6	−	−	SCOS
38	50% SCOS + 50% MA	3	−	+	SCOS/MA/spermatogenesis
39	MA	2	+	+	MA/spermatogenesis
40	SCOS + 1% spermatogenesis	6	−	+	MA/spermatogenesis
41	SCOS + 1% spermatogenesis	6	−	+	MA/spermatogenesis

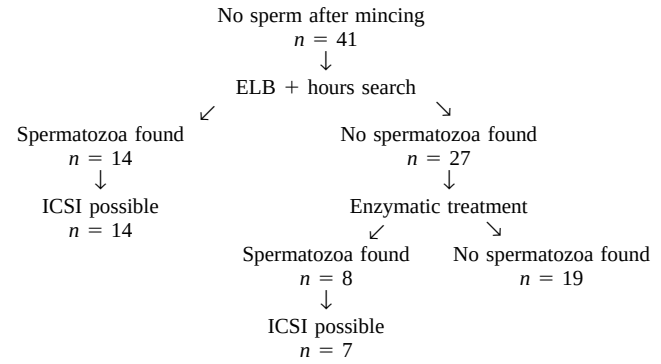
Spermatogenesis = all biopsies contained a small number of all types of germ cells.
ICSI = intracytoplasmic sperm injection; ELB = erythrocyte-lysing buffer; MA = maturation arrest; SCOS = Sertoli cell-only syndrome.
SCOS/spermatogenesis = some biopsies showed SCOS, others contained a small number of all types of germ cells.
Cytological findings = identification of different cells without staining under phase-contrast microscopy at the day of intracytoplasmic sperm injection.

In one case, however, too few spermatozoa to inject all available oocytes were found, so that spermatozoa retrieved after enzymatic dissociation of the remaining minced tissue pieces were used additionally for ICSI. In 11 of the 13 cases where enough spermatozoa were retrieved after mincing and the use of ELB, spermatozoa, even motile ones, were sometimes more easily found after enzymatic treatment of the remaining tissue pieces, but the policy was to use for ICSI the spermatozoa retrieved with enzymes only when no other spermatozoa were available (Table I).

In 27 of the 41 cases, no spermatozoa were found after hours of searching in the suspension treated by mincing and ELB. The time spent for searching depended on the number and size of the investigated biopsies (range: 1–4 h). In seven out of these 27 failures (26%), spermatozoa for ICSI, however, were retrieved after enzymatic dissociation of the residual minced tissue pieces, thus making ICSI possible despite the failure to find spermatozoa with conventional mincing. Unfortunately, in 19/41 (46%) cases, no spermatozoa were found even after enzymatic treatment of the remaining testicular tissue. In 1/41 cases, spermatozoa were found after enzymatic treatment in the 5 μl suspension examined under the upright phase-contrast microscope but no more spermatozoa were seen in the remaining cell suspension after searching under the inverted microscope (Table II).

A total of 157 metaphase-II oocytes were injected with testicular spermatozoa in 21 patients (Table III). The majority of the oocytes (109 oocytes from 14 patients) were injected with spermatozoa retrieved after mechanical mincing procedures and ELB. A total of 48 oocytes derived from seven patients

Table II. Flow sheet and success rate of sperm retrieval procedures



ELB = erythrocyte-lysing buffer; ICSI = intracytoplasmic sperm injection.

Table III. Fertilization rate, embryonic development and embryo transfer after intracytoplasmic sperm injection with testicular spermatozoa retrieved after mincing and the use of erythrocyte-lysing buffer (ELB) or retrieved after enzymatic dissociation of the residual testicular tissue

		Mincing + ELB	Enzymatic treatment
Patients and treatment cycles		14	7
Injected oocytes		109	48
Injected motile spermatozoa (%)		82 (75)	9 (19)
2PN status per injected oocyte (%)		57 (52)	22 (46)
	Range	0–100%	0–100%
1 PN status		1	2
3 PN status		2	0
Embryo quality (%) of 2 PN oocytes	Type A	3 (5)	0
	Type B	32 (56)	12 (55)
	Type C	7 (12)	6 (27)
	Type D	15 (27)	4 (18)
Number of transfers (%)		11 (85)	5 (71)
Embryos transferred		22	11
Developmental speed (%)	2 cell	3 (14)	0 (0)
	3–4 cell	9 (41)	5 (45)
	5–8 cell	10 (45)	6 (56)
Mean no. embryos transferred		2	2.2
Rise in HCG		2	2
Ongoing pregnancies		2	1

HCG = human chorionic gonadotrophin; PN = pronuclei.

were injected with spermatozoa retrieved only after further enzymatic treatment of the residual tissue fractions. Normal fertilization per survived metaphase-II oocyte was obtained in 52% of oocytes injected with spermatozoa from the conventional mincing group and in 46% of oocytes injected with spermatozoa retrieved after further enzymatic treatment of the remaining tissue. The fertilization rate within the two groups ranged from 0% to 100%.

Of the 14 patients whose oocytes were injected with mechanically retrieved spermatozoa, 11 had an embryo transfer, two of which resulted in a rise in HCG. Of the seven patients who had ICSI with testicular spermatozoa obtained only after the supplementary enzymatic treatment of the testicular biopsies, five had an embryo transfer. Two of these resulted in a rise in HCG, one of which was a confirmed ongoing pregnancy; the other was a biochemical pregnancy. Spermatozoa for ICSI would not have been found in these patients without the use of the enzymatic procedure on the remaining tissue pieces. Furthermore, it appears that enzymatic preparation would have made the procedure much easier for all the 41 patients whose biopsies revealed no spermatozoa during an initial search.

Discussion

TESE offers a valid treatment option for many azoospermic patients with severely deficient spermatogenesis. Enough spermatozoa may be recovered from a wet preparation of a testicular biopsy in many patients with non-obstructive azoospermia. In these patients, ICSI with spermatozoa retrieved from testicular biopsies resulted in good fertilization rates and pregnancies (Devroey *et al*., 1995; Silber *et al*., 1995, 1996; Kahraman *et al*., 1996; Schlegel *et al*., 1997). An excisional testicular biopsy should be offered to all azoospermic patients, irrespective of concentrations of follicle-stimulating hormone, testicular size or medical history (Tournaye *et al*., 1995), although repeated testicular surgery may cause some permanent testicular damage (Friedler *et al*., 1997; Schlegel and Li-Ming Su, 1997). No strong predictors for successful testicular sperm recovery are available except for testicular histopathology, although the accuracy of this parameter is limited (Kahraman *et al*., 1996; Tournaye *et al*., 1997). An unsuccessful sperm recovery procedure has important emotional and financial implications. Every possible effort to find spermatozoa should therefore be offered to the couple.

Mechanical methods to retrieve testicular spermatozoa are applied worldwide. However, all ICSI centres are faced with a certain proportion of sperm recovery failures, depending on the criteria for ICSI treatment allocation; while some centres treat couples only when spermatozoa are found in a diagnostic testicular biopsy, others more easily allocate couples to ICSI treatment on their own request, after careful counselling of the patients about the bad prognosis. Enzymatic preparation of testicular tissue with collagenase type IA to obtain spermatozoa for ICSI from human frozen-thawed testicular tissue has already been proposed by Salzbrunn *et al*. (1996). Pregnancy after ICSI using spermatozoa extracted by this method has been reported by Fischer *et al*. (1996) and Simons *et al*. (1997). Collagenase type IV has been found to be more efficient than collagenase type IA for testicular sperm recovery (Crabbé *et al*., 1997). Enzymatic preparations using collagenase type IV provide complete dissolution of the cells from their tissue, with a higher yield and a higher percentage viability than other enzymatic preparations. One of the reasons why collagenase type IV appeared to be the best protease to dissociate the testicular tissue might be that type IV collagenase is one of the products secreted by the Sertoli cells. Its secretion may play a role in the translocation of germ cells and spermiation, i.e. the release of mature spermatozoa into the lumen of the tubules (Matsumoto, 1996).

It is difficult to find spermatozoa in a field of debris and remaining tissue pieces when shredding and mincing of the testicular tissue is performed and, therefore, usually only the supernatant is searched for spermatozoa after short sedimentation of the tissue fractions. In some cases (14/41 in this study), sperm retrieval could be facilitated by simple

use of ELB (Nagy *et al*., 1997; Verheyen *et al*., 1997). However, when there are only tiny numbers of spermatozoa, these can easily be attached to Sertoli cells or captured in remaining tissue pieces. The results of this study indicate that sperm recovery failures can be reduced with enzymatic digestion of the remaining tissue pieces. Even motile spermatozoa were additionally observed in some of the enzymatically digested residual tissue pieces in the cases where initially after mechanical treatment of these biopsies only immotile spermatozoa were retrieved. In 30% (8/27) of patients where no spermatozoa were found after an extensive search of the supernatant of the minced suspension, spermatozoa were found after enzymatic treatment of the residual tissue pieces. This may be due to the fact that this method will release all spermatozoa, even attached ones, from the tissue. Furthermore, owing to the simplified field of view obtained by this method, ideal conditions are provided for the search for spermatozoa, for the selection of spermatozoa in terms of motility and morphology, and for the aspiration of spermatozoa and the ensuing microinjection.

A diagnostic testicular biopsy is still recommended for all patients with azoospermia from whatever cause in order to plan appropriate treatment on the day of ICSI. Unfortunately, not many studies have compared the diagnostic histopathology and the cytological findings on the day of ICSI. In our study, we were able to rescue the ICSI cycle where no spermatozoa had been retrieved after conventional mincing: in 40% (8/20) of the cases where SCOS was diagnosed and in 64% (7/11) of the cases where MA was diagnosed by histopathology from a diagnostic biopsy, previously taken or taken at the day of ICSI. Spermatozoa were found as a result of extensive searching in multiple biopsies and the use of supplementary methods such as ELB and enzymatic digestion of the residual tissue pieces.

The fertilization rate with injection of spermatozoa retrieved after supplementary enzymatic digestion of the remaining tissue pieces is comparable to that after ICSI with the spermatozoa retrieved from the pellet of the supernatant of the minced tissue. There is little evidence that fertilization would be impaired due to the enzymatic treatment of the spermatozoa, because enzymatic treatment appears to have no effect on the long-term viability of ejaculated spermatozoa (Crabbé *et al*., 1997). Salzbrunn *et al*. (1996) even suggested that, with better conservation of the cells after enzymatic dissociation of the tissue, the fertilization rate might be improved. Fischer *et al*. (1996) reported that the fertilization rate of oocytes injected with spermatozoa obtained after enzyme treatment of frozen-thawed testicular tissue of a patient suffering from obstructive azoospermia compared well with the fertilization rate of oocytes injected with fresh testicular spermatozoa. Moreover, in a first cycle on sibling oocytes where half of the oocytes were injected with motile spermatozoa obtained after mechanical mincing and the other half with motile spermatozoa obtained after further enzymatic treatment of the residual biopsy pieces, 1/6 oocytes and 6/6 oocytes were fertilized, respectively (data not included in this study). Larger series on sibling oocytes are required to clarify this issue.

In conclusion, enzymatic preparation of testicular biopsy may, on the one hand, be considered successful in reducing sperm recovery failures in patients with non-obstructive azoospermia. On the other hand, spermatozoa seem more easily recovered from testicular tissue using this method, which may increase the chance of selecting the best quality spermatozoa.

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