A laboratory modification to testicular sperm preparation technique improves spermatogenic cell yield

Sinan Ozkavukcu¹, Ebru Ibis¹, Sule Kizil², Suheyla Isbacar¹, Kaan Aydos³

Testicular sperm extraction is a common procedure used to find spermatogenic cells in men with nonobstructive azoospermia. The laboratory processing of biopsied testicular tissues needs to be performed meticulously to acquire a high yield of cells. In this study, the effectiveness of mincing the tissues after testicular biopsy was assessed using histological evaluation, as was the possible adverse effect of residual tissue on the migration of spermatogenic cells during density gradient centrifugation. Our results indicate that testicular residual tissue, when laid on the density gradient medium along with the sperm wash, hinders the spermatogenic cells' forming a pellet during centrifugation, and therefore impairs the intracytoplasmic sperm injection procedure. Whereas the mean number of recovered cells from the sperm wash medium (SWM) with residual tissue is 39.435 ± 24.849, it was notably higher (60.189 ± 28.214 cells) in the SWM without minced tissues. The remaining tissue contained no functional seminiferous tubules or spermatogenic cells in histological sections. In conclusion, the remaining residual tissue after mincing biopsied testicular tissue does not add any functional or cellular contribution to spermatogenic cell retrieval; in fact, it may block the cellular elements in the accompanying cell suspension from migrating through the gradient layers to form a pellet during centrifugation and cause loss of spermatogenic cells.

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INTRODUCTION

Azoospermia is defined as the diagnosis of a man with no spermatozoa in the sediment of a centrifuged semen sample.¹ With the introduction of intracytoplasmic sperm injection (ICSI), testicular sperm extraction (TESE) has become a revolutionary tool for this group of patients where fertilization, pregnancy and live birth have become possible. Nonobstructive azoospermia (NOA) affects 10% of infertile men and is diagnosed in 60% of azoospermic cases.² Testicular failure is evident in the etiology of genetic disorders (sexual chromosomal abnormalities, translocations and microdeletions of the Y chromosome), cryptorchidism, testicular torsion, exposure to radiation and toxins.² It has been shown that intact spermatozoa can be found in only limited parts of the testes of NOA patients. The focal exploration of active spermatogenesis can be challenging during TESE, as there is no established effective method. The morphological appearance of the seminiferous tubules may be informative, as dilated tubules are indirect signs of spermatogenic activity. However, an active-looking tubule segment may contain a series of spermatogenic stages, but no intact spermatozoa, if spermiogenesis is blocked. It has been shown that full spermatogenesis and a focus of Sertoli-cell-only can appear to be side-by-side in the same tubule.³ Therefore, multiple focal testicular tissue retrieval has been recommended to ensure the presence of sperm in testicular biopsies.³-⁵ TESE is an essential and effective method for the retrieval of immature sperm cells in cases of obstructive azoospermia and NOA. Conventional TESE allows the removal of many biopsies following a small incision, and is reported to have an average sperm retrieval rate of 50% in NOA.⁶ In microdissection TESE (microTESE), which was introduced by Schlegel in 1999,⁷ a large incision is made in the tunica albuginea and the parenchyma is examined in a stereo dissection microscope in order to visualize larger, white tubuli, that have a higher chance of containing active spermatogenesis.⁸

The biopsied sample is prepared for ICSI by mincing the tissue in order to release the seminiferous tubular contents into the medium. This procedure is crucial, as it is the only way to retrieve spermatogenic cells during ICSI. The practitioner has to mince the testicular tissue patiently, being sure to shred all tubules which are visible macroscopically or with a stereomicroscope. This done, luminal cells of the seminiferous tubules as well as interstitial cells and connective tissue elements are freed. Mincing may reduce the large size of extracted testicular tissue, nevertheless, small, solid tissue fragments, possibly lacking seminiferous tubules; remain in suspension after the procedure. The cells in the wash medium and the remaining residual fragments are usually centrifuged through a density gradient to obtain cell suspension of light, small particles, as well as the cells of interest, at the bottom of the centrifuge tube.

The aim of this study, prompted by our observations, was to investigate whether residual fragments of minced TESE material

¹Department of Obstetrics and Gynecology, Center for Assisted Reproduction, Ankara University School of Medicine, Ankara, 06100, Turkey; ²Department of Histology and Embryology, Graduate School of Health Sciences, Ankara University, Ankara, 06110, Turkey; ³Department of Urology, Ankara University School of Medicine, Ankara, 06230, Turkey.
Correspondence: Dr. S Ozkavukcu (sinozk@gmail.com)
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overlaid on the gradient media, make it difficult for spermatogenic cells to enter the gradient, thus reducing the number of spermatogenic cells collected. The histopathological nature of these tissue minces was also to be evaluated to determine if they contained any cells or tubules, and if they need be placed on the gradient column to increase cell yield.

**MATERIALS AND METHODS**

**Patient selection and surgery**

Testicular sperm extraction materials from 20 men who presented with clinical and laboratory data indicating NOA and referred to Ankara University School of Medicine, Center for Assisted Reproduction for a testicular biopsy were included in this study with their informed consent and approval from Local Ethical Committee, between August 2012 and March 2013.

Azoospermia was discovered through analysis in at least two seminal samples that were handled as described by the World Health Organization manual. Subjects underwent a full clinical evaluation including medical and reproductive histories, and physical examination of vasa deferentia, epididymides, and testes. Testicular size and texture were assessed by scrotal ultrasonography. Endocrine screening included radio-immunoassays of serum follicle stimulating hormone (FSH) and testosterone (Vitros Immunodiagnostic Products; Ortho-Clinical Diagnostics, Raritan, NJ, USA). The absence of ductal obstruction was confirmed at the time of the diagnostic biopsy or at the time of TESE. To confirm the obstruction, a scrotal exploration was done. The vas deferens was transected, and saline injected into the vas deferens to confirm the patency of the vas. The epididymis was then examined under an operating microscope to look for dilated tubules. Any epididymal fluid obtained from the tubule was examined under a light microscope for the presence of sperm. In the absence of sperm in the epididymal fluid even up to the caput, a biopsy of the testis was taken in Bouin’s fluid for histopathological examination. The diagnosis of NOA was based on the evidence of azoospermia together with the presence of small testes or elevated serum FSH and the absence of obstruction. All of the TESE procedures were performed by the same surgeon using an operating microscope. A 0.5–3.0 cm incision was made on an avascular region of the tunica albuginea, selected with the aid of an operating microscope (×8). The testicular parenchyma was directly examined at higher magnification (×40) to identify typically dilated and more opaque-white seminiferous tubules. Small volumes of testicular samples, including these tubules, were extracted by traction and excision. If no morphologically normal tubules were identified, the incision was extended to expose more areas and any tubules that differed from the remainder of the tissue that was harvested. In cases where a spermatozoon could not be extracted from one testicle, other one was searched using similar methods. Tissues were minced and investigated immediately after retrieval in an inverted microscope and the urologist was informed if the material contained spermatozoa. The operation went ahead if mature sperm cells were seen in the suspension, otherwise additional tissue pieces were harvested to increase the chance of finding spermatozoa. At the end of the procedure, the tunica albuginea was closed with a 5–0 vicryl suture (Covidien, New Haven, CT, USA).

**Testicular sperm extraction preparation and cell counting**

Retrieved tissue pieces were washed and dissected in a sperm wash medium (SWM) (G-IVF, Vitrolife, Gothenburg, Sweden) by using two 26 gauge fine-needles, attached to disposable 1.0 ml tuberculin syringes, bent parallel to the base of the culture dish to strip the cells from the seminiferous tubules. One of the syringes was used to immobilize the biopsied tissue, and the other was moved while it was pressed on the tissue to squeeze out the tubules. In cases where tubule elasticity was high, the short edges of two glass microscope slides were used to stretch and tear the seminiferous tubules. The procedure was applied for approximately 10 min until no stretched, thread-like tubules were visible, and all tubules were considered perforated. A stereomicroscope (Olympus SZ61, Tokyo, Japan) was used in cases where tubules were extremely thin or connective tissue was dominant.

To determine whether overlaying the residual minced tissues impairs cell migration during centrifugation through gradient layers; two sets of centrifuge tubes were prepared per patient. Two-layer density gradients containing silane-coated silica (PureSperm® 40/80, Nidacon, Gothenburg, Sweden) were prepared, 0.5 ml each, with a 40% (v/v) upper and an 80% (v/v) lower density layer. Minced TESE material in the accompanying medium was then separated into two groups: (i) half of the accompanying SWM with the residual minced tissues (residual tissue with medium [RTM]), (ii) other half of SWM only, without any residual tissues. For SWM group, residual tissues were eliminated by aspirating the SWM only, carefully, using a fine tip (1 mm) plastic Pasteur pipette (BRAND GMBH and CO, Cat. No.: 747770, Wertheim, Germany) while tilting the culture dish side-to-side. For RTM group, conventional large tip pipettes were used (BRAND GMBH and CO, Cat. No. 747755). In order to avoid sperm loss, no count was performed before ICSI procedure. Centrifugation was performed at 400 g for 10 min and the supernatant from both tubes were discarded. To facilitate sperm visualization by the embryologist, 5 ml of an erythrocyte-sperm separation medium (TESEparate, Korpus, Ankara, Turkey) was put on each pellet and red blood cells were lysed by gentle pipetting. After a final wash in SWM, the pellet was agitated in 0.25 ml of the same medium and placed in the ICSI dish. ICSI dishes were prepared from a 3-(N-morpholino) propanesulfonic acid (MOPS) -buffered medium (G-MOPS, Vitrolife) under mineral oil (OVOIL, Vitrolife) by making two different large pools for the RTM and SWM groups (Figure 1). Both pools were investigated and scanned for intact spermatozoa in an inverted microscope at ×400. Recovered sperm cells were transferred into another drop in appropriate numbers for the number of mature oocytes retrieved. At the end of the ICSI procedure, the cells in the remaining RTM and SWM pools were withdrawn and a volume of 150 µl from each specimen was loaded in an automated Iris iQ200 cell counter with the Body Fluids Module (Iris

![Figure 1](https://example.com/figure1.png)
Diagnostics, Chatsworth, CA, USA) which counts nucleated cells in body fluids and cell suspensions by digital imaging. The iQ200 uses a live flow cell that hydrodynamically orients the particles within the focal plane of a microscopic lens coupled to a 1.3 megapixel CCD digital camera. Each particle image is digitized and sent to the instrument processor. The module provides digital micrographs of the cell types and of any cellular elements during counting which leads to true cell-count standardization. By flow cell digital imaging, the user has the chance to define the type of cells enumerated and to confirm an accurate count by classifying the cells into the database. Epithelial cells, red blood cells, white blood cells and spermatogenic cells are easily identified by the help of on-screen viewing and counted in order to have objective data, unlike manual counting chambers. Complex and heterogeneous cell suspensions can be assessed, and cells can be classified from urine or cerebrospinal fluid. This device has been used for a long time to evaluate routine urine analysis in our hospital where it is used to detect sperm cells in urine samples too. Cells can be defined manually by data input; according to their shape, size and nuclear status, with the help of flow images taken by the module itself. Many papers and comparisons have been published on testing the module on different body fluids and reports have indicated its superiority over manual counting techniques. The quantitative data provided the number of cells that had migrated through the gradient layers and minced tissues (in case of RTM), during centrifugation.

**Histological evaluation**

Approximately, the same volume (5 mm × 5 mm × 1 mm) of tissue from the patients’ raw biopsies just after the surgery and before mincing (control), and after mincing the retrieved biopsies (residuum), was evaluated to assess how effective the mincing procedure was in removing spermatogenic cells from the seminiferous tubules. Tissues were fixed in 4% (w/v) buffered formaldehyde, embedded in paraffin and 5-μm serial sections were made on a microtome (Leica RM 2125RT, Wetzlar, Germany) for histological evaluation of the presence of intact seminiferous tubule structures and of spermatogenic cells. The sections were stained with hematoxylin and eosin (HE) and Mallory–Azan (MA). Briefly, for HE staining, sections were deparaffinized with xylol and rehydrated in decreasing concentrations of alcohol and distilled water, before hematoxylin staining for 3 min. Following eosin staining for 1 min, excess eosin was rinsed off with increasing concentrations of alcohol. For MA staining, the following procedure was applied: xylol (30 min, twice), ethyl alcohol (100%, 96%, 75%), distilled water, azocarmine G solution (60°C, 1 h), distilled water, alcohol-based aniline dye, acidic alcohol, distilled water, phosphotungstic acid (30 min), Heidenhain blue (30 min), distilled water, ethyl alcohol (75%, 96%, 100%), xylol (30 min). Slides were covered with a cover slip using Entellan (Electron Microscopy Sciences, Hatfield, PA, USA).

The number of seminiferous tubule sections present in the stained sections and the presence of any spermatogenic cells in the remaining tubular structures were assessed. Ten sections were stained from different parts of each tissue to avoid repeated counting of the same tubules; all the seminiferous tubules on the sections were counted to get mean tubule numbers per given volume tissue. The mean numbers were compared between fresh biopsies (control) and minced residuum pieces.

Altogether, cell numbers in RTM versus SWM suspensions, seminiferous tubule section counts and spermatogenic cell quantifications in tissue sections were assessed for each case and compared statistically using Student’s t-test. The relationship between sperm recovery and hormone values was evaluated using the Mann–Whitney test. Correlation analysis was performed with Spearman’s Rank correlation coefficient. Normality tests performed and coefficients of varieties were calculated using Shapiro–Wilks’ test. All tests were carried out using SPSS version 11 (SPSS Inc., Chicago, IL, USA).

**RESULTS**

A total of 20 patients with a mean age of 33.38 (range: 26–51, standard deviation [s.d.]: 7.44) years were included in this study. The mean value of FSH was 25.66 IU l⁻¹ (s.d. ± 4.58), testosterone was 174.55 ng dl⁻¹ (s.d.: ±55.14) and total testicular volume per patient was 21.20 cm³ (s.d.: ±2.59; mean volume of right testes was 10.35 ± 5.99 cm³, left testes 10.85 ± 6.33 cm³). There were no statistically significant relationship between age and positive TESE results, as the age mean was 33.70 ± 5.64 for patients without intact spermatozoa and 33.00 ± 9.67 for patients with intact spermatozoa in their TESE material (P > 0.05). There were also no relationship between age and FSH, testosterone values or total testicular volume (r = 0.124, P > 0.05; r = -0.156, P > 0.05; r = 0.159, P > 0.05, respectively).

The only notable negative correlations were between serum FSH and testosterone concentrations (r = -0.418, P = 0.067), and between serum FSH and total testicular volume (r = -0.477, P = 0.034). None of the laboratory values had a relation with sperm retrieval rates of SWM or RTM groups. Sperm retrieval rate (SWM or RTM groups) was not predictable from serum FSH or testosterone, or testicular volume.

The mean number of cells (±s.d.) in the suspensions recovered from the density gradients of RTM and SWM was 39.435 ± 24.849 and 60.189 ± 28.214, respectively which was statistically significant (P < 0.001). Of the 20 patients, spermatozoa were recovered in 12 (60%) and in 10 spermatozoa were present in both RTM and SWM pools. In the remaining two patients, spermatozoa were discovered only in the SWM pool. In the 10 cases, despite sperm cells in both pools, all spermatozoa were retrieved from the SWM by the ICSI practitioner, because of their higher quantity. This technique, along with the usage of erythrocyte-sperm separation medium, radically decreased the time spent on ICSI and time of oocyte incubation in vitro.

The effectiveness of testicular tissue processing was also tested by evaluating fixed and stained tissue pieces for tubule structures as well as the presence of spermatozoa in the residual tissue. The mean number of tubular sections in the control group, before extraction, was 32.22 ± 5.13. In the residual tissue after extraction, the mean tubule count was 4.34 ± 1.21, an 87% decrease, which was statistically significant (P < 0.001). The control tissues revealed intact seminiferous tubules filled with different grades of spermatogenic cells (Figure 2, control). MA staining, a definitive stain for connective tissue components, revealed thickening of the seminiferous basement membrane in the majority of patients due to atrophy and fibrosis. This is an end-stage pathologic display of tubular sclerosis and it was higher, but not significant in patients with no intact spermatozoa retrieved after TESE. Twelve patients who presented intact spermatozoa in their preparations also revealed healthy seminiferous tubular morphology with the presence of intact spermatozoa. The remaining patients with no intact spermatozoa found in their biopsy extracts (n = 8), demonstrated various forms of germ cell maturation arrest in histological sections. Residual minced tissues were characterized by large clumps of connective tissue bundles with a prominent vascular structure. A few tubular structures were occasionally present in these clumps with minimal cellular content loosely attached to the tubular wall (Figure 2, residuum).
In the light of facts discussed above, the TESE procedure may provide large biopsies, which are transferred immediately to the embryology or andrology laboratory for sperm exploration following tissue dissection. Nevertheless, it should be the ultimate goal for a surgeon to extract a minimum volume of tissue by using advanced surgical techniques, such as microTESE, in order to avoid subsequent testicular dysfunction. Men suffering NOA may have an increased chance of having their active seminiferous tubule segments retrieved when the conventional TESE procedure is performed, especially in cases of maturation arrest. Testicular tissue biopsies are examined immediately by an experienced embryologist, and then minced into small pieces in order to release the testicular spermatozoa throughout the tubules into the surrounding culture medium. This procedure must be continued until no string-like tubules remain in the sample. There are additional experimental techniques to ensure tubular wall break down and spermatogenic content loss, such as grinding the tubules against a cell strainer, using motorized mini-tissue grinders and exposure to collagenase. After sufficient disintegration of the seminiferous tubules, the sample must be processed for the ICSI procedure, for which residual tissues, dense intercellular matrix components, red blood cells, leukocytes, intertubular space components, large cellular droplets and reactive oxygen species must be eliminated from the suspension. The embryologist has to examine the sample for a single spermatozoon and be able to retrieve it effectively; however, without eliminating surplus tissue elements, an efficient performance at ICSI workstation is challenging. The temperature and pH of working solutions change with the increased duration of ICSI when spermatozoa are sought in the TESE material. In order to enhance sperm retrieval and increase micromanipulation efficiency; gradient centrifugation and the application of erythrocyte-lysing buffer are often used. Enzymatic digestion of seminiferous tubules is a time-consuming procedure (about 4 h) and has produced conflicting results about safety. It is not superior to mechanical preparation. After collagenase treatment, the production of cytokines from macrophages may increase, influencing spermatozoa negatively.

It has been published that testicular spermatozoa of NOA patients are more likely to have aneuploidy than those from normozoospermic control donors. Overall disomy rates are almost four-fold, and diploidy rates are almost three-fold higher in testicular spermatozoa of NOA patients than the ejaculated spermatozoa of the control group. It is known that density gradient centrifugation is an efficient tool to eliminate aneuploidic spermatozoa. Brahem et al. found that the 90% density layer possessed a lower proportion of aneuploid spermatozoa than those remaining in the 70% or 50% layers. They also showed that diploid spermatozoa were significantly fewer in the 90% layer than in the other two gradient layers. Kovanci et al. has demonstrated that mature spermatozoa selected by 80% Percoll showed less total disomy, density diploidy rates are almost four-fold, and increased DNA fragmentation impairs the outcome of assisted reproduction. Density gradient centrifugation is a superior sperm processing method with the ability to select spermatozoa with good morphology, motility, viability and progressive motility. This technique has a beneficial role in selecting sperm cells with intact DNA and properly condensed chromatin, and decreases the rates of DNA fragmentation in spermatozoa recovered from both fertile and infertile male populations as well as in frozen-thawed spermatozoa. Collagen and extracellular matrix components...
Modification to TESE preparation increases cell yield

S Ozkavukcu et al

inversely affect the ICSI procedure in the final preparations, as they make the solution dense and hinder micro-manipulation. Gradient centrifugation has another advantage, eliminating compounds that could be freed during tissue grinding. This observation was documented by one group where assisted reproduction outcomes in TESE cases were compared with percutaneous epididymal sperm aspiration results. Hammitt et al. eliminated debris by density gradient centrifugation after TESE and had similar ongoing pregnancy and delivery rates to those from centrifuging only in SWM. On the other hand, sperm retrieval was easier when density gradient centrifugation was used. With density gradient centrifugation the only sediment, other than spermatogenic cells, is comprised of red blood cells, a problem overcome by the application of an erythrocyte-sperm separation medium, which eliminated the majority of red blood cells in our study.

In our work, histological examination of fresh biopsies demonstrated that complete spermatogenesis was present in the seminiferous tubule sections in all cases where mature spermatozoa retrieval was successful. In many studies, testicular histopathology is presented as the best predictor of positive sperm retrieval in men with NOA. Our results are consistent with those in the literature in that levels of FSH and testosterone, along with the testicular volume, are less accurate predictors of positive sperm retrieval than histopathologic data.

There has been a practical concern that density gradient centrifugation of TESE material may trap some spermatogenic cells during centrifugation. From our observations, whether TESE is performed with or without microdissection, lumps of tissue consisting of connective tissue remain as a residue after mincing. Our findings indicate that these residues do not contain any functional seminiferous tubules or sperm cells, but they block cells from migrating through the density gradient column when layered over the gradient medium with the wash medium. When nonfunctional tissue debris was removed from the centrifugation (SWM group), the number of spermatozoa in the final suspension was increased, as were other forms of spermatogenic cells such as spermatids and spermatocytes. This comparison was made from the suspensions prepared from the same patient (RTM vs SWM groups).

CONCLUSIONS

Density gradient centrifugation has no inverse effect on spermatozoa retrieval as long as a detailed mincing of the testicular tissue is performed. The mincing of the biopsied material must be effectively carried out in order to be sure that all seminiferous tubule structures are successfully disrupted and intratubular cells are freed into the suspension. According to our findings, after mincing, the remaining residual tissues do not add any functional or cellular contribution to spermatogenic cell retrieval; they may actually block the cellular elements in the accompanying cell suspension from migrating down the gradient layers to form a cell pellet during centrifugation and that may cause loss of spermatogenic cells.

AUTHOR CONTRIBUTIONS

EI, SK, SI, KA performed the research, analyzed the data. SO performed the research, analyzed the data, designed the research study and wrote the paper. KA has performed all the surgery. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Modification to TESE preparation increases cell yield
S Ozkavukcu et al

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