



Open Access

ORIGINAL ARTICLE

Sperm Biology

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.

Asian Journal of Andrology (2014) 16, 852–857; doi: 10.4103/1008-682X.132468; published online: 01 July 2014

Keywords: assisted reproductive techniques; azoospermia; gradient density centrifugation; sperm yield; testicular sperm extraction

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)

Received: 09 November 2013; Revised: 14 January 2014; Accepted: 04 March 2014

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.¹ 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 ($\times 8$). The testicular parenchyma was directly examined at higher magnification ($\times 40$) 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-IVE, 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.¹

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.⁹ 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.¹⁰ 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 $\times 400$. 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 μ l from each specimen was loaded in an automated Iris iQ200 cell counter with the Body Fluids Module (Iris

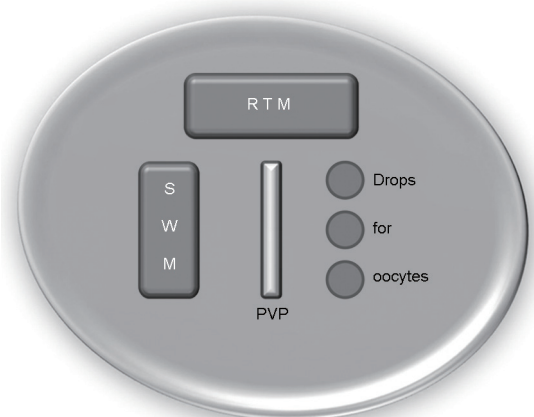


Figure 1: Intra-cytoplasmic sperm injection (ICSI) dish setup. Two different suspension pools are formed and blindly investigated for mature spermatozoa. The pools are drawn back for nucleated cell count after ICSI.

Diagnostics, Chatsworth, CA, USA) which counts nucleated cells in body fluids and cell suspensions by digital imaging.¹¹ 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.¹² 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.¹³ 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.¹⁴ Complex and heterogeneous cell suspensions can be assessed, and cells can be classified from urine or cerebrospinal fluid.^{11,13} 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.^{11,13,14} 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- μ m 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-Wilk's 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 24.56 IU l⁻¹ (s.d. \pm 4.56), testosterone was 184.55 ng dl⁻¹ (s.d.: \pm 55.14) and total testicular volume per patient was 21.20 cm³ (s.d.: \pm 2.59; mean volume of right testes was 10.35 \pm 5.99 cm³, left testes 10.85 \pm 6.53 cm³). There were no statistically significant relationship between age and positive TESE results, as the mean age was 33.70 \pm 5.64 for patients without intact spermatozoa and 33.00 \pm 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 (\pm s.d.) in the suspensions recovered from the density gradients of RTM and SWM was 39.435 \pm 24.849 and 60.189 \pm 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 \pm 5.13. In the residual tissue after extraction, the mean tubule count was 4.34 \pm 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 sclerosis¹⁵ 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 lumps 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).

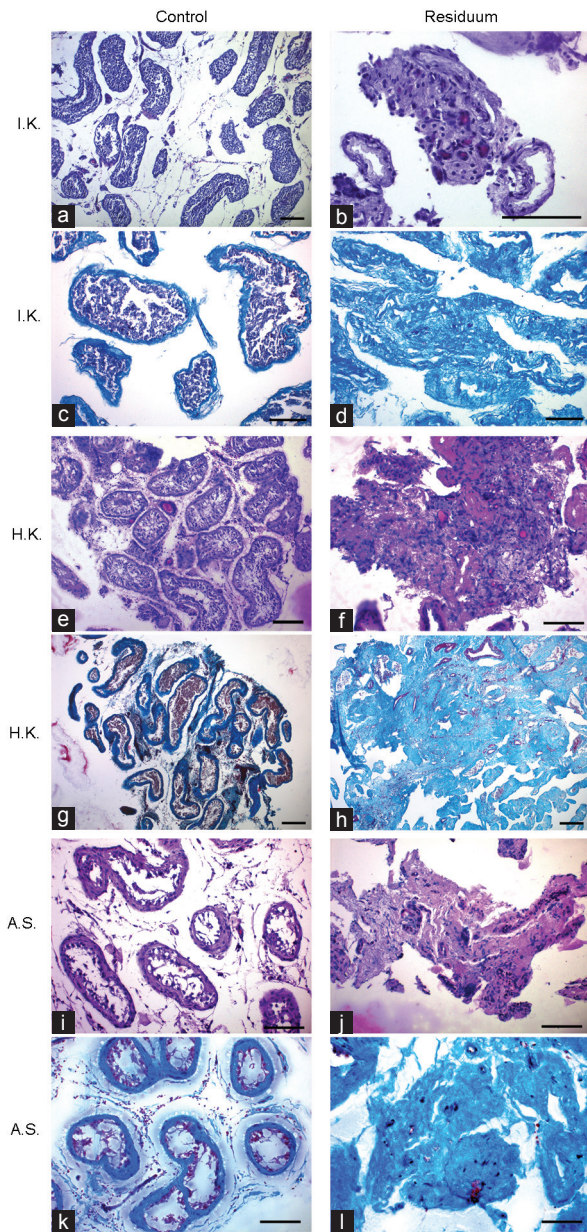


Figure 2: Comparison of control (a, c, e, g, i, and k) versus minced residual tissues (b, d, f, h, j and l). HE (a, b, e, f, i, and j) and MA (c, d, g, h, k, and l) stain were used in order to determine spermatogenic cells and connective tissue elements. Scale bars = 100 μ m.

DISCUSSION

Microdissection TESE has become a popular technique for exploring testicular tissue and sperm retrieval. Even though, its superiority over conventional TESE is uncertain, some benefits have been published. Okada *et al.*¹⁶ and Ghalayini *et al.*¹⁷ found a better sperm retrieval rate by using the microTESE technique in Sertoli-cell-only Syndrome than in conventional TESE. In a systematic review published by Deruyver *et al.*⁸ overall sperm retrieval rate was significantly higher in microTESE group (42.9%–63%) than conventional TESE (16.7%–45%). The only occasion where microTESE is not favourable seems to be in patients with uniform histological patterns such as maturation arrest, where studies have not shown any significant difference between two procedures.⁸ Our microTESE sperm retrieval rate of 60% here is consistent with that in the literature.

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.¹⁸ 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.^{19,20} 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 centrifugation²¹ and the application of erythrocyte-lysing buffer are often used.²² 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.⁹ After collagenase treatment, the production of cytokines from macrophages may increase, influencing spermatozoa negatively.^{23,24}

It has been published that testicular spermatozoa of NOA patients are more likely to have aneuploidy than those from normozoospermic control donors.²⁵ 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.*²⁶ 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 two other gradient layers. Kovanci *et al.*²⁷ has demonstrated that mature spermatozoa selected by 80% Percoll showed less total disomy, total diploidy and total aneuploidy frequencies than those of neat semen of the same patient. Elimination of spermatozoa with deoxyribonucleic acid (DNA) fragmentation is another goal for achieving satisfactory outcomes in IVF. The testicular sperm population has an elevated rate of fragmented DNA,²⁸ and increased DNA fragmentation impairs the outcome of assisted reproduction.²⁹ Density gradient centrifugation is a superior sperm processing method with the ability to select spermatozoa with good morphology, motility, viability and progressive motility.³⁰ This technique has a beneficial role in selecting sperm cells with intact DNA and properly condensed chromatin,³¹ and decreases the rates of DNA fragmentation in spermatozoa recovered from both fertile and infertile male populations^{32,33} as well as in frozen-thawed spermatozoa.^{34,35} Collagen and extracellular matrix components

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.*³⁶ 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.³⁷⁻⁴⁰ 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.³⁷

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.

REFERENCES

- 1 WHO. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010.

- 2 Schlegel PN, Palermo GD, Goldstein M, Menendez S, Zaninovic N, *et al.* Testicular sperm extraction with intracytoplasmic sperm injection for nonobstructive azoospermia. *Urology* 1997; 49: 435-40.
- 3 Turunc T, Gul U, Haydardeoglu B, Bal N, Kuzgunbay B, *et al.* Conventional testicular sperm extraction combined with the microdissection technique in nonobstructive azoospermic patients: a prospective comparative study. *Fertil Steril* 2010; 94: 2157-60.
- 4 Marconi M, Keudel A, Diemer T, Bergmann M, Steger K, *et al.* Combined trifocal and microsurgical testicular sperm extraction is the best technique for testicular sperm retrieval in "low-chance" nonobstructive azoospermia. *Eur Urol* 2012; 62: 713-9.
- 5 Ishikawa T. Surgical recovery of sperm in non-obstructive azoospermia. *Asian J Androl* 2012; 14: 109-15.
- 6 Donoso P, Tournaye H, Devroey P. Which is the best sperm retrieval technique for non-obstructive azoospermia? A systematic review. *Hum Reprod Update* 2007; 13: 539-49.
- 7 Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. *Hum Reprod* 1999; 14: 131-5.
- 8 Deruyver Y, Vanderschueren D, Van der Aa F. Outcome of microdissection TESE compared with conventional TESE in non-obstructive azoospermia: a systematic review. *Andrology* 2014; 2: 20-4.
- 9 Esteves SC, Varghese AC. Laboratory handling of epididymal and testicular spermatozoa: what can be done to improve sperm injections outcome. *J Hum Reprod Sci* 2012; 5: 233-43.
- 10 Celik-Ozenci C, Sati L, Celik S. Effect of erythrocyte-sperm separation medium treatment on sperm motility, viability, morphology, acrosome reaction, hyaluronic acid binding, chromatin condensation, and DNA fragmentation (Abstract). *Fertil Steril* 2013; 100: S449-50.
- 11 Walker TJ, Nelson LD, Dunphy BW, Anderson DM, Kickler TS. Comparative evaluation of the Iris iQ200 body fluid module with manual hemacytometer count. *Am J Clin Pathol* 2009; 131: 333-8.
- 12 Boven LA, Kemperman H, Demir A. A comparative analysis of the Iris iQ200 with manual microscopy as a diagnostic tool for dysmorphic erythrocytes in urine. *Clin Chem Lab Med* 2012; 50: 751-3.
- 13 Butch AW, Wises PK, Wah DT, Gornet TG, Fritsche HA. A multicenter evaluation of the Iris iQ200 automated urine microscopy analyzer body fluids module and comparison with hemacytometer cell counts. *Am J Clin Pathol* 2008; 129: 445-50.
- 14 Wah DT, Wises PK, Butch AW. Analytic performance of the iQ200 automated urine microscopy analyzer and comparison with manual counts using Fuchs-Rosenthal cell chambers. *Am J Clin Pathol* 2005; 123: 290-6.
- 15 Cerilli LA, Kuang W, Rogers D. A practical approach to testicular biopsy interpretation for male infertility. *Arch Pathol Lab Med* 2010; 134: 1197-204.
- 16 Okada H, Dobashi M, Yamazaki T, Hara I, Fujisawa M, *et al.* Conventional versus microdissection testicular sperm extraction for nonobstructive azoospermia. *J Urol* 2002; 168: 1063-7.
- 17 Ghalayini IF, Al-Ghazo MA, Hani OB, Al-Azab R, Bani-Hani I, *et al.* Clinical comparison of conventional testicular sperm extraction and microdissection techniques for non-obstructive azoospermia. *J Clin Med Res* 2011; 3: 124-31.
- 18 Dabaja AA, Schlegel PN. Microdissection testicular sperm extraction: an update. *Asian J Androl* 2013; 15: 35-9.
- 19 Popal W, Nagy ZP. Laboratory processing and intracytoplasmic sperm injection using epididymal and testicular spermatozoa: what can be done to improve outcomes? *Clinics (Sao Paulo)* 2013; 68 Suppl 1: 125-30.
- 20 Aydos K, Demirel LC, Baltaci V, Unlü C. Enzymatic digestion plus mechanical searching improves testicular sperm retrieval in non-obstructive azoospermia cases. *Eur J Obstet Gynecol Reprod Biol* 2005; 120: 80-6.
- 21 Li P, Li S, Peng Z, Ma Q, Huang Z, *et al.* [Primary study on the method for separating mature sperms from human testicular tissue]. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi* 2005; 22: 371-3.
- 22 Nagy ZP, Verheyen G, Tournaye H, Devroey P, Van Steirteghem AC. An improved treatment procedure for testicular biopsy specimens offers more efficient sperm recovery: case series. *Fertil Steril* 1997; 68: 376-9.
- 23 Bryniarski K, Szczepanik M, Ptak M, Ptak W. The influence of collagenase treatment on the production of TNF-alpha, IL-6 and IL-10 by testicular macrophages. *J Immunol Methods* 2005; 301: 186-9.
- 24 Bryniarski K, Szczepanik M, Ptak M, Ptak W. Modulation of testicular macrophage activity by collagenase. *Folia Histochem Cytobiol* 2005; 43: 37-41.
- 25 Vozdova M, Heracek J, Sobotka V, Rubes J. Testicular sperm aneuploidy in non-obstructive azoospermic patients. *Hum Reprod* 2012; 27: 2233-9.
- 26 Brahem S, Letaief K, Ben Ali H, Saad A, Mehdi M. Efficacy of the density gradient centrifugation method in eliminating sperm with aneuploidy. *Andrologia* 2013; 45: 158-62.
- 27 Kovanci E, Kovacs T, Moretti E, Vigue L, Bray-Ward P, *et al.* FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention. *Hum Reprod* 2001; 16: 1209-17.
- 28 Meseguer M, Santiso R, Garrido N, Gil-Salom M, Remohí J, *et al.* Sperm DNA fragmentation levels in testicular sperm samples from azoospermic males as assessed by the sperm chromatin dispersion test. *Fertil Steril* 2009; 92: 1638-45.

- 29 Simon L, Proutski I, Stevenson M, Jennings D, McManus J, *et al*. Sperm DNA damage has a negative association with live-birth rates after IVF. *Reprod Biomed Online* 2013; 26: 68–78.
- 30 Ghaleno LR, Valojerdi MR, Janzamin E, Chehrazi M, Sharbatoghli M, *et al*. Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: a flow cytometric study. *Arch Gynecol Obstet* 2014; 289: 173–80.
- 31 Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, *et al*. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Hum Reprod* 2000; 15: 1112–6.
- 32 Zhang XD, Chen MY, Gao Y, Han W, Liu DY, *et al*. The effects of different sperm preparation methods and incubation time on the sperm DNA fragmentation. *Hum Fertil (Camb)* 2011; 14: 187–91.
- 33 Brahem S, Mehdi M, Elghezal H, Saad A. Semen processing by density gradient centrifugation is useful in selecting sperm with higher double-strand DNA integrity. *Andrologia* 2011; 43: 196–202.
- 34 Morató R, Soares JM, Orero G, Mogas T, Miró J. Pre-selection by double layer density gradient centrifugation improves the fertilising capacity of frozen-thawed, capacitated stallion sperm. *Anim Reprod Sci* 2013; 139: 62–8.
- 35 Brugnon F, Ouchchane L, Pons-Rejraji H, Artonne C, Farigoule M, *et al*. Density gradient centrifugation prior to cryopreservation and hypotaurine supplementation improve post-thaw quality of sperm from infertile men with oligoasthenoteratozoospermia. *Hum Reprod* 2013; 28: 2045–57.
- 36 Hammitt DG, Ferrigni RG, Sattler CA, Rebert JA, Singh AP. Development of a new and efficient laboratory method for processing testicular sperm. *J Assist Reprod Genet* 2002; 19: 335–42.
- 37 Abdel Raheem A, Garaffa G, Rushwan N, De Luca F, Zacharakis E, *et al*. Testicular histopathology as a predictor of a positive sperm retrieval in men with non-obstructive azoospermia. *BJU Int* 2013; 111: 492–9.
- 38 Vicari E, Grazioso C, Burrello N, Cannizzaro M, D'Agata R, *et al*. Epididymal and testicular sperm retrieval in azoospermic patients and the outcome of intracytoplasmic sperm injection in relation to the etiology of azoospermia. *Fertil Steril* 2001; 75: 215–6.
- 39 Schoor RA, Elhanbly S, Niederberger CS, Ross LS. The role of testicular biopsy in the modern management of male infertility. *J Urol* 2002; 167: 197–200.
- 40 Mercan R, Urman B, Alatas C, Aksoy S, Nuhoglu A, *et al*. Outcome of testicular sperm retrieval procedures in non-obstructive azoospermia: percutaneous aspiration versus open biopsy. *Hum Reprod* 2000; 15: 1548–51.

How to cite this article: Ozkavukcu S, Ibis E, Kizil S, Isbacar S, Aydos K. A laboratory modification to testicular sperm preparation technique improves spermatogenic cell yield. *Asian J Androl* 01 July 2014. doi: 10.4103/1008-682X.132468. [Epub ahead of print]