

A new concept for the extraction of testicular spermatozoa as a tool for assisted fertilization (ICSI)

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A new method is described for the enzymatic preparation of testicular tissue to obtain vital spermatozoa for ICSI. The tissue obtained from both testes by biopsy is studied by means of the semi-thin section method as well as being cryo-preserved. If spermatid formation is assured by semi-thin section histology, vital spermatozoa from the cryo-preserved portion of testicular tissue are enzymatically prepared. In this way it is possible to optimize the reproductive medical treatment for those couples in whom an extracorporal fertilization of oocytes by means of testicular spermatozoa is being considered.

Key words: assisted fertilization/human/ICSI/spermatozoa/testis

Introduction

Through the use of direct intracytoplasmic injection of a spermatozoon into the oocyte (ICSI), fertilization and pregnancy can be achieved even for severe male infertility cases with very low sperm counts in the ejaculate, or with marked structural malformation of the spermatozoa. Fertilization rates lie between 64.2 and 66% (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993; Redgment *et al.*, 1994). Where the ejaculate does not contain enough vital spermatozoa or no spermatozoa at all, ICSI can also be performed using spermatozoa from the epididymis (Liu *et al.*, 1994) or even directly extracted from testicular tissue (TESE) (Devroey *et al.*, 1994; Silber *et al.*, 1995a,b). In the latter case, spermatozoa are obtained following mechanical shredding of the testicular tissue. Fertilization rates are given as about 45% (Devroey *et al.*, 1994) or 46 and 49% (Silber *et al.*, 1995a,b).

When using fresh testicular tissue, it is often difficult to coordinate procedures with the gynaecological stimulation protocol. Without the prior histological evaluation of the spermatogenetic activity of the testicular tissue, there is a high risk that the women may have been treated in vain.

Here we present a new concept with which the preparation of male haploid gametes from testicular tissue can be optimized for use in ICSI.

Materials and methods

As a model for the development of the methodology suitable for isolating spermatozoa, testicular tissues were initially used which were derived by orchiectomy from five patients (aged 68–76 years) being treated for carcinoma of the prostate. The orchiectomies were performed as part of the supplementary therapeutic programme prior to hormone therapy. Biopsies were imitated on testes which had been obtained by operation maximally 2 h before. Following a small incision in the tunica albuginea, a small piece of the tissue subsequently bulging from the incision was cleanly excised using a microsurgical scissor.

Four similar fragments, measuring 3×3×3 mm, of testicular tissue were removed from each testis and treated as follows.

One tissue sample was immersed in 5.5% glutaraldehyde for 2 h and postfixed in OsO₄ for 2 h, followed by dehydration in ascending alcohols and propyleneoxide, and embedded in Epon 812 (Holstein and Wulfhekel, 1971). Semi-thin sections were cut on a Reichert ultramicrotome with diamond knives and stained with Toluidine Blue/Pyronine (Ito and Winchester, 1963). This material was used for the histological evaluation of spermatogenesis (Figure 1).

The diameter of the seminiferous tubules, the height of the germinal epithelium, the thickness of the lamina propria, the occurrence of type A pale and type A dark spermatogonia, of spermatocytes I and of spermatid stages 1–8 (Holstein and Roosen-Runge, 1981) were assessed. The results of this quantitative analysis are given in Table I.

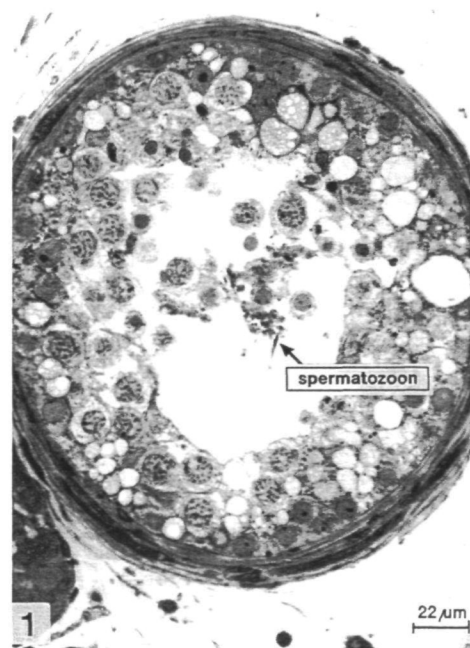


Figure 1. Semi-thin section of a seminiferous tubule (75 year old man with carcinoma of the prostate). The lamina propria appears of normal thickness, the germinal epithelium is vacuolated and the number of germ cells is reduced. In this tubule only one spermatozoon is visible.

Table 1. Histological evaluation of spermatogenesis

Sample no.	Tubular diameter (μm)	Height of germinal epithelium (μm)	Thickness of lamina propria (μm)	Spermatogonia A pale	Spermatogonia A dark	Spermatocytes I	Spermatatids 1–5	Spermatids 6–8	Additional features
0	220	90	6	++	++	++	++	++	regular spermatogenesis
1	250	70	7	++	++	++	++	++	focal tubular atrophy
2	240	90	7	++	+	++	+	+	–
3	245	80	7	++	++	++	++	++	diverticula, multifocal occurrence of atrophic tubules
4	280	65	7	++	++	+	+	+	–
5	210	80	10	++	++	+	+	+	–

++ present, normal quantity.

+ present, reduced quantity.

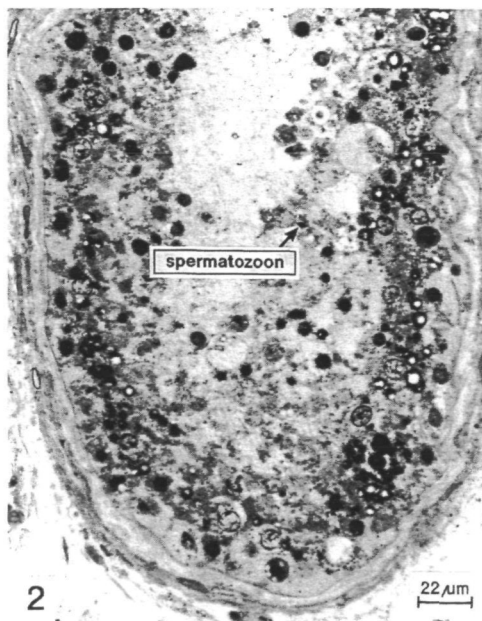


Figure 2. Semi-thin section of a seminiferous tubule (same material as in Figure 1) after cryo-conservation and thawing. The organization of the germinal epithelium and the structure of germ cells are disrupted. One sperm head, however, is still identifiable.

The first column in this table shows data from a patient with normal spermatogenesis.

Three tissue samples were individually placed in 0.5 ml Sperm-Freeze (Medicult, Hamburg, Germany) and frozen immediately using a Nicoolbag 10 (Air Liquide, Wiesbaden, Germany) apparatus. After 60 min in nitrogen gas (cooling to -60°C in the first 5 min and exponentially to -120°C in the following 55 min), samples were stored in liquid nitrogen.

If the results of the histological examination of the first tissue fragment showed that the spermatogenic activity was sufficient to give rise to mature spermatids, then one of the frozen samples was used for the isolation of germ cells.

For germ cell isolation, the frozen sample lying in a small glass container was first thawed in warm water at 37°C and then immediately transferred to 1 ml of Sperm Prep Medium (Medicult) prewarmed at 37°C . The histological quality of the sample after this procedure is pictured in Figure 2. This sample was then incubated for 2 h in a

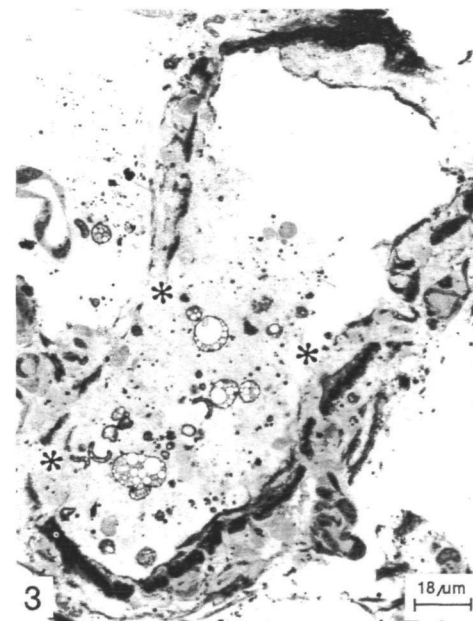


Figure 3. Semi-thin section of a seminiferous tubule (same material as in Figure 1) following cryo-conservation and incubation with collagenase. Note the disruption of the lamina propria (asterisk). Within the tubule the cellular organization has broken down. Only a few cellular remnants and lipid droplets are recognizable. Spermatozoa and immature germ cells are washed away.

gas-controlled incubator at 37°C and supplemented with 0.8 mg collagenase, type AI (Sigma, Heidelberg, Germany) and 0.2 μg trypsin inhibitor (Sigma), which had been dissolved in 1 ml of prewarmed Sperm Prep Medium and sterile filtered. The sample was digested for further 2 h in the gas-controlled incubator. Finally, the sample was removed from the incubation solution and prepared as above for semi-thin sectioning (Figure 3). The digest solution was then centrifuged for 10 min at 800 g and 37°C . After removal of the supernatant, the resulting pellet was analysed microscopically. In order to evaluate the method for isolating the germ cells, native pellet preparations were examined under the light microscope at a $\times 400$ magnification. The number of mature germ cells were counted in 10 fields of view.

At the same time the number of vital spermatozoa was also

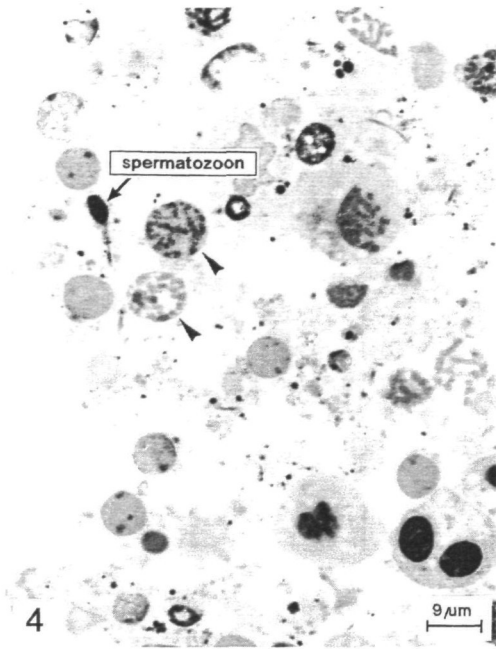


Figure 4. Semi-thin section of pelleted cell material (same material as in Figure 1) following cryo-conservation, incubation with collagenase and centrifugation. One mature spermatid (arrow) and several germ cells of earlier developmental stages (arrowheads) are visible.

Table II. Evaluation of isolated mature germ cells

Sample no.	Number of mature germ cells per 10 visual fields	Number of vital spermatozoa per 10 visual fields
0	295	112
1	261	64
2	128	32
3	58	15
4	30	9
5	47	17

evaluated. Vital germ cells were considered to be those showing a degree of independent movement or progressive movements. After repeating the described procedure several times for every specimen, the obtained motile spermatozoa were discarded. In addition, in some cases the vitality of spermatozoa was checked using the Eosin test. A portion of this pellet was prepared for the semi-thin sectioning technique (Figure 4). The results are summarized in Table II.

In preliminary examinations the described method for isolation of spermatozoa was also applied to fresh testicular tissue. No differences in the results were observed.

Discussion

The concept described here for the isolation of spermatozoa from testicular tissue biopsies (TESE) offers several advantages. The method of obtaining the biopsies guarantees a minimal traumatization of the tissue. The histological examination of the tissue by means of the semi-thin

sectioning technique allows a very precise evaluation of spermatogenesis down to highly detailed cytological analysis. If necessary, even an electron microscopical evaluation is possible. For instance, in cases of ductal obstruction, the histological examination may show normal spermatogenesis. In cases of focal atrophy, one can find tubular shadows and seminiferous tubule with normal spermatogenesis both in the same section. If the spermatogenesis is disturbed, perhaps caused by a genetic defect, only a few, defect spermatozoa are found. In cases of germinal cell aplasia ('classical' Sertoli-only syndrome according to Del Castillo (Del Castillo *et al.*, 1947)) or spermatogenetic arrest, no spermatozoa are found in the section. The testicular biopsy comprises the only valid option to clarify the type of failure, and thus to avoid unnecessary stimulation treatment in women. With percutaneous approaches (Tsirigotis and Craft, 1995), there is no possibility for histological examination. If the preliminary histological analysis suggests that it is doubtful whether sufficient spermatozoa can be obtained for ICSI, then one of the extra cryo-conserved biopsy fragments can be used just to test the TESE technique. Freezing of isolated spermatozoa from testicular tissue is difficult because of the low number (Silber *et al.*, 1995a). By using cryo-conservation of the tissue samples, the spermatozoa are isolated after thawing, and one can avoid the complications of temporal coordination of TESE and the ICSI treatment. Because of the ease of transport of the frozen samples, there is equally a considerable degree of spatial independence between the two techniques. The simultaneous cryo-conservation of several biopsy samples also permits repeated treatment cycles without recourse to a new biopsy operation, as already discussed by Tucker *et al.* (1995). As has been shown for some time in the context of conventional cell culture (Chemes *et al.*, 1992), the enzymatic preparation using collagenase provides a gentle dissolution of the cells from their tissue. This has been confirmed here also for testicular tissue, with high yields of mature, vital spermatids or spermatozoa. When shredding the testicular tissue to isolate spermatozoa (Silber *et al.*, 1995a), it is difficult to find vital spermatozoa in a field of debris. Because of the simplified field of view obtained with this new method of germ cell isolation, ideal conditions are provided also for the aspiration of spermatozoa and the ensuing microinjection.

It seems, moreover, possible that with the improved conservation of the cells, also the fertilization rates might be improved to approach those obtained using ICSI with ejaculated spermatozoa.

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