

Adapted methods for scanning electron microscopy (SEM) in assessment of human sperm morphology

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ABSTRACT

Infertility is a widespread problem, and in some cases, the routine basic semen analysis is not sufficient to detect the cause of male infertility. The use of the scanning electron microscope (SEM) could provide a detailed insight into spermatozoa morphology, but it requires specific sample preparation techniques. The purpose of this study was to select, adjust, and optimize a method for the preparation of spermatozoa samples prior to SEM analysis, and to establish the protocol required for its use in clinical practice. We examined sperm samples of 50 men. The samples were fixed with modified iso-osmolar aldehyde solution followed by osmium post-fixation. In the first method, dehydration of the cells and subsequent critical point drying (CPD) were performed on a coverslip. In the second method, the samples were dehydrated in centrifuge tubes; hexamethyldisilazane (HMDS) was used as a drying agent instead of CPD, and the samples were air-dried. The third procedure was based on a membrane filter. The samples were dehydrated and dried with HMDS in a Gooch crucible, continuously, without centrifugation or redispersion of the sample. Our results showed that the fixation with modified iso-osmolar aldehyde solution followed by osmium post-fixation, and combined with dehydration and CPD on a coverslip, is the most convenient procedure for SEM sample preparation. In the case of small-size samples or low sperm concentration, dehydration and drying with HMDS on the membrane filter enabled the best reliability, repeatability, and comparability of the results. The presented procedures are suitable for routine use, and they can be applied to confirm as well as to correct a diagnosis.

KEY WORDS: Scanning electron microscope; SEM; preparation techniques; human sperm morphology; infertility; clinical practice; semen; CPD; critical point drying; HMDS; hexamethyldisilazane

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INTRODUCTION

Poor quality of semen is a common cause of male infertility [1]. Approximately, in one out of five couples who are having fertility problems, there is an issue with the male partner [2,3]. The semen analysis or spermiogram includes physical, morphological, and biochemical analysis of the ejaculate. For a correct diagnosis, all semen parameters should be considered [4]. The semen analysis is also important in determining the fertilizing potential of spermatozoa [5-7]. Generally, spermiogram is a part of andrological examination and currently remains the most appropriate test for assessing the male fertility [8].

Due to the fact that, in some men, the semen analysis shows normal findings despite the presence of infertility, this

method is not always suitable to detect the cause of male infertility [9]. Although viable sperm cells can be analyzed by light microscopy, the resolution of a light microscope is low and various morphological defects that may occur in the organelles of a spermatozoon cannot be identified [5,10]. In this case, the use of a scanning electron microscope (SEM) could provide more information. In general, electron microscopy is used for the identification and classification of structural and morphological characteristics of spermatozoa [11,12].

The surface of a sperm cell can be viewed at the nanometer resolution with SEM and in a wide range of magnification [13]. A three-dimensional (3-D) image with large visual depth can be obtained using lenses with a large depth of field [14]. When preparing a sample for a SEM analysis, different characteristics of the biological specimen should be considered, as well as appropriate methods for sample preparation. The basic requirements for SEM sample preparation are: first, the sample must not be affected by vacuum and exposed to electron beam; second, there has to be a sufficient amount of

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secondary electron emission (e_2); and third, the examination surface need to be clean [15,16].

Electron microscopy enables the identification of systematic and nonsystematic spermatozoa defects as well as changes of the organelles in the head and tail of spermatozoon [5].

The use of SEM for diagnostic purpose is still relatively rare. However, due to the higher resolution compared to light microscopy and wider range of magnification, SEM could be a useful additional tool in diagnosing difficult cases of male infertility [10].

The purpose of this study was to select, adjust, and optimize a method for the preparation of sperm samples prior to SEM analysis, and to establish the protocol required for its use in clinical practice.

MATERIALS AND METHODS

Samples

Fifty Slovenian male partners of infertile couples attending the outpatient clinic for infertility at the Department of Obstetrics and Gynecology, University Medical Centre Ljubljana from May 2014 to December 2015, were consecutively enrolled in this study. The sperm samples were included into the routine testing for diagnosing male infertility. According to Kruger's strict criteria for the evaluation of sperm morphology, the 50 men were classified into two groups: 5 men with normal sperm morphology and 45 men with abnormal sperm morphology. The initial semen analysis was performed following the standard procedure [8,9]. The semen samples were collected by masturbation into sterile containers after 3 days of sexual abstinence, and were examined after liquefaction for 30 minutes at 37°C. A portion of the samples was assessed using optical microscopy for basic diagnostics of male infertility according to the World Health Organization (WHO) guidelines [1], while the remaining samples were analyzed by SEM. For SEM analysis, each sperm sample was prepared by centrifugation (300 g) for 20 minutes on 100%/40% density gradient of PureSperm (NidaCon, Sweden) followed by a swim-up technique, where high-quality spermatozoa were extracted from the sample.

The study was approved by the National Medical Ethics Committee. After an informed consent was obtained, a detailed interview was made.

Fixation of samples for SEM analysis

Each spermatozoa sample obtained with the swim-up technique was immediately centrifuged at 400 g for 10 minutes at room temperature. The supernatants were carefully removed and pellets were suspended and fixed in aldehyde solution (primary fixation): 1% glutaraldehyde and 0.4%

formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 300-400 mOsm) for 30 minutes at 4°C. After the primary fixation, the samples were washed 3 times in 0.1 M sodium cacodylate buffer over 5 minutes, centrifuged at 400 g for 10 minutes, and post fixed with 1% OsO₄ in 0.1 M sodium cacodylate buffer for 30 minutes. The cells were washed again 3 times and centrifuged.

SEM sample preparation on a coverslip

The samples were placed on glass coverslips and treated with Alcian blue (Alcian blue 8 GX, Sigma) for better attachment. The samples on the coverslips were dehydrated in ethanol series (50%, 70%, 80%, 90%, 96%, and absolute alcohol), and critical point dried [CPD] (Balzers CPD 030 Critical Point Dryer; Bal-Tec AG, Balzers, Liechtenstein) in carbon dioxide.

SEM sample preparation in centrifuge tubes

The dehydration of the spermatozoa samples on the coverslips was replaced by dehydration in centrifuge tubes due to the loss of the cells that did not attach to the coverslip surface. The sperm pellet was suspended in a graded ethanol series and after each step centrifuged at 400 g for 10 minutes. Furthermore, CPD was replaced by hexamethyldisilazane (HMDS). In the drying process, the dehydrated sperm pellets were immersed in HMDS, placed on the glass coverslips covered with Alcian blue, and air-dried at room temperature.

SEM sample preparation on a membrane filter

In these procedures, the spermatozoa were processed on a membrane filter instead of coverslips. We used an improvised glass filtration system in a filter holder (Millipore glass filtration system; Merck Millipore, Burlington, Massachusetts, USA). After the fixation and post-fixation in centrifuge tubes, the samples were loaded on a membrane filter (Millipore, 0.2 μ) in a filter unit. The cells were concentrated on the filter by vacuum filtration. The samples were subsequently dehydrated in a series of ethanol dilutions in increasing concentrations (50%, 70%, 80%, 90%, 96%, and absolute alcohol) and dried with HMDS. Following the last wash with HMDS, the filter was removed from the filter unit and allowed to air-dry for 30 minutes.

Sputter coating and SEM analysis

All dried specimens were glued to the specimen stubs by carbon adhesive discs, then Pt sputtered (Bal-Tec SCD 050 Sputter Coater; Bal-Tec AG, Balzers, Liechtenstein), and examined with a field emission SEM type FESEM, 7500 F (Tokyo, Japan).

RESULTS

Preparation techniques

SEM analysis of spermatozoa prepared on coverslips and CPD

The CPD was successfully performed as shown on the SEM micrographs in Figure 1A and B. The combination of modified iso-osmolar aldehyde solution followed by osmium post-fixation and CPD yielded the best results. CPD enables the fluid to transform to the gaseous phase without any phase boundary, meaning that there are no forces of surface tension that could damage the structure of the sample (Figure 1A and B). The spermatozoa revealed intact surfaces of the head (Figure 1B), neck, and tail regions (Figure 1A), while the artifacts typical for drying were barely noticeable.

SEM analysis of spermatozoa prepared in centrifuge tubes and dried with HMDS

On Figure 2A and B, the characteristic SEM micrographs show spermatozoa fixed with a modified iso-osmolar aldehyde solution, followed by osmium post-fixation and drying with HMDS, an attractive alternative to CPD. The SEM micrographs show larger, more visible lesions and damage to the surface of the tail structures (Figure 2A and B) that are, nevertheless, still small to get a satisfactory result.

SEM analysis of spermatozoa prepared on a membrane filter and dried with HMDS

On Figure 3A and B, the SEM micrographs show spermatozoa trapped on the membrane filter. The samples were dehydrated and dried with HMDS in a Gooch crucible, continuously, without any centrifugation or redispersion of the sample. Spermatozoa were concentrated in the pores as well as around the pores of the membrane (Figure 3A), and the fine structure of the filters prevented damage and loss of spermatozoa (Figure 3B). A disadvantage of this procedure is that various impurities, which can cover the surface of the specimen, remain on the filter together with the cells. The use of high-purity chemicals and reagents can help prevent the contamination of the samples.

SEM usefulness in clinical practice

Sperm samples often contain other, non-sperm, cells or their parts (Figure 4A and B). SEM micrographs of bacteria (Figure 5A and B) and leukocytes (Figure 4B), that are present in sperm samples, can give clues about the presence of infection that may affect the quality of semen.

Our SEM micrographs showed various deformities of the head of spermatozoa (Figure 6A and B). The structures observed on the heads may correspond to vacuoles that are seen when using the transmission electron microscopy.

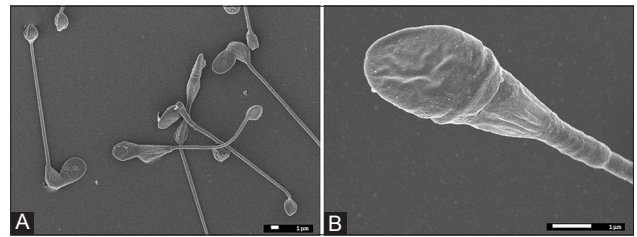


FIGURE 1. (A and B) Scanning electron micrographs of spermatozoa prepared on glass coverslips and critical point dried (CPD).

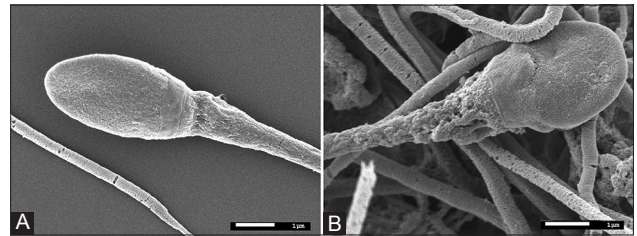


FIGURE 2. (A and B) Scanning electron micrographs of spermatozoa prepared in centrifuge tubes and dried with hexamethyldisilazane (HMDS).

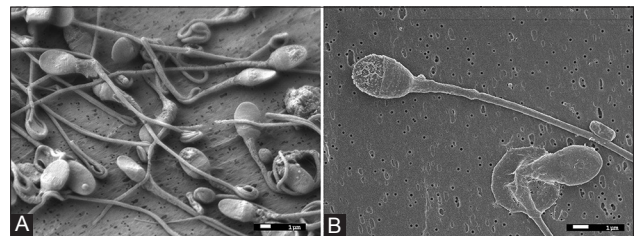


FIGURE 3. (A and B) Scanning electron micrographs of spermatozoa prepared on a membrane filter and dried with hexamethyldisilazane (HMDS).

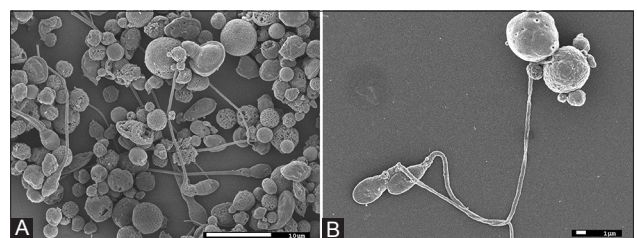


FIGURE 4. Scanning electron micrographs of non-sperm cells (A and B) and leukocytes (B).

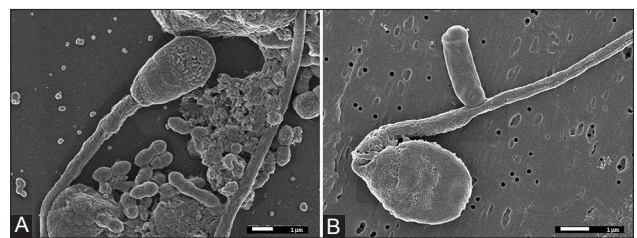


FIGURE 5. Scanning electron micrographs of bacterial clusters (A) and the apparent attachment of bacteria to the tail of spermatozoa (B).

On Figure 7A and B, the SEM micrographs show different types of spermatozoa with thin necks. In the case when the neck and midsection of the tail are very thin, the cellular

membrane may disappear in those parts and reveal a bundle of fibrils and mitochondria (Figure 8A). The thin area showed a decreased number of mitochondria (Figure 8B), which was probably the cause of the decreased sperm motility or even the complete inability of the spermatozoon to move.

DISCUSSION

Fixatives for electron microscopy have been greatly improved over the last 20 years. These fixatives have several roles, including: to stop the cell metabolism, to fix the cell organelles and molecules in their current position, and to make the material accessible and stable during subsequent processing [17]. In our study, the modified fixation procedure consisting of primary fixation with aldehydes and osmium post-fixation enabled the best fixation of spermatozoa. Fixatives containing both paraformaldehyde and glutaraldehyde provide significantly better fixation than aldehyde alone [18]. Formaldehyde rapidly penetrates tissues and moderately stabilizes proteins which are then permanently fixed by glutaraldehyde [19,20].

In the first sample preparation method, we placed the spermatozoa samples on glass coverslips, dehydrated in a

graduated ethanol series, and dried at critical point. This method was used due to the fact that CPD is most commonly used to dry samples prior to SEM analysis [21-23]. Our results showed that the fixation with modified iso-osmolar aldehyde solution followed by osmium post-fixation, and combined with dehydration and CPD on a coverslip, is the most convenient procedure for SEM sample preparation. Nevertheless, a downside of this method is a significant loss of the material, resulting from its inability to attach to the coverslip surface. Subsequently, a significant portion of the samples is washed away, despite adhesives, such as Alcian blue [24], are applied to the coverslips [16,25,26].

In the second method, we used centrifuge tubes for the dehydration instead of the coverslips. Moreover, CPD was replaced with HMDS. A study showed that images of better quality can be obtained with HMDS compared to CPD technique [27]. Furthermore, no specialized equipment is required for HMDS, making this method time- and cost-effective. However, it has no advantages in terms of the loss of the cells compared to the CPD-based procedure [23]. Finally, we tested the third method for SEM sample preparation in which the samples were placed on the membrane filter instead of coverslips. This, relatively new method, turned out to be the most useful in terms of sample preservation, without any centrifugation or redispersion of the sample [28,29]. The filter-based method is convenient for small-size samples or those with low sperm concentration, as it prevents excessive loss and damage to the sample.

Overall, our results confirm the premise that SEM analysis is a useful and valuable method in clinical practice [10]. Previous experiments [30] have indicated potential defects that were not visible with a light microscope analysis of semen but were obvious when analyzed with SEM. However, the criteria for morphological analysis with light microscopy are well established, according to the WHO guidelines [1], while there is no such standardized protocol for the SEM micrograph analysis.

In our study, the SEM micrography showed several unusual deformations on the surface of the spermatozoa or absence of mitochondria, that were not visible when the standard microscope was used. In addition, we found other, non-sperm, cells in our specimens that may reflect the quality of semen samples [31,32]. Finally, our semen samples showed a high variability in the sperm morphology and open a number of questions about the spermatozoa functionality [31].

CONCLUSION

In this study, we developed and optimized a method for sperm sample preparation for SEM analysis. This method is highly reproducible and gives high-quality images. Among the

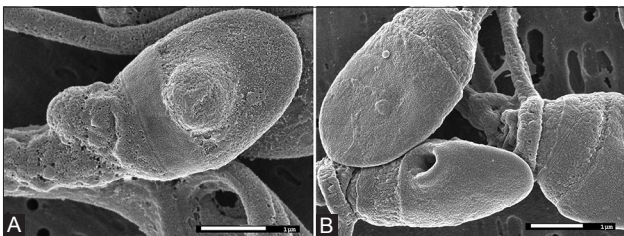


FIGURE 6. (A and B) Scanning electron micrographs showing vacuole-like structures on the head of spermatozoa.

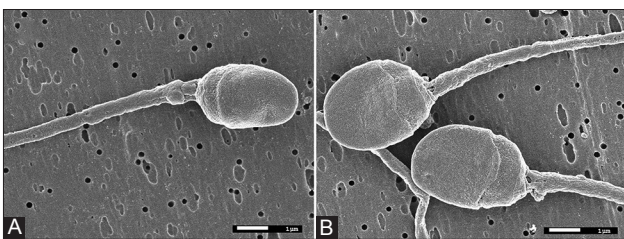


FIGURE 7. (A and B) Scanning electron micrographs of spermatozoa showing a significantly thinner midsection of the tail and absence of mitochondria.

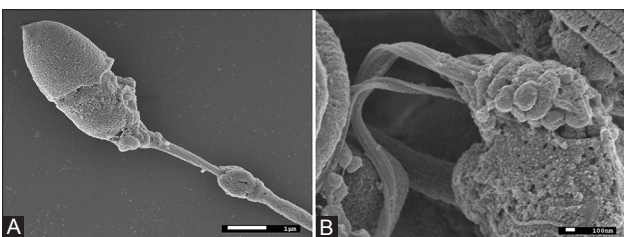


FIGURE 8. Scanning electron micrographs of spermatozoa with exposed necks and midsection of the tail (A), as well as individual myofibrils (B).

three tested protocols, the most suitable preparation method for the analysis of spermatozoa morphology was the fixation with modified iso-osmolar aldehyde solution followed by osmium post-fixation, and combined with dehydration and CPD on a coverslip. In the case of small-size samples or low sperm concentration, the dehydration and drying with HMDS on the membrane filter enabled the best reliability, repeatability, and comparability of the results, as well as the generation of high-quality SEM images. The presented procedures are suitable for routine use, and they can be applied to confirm as well as to correct a diagnosis.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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