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ASSESSMENT OF HUMAN SPERM MORPHOLOGY BY STRICT CRITERIA: COMPARISON OF WET PREPARATION VERSUS STAINED WITH THE MODIFIED DIFF-QUIK METHOD

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Routine semen examination remains an important tool for the diagnosis and treatment in human subfertility. Of all semen parameters, sperm morphology seems to be one of the most powerful indicators of a man's fertilizing potential in vitro and in vivo. Lack of standardization of sperm morphology assessments remains the main reason for the usefulness of this parameter. The aim of this study was to analyze the agreement between the wet-stained preparations versus those stained with modified Diff-Quik for sperm morphology. A total of 100 unselected semen samples from infertile couples were analyzed. Sperm morphology was evaluated with unstained specimens and following modified Diff-Quik staining according to the strict (Kruger classification) criteria by two different examiners (intralaboratory blind assessment). Mean percentages of morphologically normal spermatozoa were identical on wet and stained preparation slides (4.79 vs. 4.61, $p > .05$). Wide divergence of results was found with respect to the percentage of sperm with head and midpiece defects with the two different preparations ($p > .001$). The percentage of sperm tail defects was similar in both methods ($p > .05$). Simple linear regression analysis between the two methods revealed very good correlation for the morphologically normal spermatozoa ($r = .83$), but poor correlation for the sperm head, midpiece, and tail defects ($r = .25$, .25, and .28, respectively). Wet preparation is suitable only for the morphologically normal spermatozoa, but to determine the percentage of the defective spermatozoa, staining the smear is recommended.

Keywords sperm morphology, strict criteria, Diff-Quik, wet preparation

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Sperm concentration, motility, and morphology are generally considered the three most important parameters for assessment of male infertility. In humans, normal fertile ejaculates contain spermatozoa exhibiting considerable variation in the size and shape of the head and acrosome, midpiece, and tail. The assessment of sperm morphology, an integral component of basic semen evaluation, is useful in the management of the infertile male [13]. A good correlation between normal sperm morphology and in vitro and in vivo fertilization has been reported by many investigators [3, 10, 14]. Sperm morphology assessed by strict criteria (Kruger classification) [8] has been shown to have a high predictive value for the outcome of assisted reproductive technologies and intrauterine insemination [6, 7, 10]. The World Health Organization [18] has recommended that strict criteria should be applied when assessing the sperm morphology. A large inter- and intralaboratory variability still exists in the assessment of sperm morphology by strict criteria. This variability is a result of various factors, including different semen and sperm preparation techniques and differences in interpretation and technician experience [4]. Whether or not to stain the smears is another point of confusion. Alternatively, human sperm morphology can be observed using a wet preparation examined under phase-contrast microscopy by immobilizing the sperm on the slide. Such methodology has the advantages of being rapid to perform, minimizing preparative artifacts, and not requiring the use of fixative/stains. Lack of standardization of sperm morphology assessment remains the main reason for the debatable usefulness of this parameter in the laboratory evaluation of semen.

This prospective study was designed to compare wet preparation versus one stained with modified Diff-Quik for sperm morphology according to the strict criteria in our andrology laboratory.

MATERIALS AND METHODS

A total of 100 unselected semen samples from infertile couples undergoing evaluation for infertility were examined. Semen samples were obtained by masturbation after 2–5 days of sexual abstinence. Semen analysis was performed no sooner than 30 min and no later than 60 min after collection. Following liquefaction, sperm concentration was counted using the Makler Chamber (Sefi Medical, Israel). Sperm motility was assessed in a drop of semen on a covered slide at ×400–600 magnification.

For the wet preparation [18], 10 μL of semen is placed onto a clean glass slide and covered with a clean coverslip. Wet preparation evaluation was performed immediately with the aid of a phase-contract microscope at ×200–600 magnification.

For modified Diff-Quik staining [17], smears were prepared for morphological evaluation using slides precleaned with 70% ethanol. A 5-μL aliquot of semen was placed on each slide, which was air-dried at 37°C in a warm tray. Then, slides were stained with modified Diff-Quik [9]. Slides were fixed with 96% ethanol and stained with hematoxylin for nuclear morphology, then thiazine dye mixture (azure and methylene blue 1:1).

Slides were placed vertically to drain excess water and to allow them to air-dry. At least 100 cells were assessed in several randomly selected fields per slide with bright field illumination and ×100 oil immersion objective. Morphological abnormalities were classified into 3 main categories: defects of the sperm head, the midpiece, and the tail. Wet and stained slides were numerically coded and manually read in a blind fashion by

2 independent technicians using strict criteria (intralaboratory blind assessment). Both of the 2 examiners have more than 5 years experience in our andrology laboratory.

Statistical Analysis

Data are presented as means ± standard deviation. Statistical comparisons between group means were performed using a paired t test. Simple linear regression analysis was used to estimate the degree of correlation between the two preparation methods. All calculations were carried out on personal computers using SPSS for Windows 9.0 (Chicago, IL).

RESULTS

Sperm parameters are summarized in Table 1. Figures 1–4 and Table 2 summarize comparisons between the 2 preparation methods. The mean percentages (± SD) of morphologically normal spermatozoa were similar on wet and stained slides (4.8 ± 2.2 and 4.6 ± 2.5%, respectively) ($p > .05$). The mean percentages of sperm head defects were significantly different in wet (67 ± 5.3) and stained (71 ± 5.1) preparation groups ($p > .000$). The wet preparation group (16.4 ± 2.9) had significantly more midpiece abnormalities than the stained group (14.2 ± 3.7) ($p < .000$). The mean percentage of sperm tail defects was similar in wet (10.5 ± 3.6) and stained (9.8 ± 3.6) groups (p: .09).

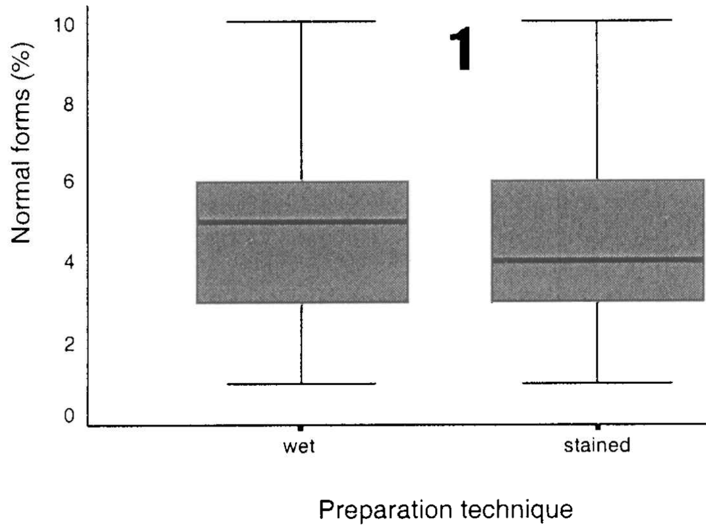
The degree of correlation between the preparation methods was analyzed by simple linear regression (Table 3). For the morphologically normal spermatozoa, there was a very strong agreement between the results obtained by wet preparation and stained samples according to the strict criteria (r: .83). However, with respect to the precentage of sperm head and midpiece defects according to the strict criteria a relatively low level of agreement was obtained between wet preparation and stained samples (r values of .25 and .25, respectively). The 2 methods were found to be better correlated in assessing spermatozoa with tail defects the spermatozoa with head and midpiece defects (r: .28).

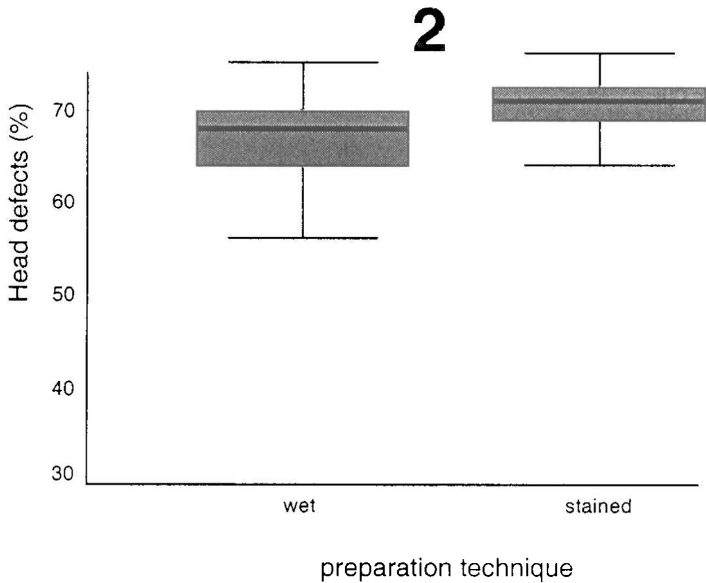
DISCUSSION

In contrast to the sperm motility, the analysis of sperm morphology still remains a technical and interpretative challenge. According to the WHO manual for andrology laboratories [18], the spectrum of recommended techniques for preparing morphology specimens has been expanded to 5 preparations (Papanicolaou, Giemsa, Shorr,

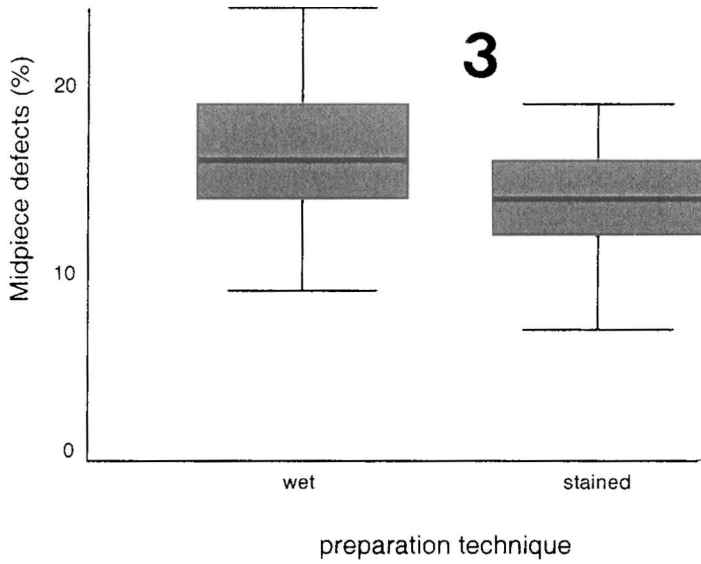
Table 1. Semen characteristics of the study group (n: 100)

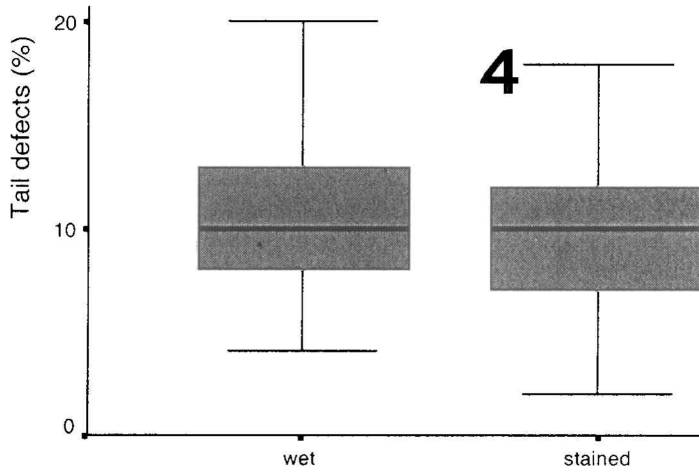
	Means ± SD	Range
Volume (mL)	2.87 ± 1.02	1–10
Sperm concentration ($\times 10^6$)	52 ± 3.9	3–220
Motility	29 ± 11.9	5–66
Morphology (stained)(%)		
| < 4%	37	
4–14%	62	





Figures 1–4. The box-and-whisker plots show the group means of the median (bold line in box), the 25th and 75th percentiles (bottom and top line of box), and the 10th and 90th percentiles (bar on lower and upper whiskers).





Figures 1–4. (*Continued*).

Table 2. Comparisons of wet preparation and modified Diff-Quik staining for assessing sperm morphology using the strict criteria

	Wet	Stained	*p*
Normal forms	4.8 ± 2.2	4.6 ± 2.5	.19
Head defects	67 ± 5.3	71 ± 5.1	.000
Midpiece defects	16.4 ± 2.9	14.2 ± 3.7	.000
Tail defects	10.5 ± 3.6	9.8 ± 3.6	.09

Note. Values are means ± SD.

Bryan-Leishman, and unstained "wet"). The two more widely utilized stains are the classic Papanicolaou and the more recently introduced Diff-Quik method [5]. Meschede et al. [12] compared Papanicolaou stain, Shorr stain, and the wet preparation protocols by the WHO criteria for sperm morphology assessment. They found that mean percentages of morphologically normal spermatozoa were identical in the stained methods, but were significantly lower in wet preparations. Ombelet et al. [15] designed a questionnaire to assess different methodologies of sperm morphology and found that 21% of the laboratories used wet preparation, while 22.9% stained the smears with Diff-Quik and 40.1% of them used strict criteria for the evaluation of sperm morphology.

Kruger et al. [9] found that the Diff-Quik staining method provided similar results with the Papanicolaou method in terms of the morphologically normal spermatozoa percentage. Even when the liquefied sample, Diff-Quik staining, and the strict criteria are used, there are still differences in sperm morphology assessments between laboratories [14, 16].

Wet preparation techniques were introduced into sperm morphology assessment with the declared intention of avoiding morphological artifacts induced by fixation and staining [18]. However, wet unstained preparations have some pitfalls: lack of experience in using these techniques; the slides cannot be stored for reevaluation and quality control; and the necessity of high-quality phase-contrast microscopes. Results of our andrology laboratory have demonstrated a very strong agreement for morphology analysis between wet and stained samples in terms of percentage of normal forms. However, the level of agreement was lower when the two methods were compared for the percentage of sperm with head, midpiece, and tail defects. The degree of correlation (*r*: .83) between stained and wet preparations with respect to the percentages of normal forms appeared to be acceptable. Concerning the percentage of spermatozoa with head, midpiece, and tail defects, the

Table 3. Correlation by simple linear regression analysis between wet preparation and modified Diff-Quik staining for assessing sperm morphology using the strict criteria

Wet-stained	Correlation Coefficient (*r*)	*p*
Normal forms	.83	.000
Head defects	.25	.01
Midpiece defects	.25	.01
Tail defects	.28	.004

impact of preparation methods was more pronounced and correlation between the two methods was so poor as to be considered irrelevant for routine practice. Ali and Grimes [1] evaluated sperm morphology with unstained specimens and following Papanicolaou staining and they demonstrated a significant difference in the percentage of morphologically normal spermatozoa in stained and unstained semen smears. A very strong correlation for morphology assessment has been reported using Papanicolaou and Diff-Quik stains in both unwashed and washed samples [11]. Norfolk laboratory has demonstrated a very good correlation between manual analysis of liquefied and washed samples with Diff-Quik staining [2].

We have concluded that wet preparation is a fast, simple, and reliable method *only* for the morphologically normal spermatozoa. For determining the percentage of the defective spermatozoa, staining of the smears are recommended.

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