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Original Article

Pathology Section

Comparison of Special Stains for Analysing the Morphology of Sperm: A Cross-sectional Study

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ABSTRACT

Introduction: In India, male infertility constitutes 40% of infertility cases in males, which has become a serious problem in developing countries. Numerous studies and advanced techniques have been identified in assessing sperm morphology. The detection of sperm morphology is a simple and widely accepted method for determining sperm viability. It assists in selecting appropriate treatments for male infertility in assisted reproductive techniques. Infertility issues can arise in healthy males due to altered sperm morphology.

Aim: To analyse the morphology of sperm using basic histopathology stains such as Haematoxylin and Eosin (H&E), Giemsa (G), Eosin-Nigrosin (EN), and Papanicolaou (Pap) stains.

Materials and Methods: A cross-sectional study was conducted from April 2022 to June 2022 at the Department of Clinical Pathology, Sree Balaji Medical College and Hospital, Chennai, Tamil Nadu, India. Thirty semen samples were collected from healthy males and stained with H&E, Giemsa, Eosin-Nigrosin, and Papanicolaou stains. The results were observed and tabulated based on sperm shape, size, and motility using Kruger's strict morphology method. A comparison was made to identify the fastest and most cost-effective method among the four stains. Descriptive statistics were used for the comparison.

Results: Among the 30 semen samples, H&E and Papanicolaou stain methods were found to be rapid and cost-effective for analysing sperm morphology compared to the other stains (p-value <0.001). The findings included cases of Normozoospermia (18), Oligozoospermia (9), Necrozoospermia (1), and Teratozoospermia (2). In the Papanicolaou stained samples, the mean sperm head length (µm) was 8.92a±0.5, head width (µm) was 4.48a±0.33, head perimeter (µm) was 27.69a±1.85, head area (µm²) was 33.96a±3.74, tail length (µm) was 44.31a±2.02, sperm length (µm) was 53.24a±2.18, with no cytoplasmic droplets greater than half the size of the head and two vacuoles observed. Papanicolaou stain was the least expensive stain (9.50rs) compared to the other stains used for assessing sperm morphology.

Conclusion: Papanicolaou stain is a simple and cost-effective method for analysing sperm morphology compared to other special stains. However, EN Stain is used to detect the morphology of live sperm. Sperm morphology assessment is necessary to monitor the causes of male infertility and determine appropriate treatment modalities.

Keywords: Semen sample, Sperm analysis, Sperm viability

INTRODUCTION

In India, male infertility affects 40% of males [1,2]. The problem of infertility arises due to many medical conditions like diabetes, hypertension, thyroid disorder, coronary heart disease, etc. Semen analysis is an important technique in determining both the qualitative and quantitative analysis of semen. Sperm morphometric analysis is important for identifying abnormal sperm and determining the percentage of normal sperm. Studying the analysis of sperm morphology is a crucial step in evaluating the qualitative and quantitative aspects of a semen sample. Special stains play a significant role in visualising sperm morphology, aiding in the identification of abnormal and normal sperm percentages. Sperm morphometric analysis is crucial in both in-vitro and in-vivo male fertilisation treatments [1].

The determination of sperm morphology through a smear depends on smear preparation, fixation, and staining procedures, which can affect the reported sperm morphology. Several semen staining procedures have been identified for detecting sperm morphology using basic histopathology stains [2]. In some special stains, changes may be noted during the measurement, count, and morphology of sperm due to the processing of the staining procedure, which can cause shrinkage of the sperm [3]. Therefore, it is important to develop rapid, simple, and inexpensive special staining methods that do not alter sperm morphology. In the present study, authors utilised H&E, Giemsa, Eosin-Nigrosin, and Papanicolaou stains to identify cost-effective, reliable, and rapid methods for assessing sperm morphology among histopathology stains. The results were analysed to determine the ideal staining procedure (in comparison with H&E) based on Kruger's strict morphology assessment criteria, cost of stains, and staining duration in sperm morphology analysis.

The objectives of the present study were to assess the morphology of sperm using histopathology special stains and to identify a faster and easier staining method for detecting sperm morphology.

MATERIALS AND METHODS

A cross-sectional study was conducted from April 2022 to June 2022 in the Department of Clinical Pathology at Sree Balaji Medical College and Hospital, Chennai, Tamil Nadu, India. Thirty semen samples were collected from healthy males after a physical examination and with their informed consent (IEC NO Ref.No.002/SBMCH/IHEC/2022/1612).

Inclusion criteria: Semen samples were collected from individuals in the reproductive age group (less than 50 years).

Exclusion criteria:

- Semen samples were not collected from individuals who did not follow the recommended sexual abstinence period (3-8 days).
- Individuals with a history of diabetes, hypertension, coronary heart disease, and thyroid disorder were excluded.

Study Procedure

Four smears were prepared from each semen sample and stained with H&E, Pap, Giemsa, and Eosin-Nigrosin stains. Among these, H&E staining was considered the gold standard technique. The results were analysed for all four special stains, and two pathologists observed and tabulated the results using Kruger's strict morphology method [Table/Fig-1] [4]. Sperm morphology was identified and graded as follows: A-Very clear visibility, B-Good visibility, C-Fairly seen, and D-Not clearly seen sperms.

Morphology	Krugers's strict criteria
Head	Smooth and perfect oval 4-6 µm×2.5-3.5 µm well defined acrosome (40-70% of sperm head)
Midpiece	No midpiece defects Slender, regular Width <1 µm, length 1.5x head size
Tail	No tail defects Uniform, 10x head length, 45 µm long
Cytoplasmic droplets	No cytoplasmic droplets >1/2 size of the head
Vacuoles	Upto 4

[Table/Fig-1]: Kruger's strict criteria.

STATISTICAL ANALYSIS

The data were analysed using Statistica 10 PL (StatSoft, USA). All results are expressed as mean±Standard Deviation (±SD). The significance of the differences between the groups was assessed using Tukey's test at $p \leq 0.05$.

RESULTS

Semen samples were collected from 30 healthy men in the age group of 18-50 years. The samples were obtained from the clinical pathology lab at the hospital after a physical examination and informed consent from the patients. The collected semen was allowed to undergo liquefaction for 30 minutes to one hour at room temperature. The pathologist observed the sperm morphology and made comments, noting cases of Normozoospermia-18, Oligozoospermia-9, Necrozoospermia-1, and Teratozoospermia-2 [Table/Fig-2].

Sperm morphology	Number of patients (N=30)	Percentage
Normozoospeermia	18	44.97%
Oligozoospermia	9	28.12%
Necrozoospermia	1	9.09%
Teratozoospermia	2	16.25%

[Table/Fig-2]: Sperm morphometric analysis of the 30 semen samples.

The sperm morphology was observed by two pathologists in all four smears. The observers were requested to score the sperm morphology according to Kruger's strict criteria and visualisation of morphology of the sperm under a microscope [Table/Fig-3]. The reports were compared, and statistical analysis was conducted. The interobserver variability and interobserver reliability between the two pathologists were calculated to be 0.009 (based on SEM) [Table/Fig-4]. According to the pathologists' opinion in H&E, the sperm morphology and parts of the sperm were visualised very clearly in the haematoxylin and Pap stains compared to the other two stains, Giemsa and Eosin-Nigrosin. The cost and staining duration were noted for each stain [Table/Fig-5].

The head, body, and tail morphology of the sperm were visualised in the H&E stain under the microscope, providing details on the identification of morphological structure and aiding in the evaluation of abnormal sperm [Table/Fig-6]. The smear stained with H&E stain showed clear sperm morphology of the head, body, and tail with an assessment score of 85% at 100× magnification. The sperm stained with May-Grunwald Giemsa showed a blue-purple colour, with prominent visualisation of the head morphology and acrosomal condensation, while the body and tail parts were unclearly seen [Table/Fig-7]. The smear stained Giemsa stain (G stain) showed clear sperm morphology of the head, body, and tail with an assessment score of 75% at 100× magnification. EN stain helped in differentiating between dead and live sperm and stained the sperm with different colours. Viable sperm appeared white due to an intact cell membrane that did not pick up the stain colour, while dead sperm were stained pink [Table/Fig-8]. The smear stained with EN stain showed clear sperm morphology of the head, body, and tail with an assessment score of 70% at 100× magnification. Pap stain prominently visualised the head morphology with acrosomal condensation under the microscope. The body and tail morphology were also prominently seen, aiding in the identification of immature germ cells [Table/Fig-9]. The smear stained with Pap stain showed clear sperm morphology of the head, body, and tail with an assessment score of 75% at 100× magnification.

DISCUSSION

Morphometric analysis of sperm is an important step in determining male infertility. Medical conditions such as diabetes, which decrease spermatogenesis and testosterone levels, can also decrease semen volume in healthy males. Patients with hypertension and coronary heart disease may experience hormonal imbalances associated with these conditions, resulting in decreased semen volume, sperm motility, sperm count, and increased immotile sperm count. Thyroid

Items	H&E	Giemsa stain	Eosin nigrosin stain	Pap stain
Head length (µm)	8.92^{a}±0.5	6.92^{a}±0.5	7.31^{b}±0.47	9.31^{b}±0.47
Head width (µm)	4.48^{a}±0.33	5.48^{a}±0.33	4.81^{b}±0.3	4.81^{b}±0.3
Head perimeter (µm)	27.69^{a}±1.85	17.69^{a}±1.85	18.73^{b}±1.74	28.73^{b}±1.74
Head area (μm^2)	33.96^{a}±3.74	28.96^{a}±3.74	27.43^{b}±3.74	37.43^{b}±3.74
Tail length (µm)	44.31^{a}±2.02	40.31^{a}±2.02	43.17^{b}±1.66	45.17^{b}±1.66
Sperm length (µm)	53.24^{a}±2.18	50.24^{a}±2.18	48.46^{b}±1.87	54.46^{b}±1.87
Cytoplasmic droplets	No cytoplasmic droplets >1/2 size of the head	No cytoplasmic droplets >1/2 size of the head	No cytoplasmic droplets >1/2 size of the head	No cytoplasmic droplets >1/2 size of the head
Vacuoles	2	0	0	2
Mid piece	No midpiece defect	No midpiece defect	No midpiece defect	No midpiece defect

[Table/Fig-3]: Sperm morphometric parameters by staining method (means±SD) according to Kruger's strict criteria.
a,b differences between average values, represented by different letters in the same row, are significant ($p \leq 0.05$)

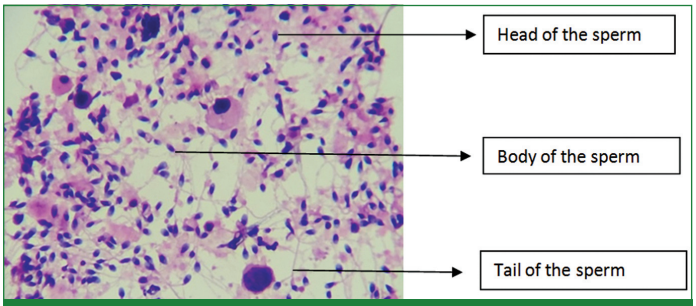
	Head				Acrosome				Middle piece				Tail			
Stain used	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Report 1 (pathologist 1)																
Haematoxylin and Eosin	20	8	1	1	20	8	2	0	19	9	1	1	20	8	1	1
Giemsa	19	7	2	2	18	8	2	2	18	7	3	2	18	8	3	1

Eosin-Nigrosin	17	5	4	4	5	4	11	10	5	10	15	0	5	9	15	1
Rapid Papanicolau	18	5	5	2	17	4	6	3	17	5	5	3	17	5	5	2
Report 2 (pathologist 2)																
Haematoxylin and Eosin	20	7	2	1	20	7	1	1	19	9	2	1	20	7	2	1
Giemsa	18	5	2	2	18	8	2	2	19	7	3	1	18	9	2	2
Eosin-Nigrosin	16	6	3	4	5	4	11	9	6	10	15	0	5	9	15	1
Rapid Papanicolau	19	7	6	2	17	4	6	4	16	5	5	3	17	5	5	2

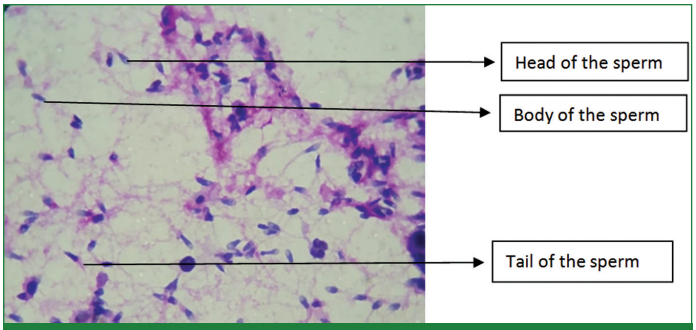
[Table/Fig-4]: Tabulated results of 30 men's semen samples by two pathologists by Kruger's strict scoring.
A- Very clear visibility; B- Good visibility; C- Fairly seen; D- Not clearly seen; Interobserver variability between two pathologist was 0.009. (calculated based on SEM , value indicate no to slight observer variability); Interobserver reliability between two pathologist was 0.009 (calculated based on SEM, value indicate no to slight observer variability)

S. No.	Stains	Cost of the stain	Staining duration
1	H&E	Rs 10 (20 drops)	45 min
2	Giemsa	Rs 25 (20 drops))	1 hr 10 min
3	Eosin nigrosin	Rs 27 (20 drops)	1 hr 5 min
4	Pap stain	Rs 9.50 (20 drops)	40 min

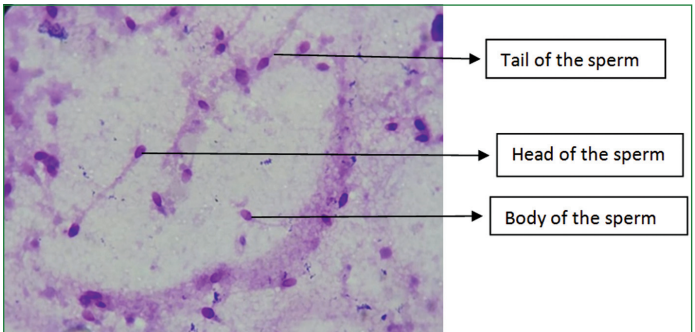
[Table/Fig-5]: Comparison of special stains according to cost and time duration of stain.



[Table/Fig-6]: Smear stained with H&E stain shows clear sperm morphology- head, body and tail with assessment score of 85% in 100X.



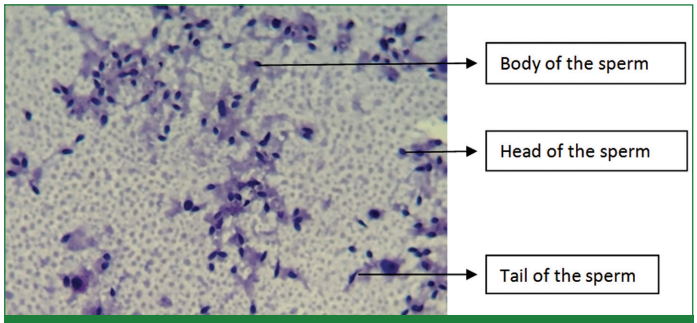
[Table/Fig-7]: Smear stained with Giemsa stain shows clear sperm morphology- head, body and tail with assessment score of 75% in 100X.



[Table/Fig-8]: Smear stained with Eosin-nigrosin stain shows clear sperm morphology- head, body and tail with assessment score of 70% in 100X.

disorders can also lead to hormonal imbalances and decreased semen volume, semen density, and sperm motility. Morphometric analysis plays a crucial role in increasing the success rate of fertility treatments. It is important in early embryonic development and in selecting IVF techniques, as it helps identify normal sperm and their motility capacity [5-7].

Pap stain is suitable for automated sperm morphology analysis, but it involves a time-consuming process with more than 15 steps and



[Table/Fig-9]: Smear stained with Pap stain shows clear sperm morphology- head, body and tail with assessment score of 75% in 100X.

10 chemical solutions. The dehydration process during staining can cause cell shrinkage [8].

A faster method for determining sperm morphology and chromatin status is the assessment done by Diff-Quick stain and G Stain procedures. These staining methods involve only two staining solutions, which can result in sperm swelling and colouring of the background areas of the sperm. Therefore, G Stain is recommended by the WHO for sperm analysis [9]. EN stain is used to detect "live-dead" sperm and assess sperm morphology. It stains the head and body of the sperm, while lightly staining the tail [10]. Berman NG et al., conducted a study highlighting the importance of assessing sperm morphology in artificial fertility treatment [9]. Although there is now a special grading system and emphasis on morphometric analysis, many small hospitals and labs still rely on manual methods for identifying sperm morphology. Guzick DS et al., emphasised the significance of using special stains for accurate analysis of sperm morphology in In-vitro Fertilisatiin (IVF) treatment and advanced technologies [11,12]. Sperm morphology is classified according to the Tygerberg Classification by Kruger and the World Health Organisation (WHO) semen analysis criteria. According to Kruger, normal sperm should not show any abnormalities. In normal sperm, the head has smooth or oval boundaries, with 60-70% acrosomal part, a length of 3-5 µm, and a width of 2.3-3.8 µm. The tail is 40-45 µm in length and is thin, flat, and not wrinkled. During the staining process, a smear may result in minimal alteration in sperm morphology. Pap stain is considered a good stain for sperm morphometric analysis, as demonstrated by a study conducted by Aksoy E et al., which evaluated both the WHO and Kruger's strict scoring system [5,13].

In a study by Garcia-Herreros M et al., it was concluded that H&E stain is the best method for evaluating sperm heads, acrosome condensation, and body and tail morphology, which aligns with our study [11]. Titford M and Victor B highlighted in their study on histological techniques that G stain is considered the best stain for evaluating sperm morphology and expecting favourable results in IVF techniques. In the present study, G stain showed clear sperm head morphology, while the body and tail were faintly stained, which is consistent with other studies [14,15]. EN stain identifies dead sperm, allowing for the calculation of percentages of oligozoospermia and necrozoospermia. Pink-coloured sperm is considered non viable due to damaged cell membranes, while live sperms appear white due to intact cell membranes [15,16]. This is very useful in selecting IVF treatment [17,18].

Items	Our study report with pap stain	Brito LFC et al., Greene LM et al., study [19,20]	Akshoy E et al., [5]	Ahmad MO et al., [21]
Head length (µm)	8.92[a]±0.5	5.62[a]±0.5	6.92[a]±0.5	5.02[a]±0.5
Head width (µm)	4.48[a]±0.33	4.99[a]±0.33	5.48[a]±0.33	4.23[a]±0.33
Head perimeter (µm)	27.69[a]±1.85	16.59[a]±1.85	17.69[a]±1.85	15.89[a]±1.85
Head area (µm^2)	33.96[a]±3.74	29.16[a]±3.74	28.96[a]±3.74	28.16[a]±3.74
Tail length (µm)	44.31[a]±2.02	39.76[a]±2.02	40.31[a]±2.02	37.76[a]±2.02
Sperm length (µm)	53.24[a]±2.18	49.24[a]±1.18	50.24[a]±2.18	48.24[a]±1.18
Cytoplasmic droplets	No cytoplasmic droplets >1/2 size of the head	No cytoplasmic droplets >1/2 size of the head	No cytoplasmic droplets >1/2 size of the head	No cytoplasmic droplets >1/2 size of the head
Vacuoles	2	0	0	0
General opinion	Very good	Good	Good	Good
Duration for completing the staining procedure	40 minutes	1 hour 30 minutes	1 hour 10 minutes	1 hour 30 minutes

[Table/Fig-10]: Comparision of our pap stains result of present study with previous studies [5,19-21].

Pap stain was considered a good stain for morphometric analysis, but it has the drawback of increased time consumption, as mentioned in studies by Greene LM et al., and Brito LFC et al., In the present study, authors used rapid Pap stain, which identifies nuclear and cytoplasmic stains while reducing time consumption [19,20]. The time factor and staining quality in our study were consistent with the study by Aksoy E et al., morphometric analysis yielded good results with very clear visualisation of the head, acrosome, body, and tail under the microscope in smears stained with rapid Pap stain [Table/Fig-10] [5,19-21].

Limitation(s)

The study duration was limited to three months, and the sample size was small.

CONCLUSION(S)

Papanicolaou stain was found to be a simple and cost-effective stain for analysing sperm morphology compared to other special stains. G Stain reduced the time duration for the staining procedure, while Eosin-Nigrosin stain was effective in detecting the morphology of live sperm. H&E stain was considered the gold standard stain in the present study. Sperm morphology assessment is necessary for monitoring the causes of infertility in males and identifying appropriate treatment modalities for male infertility.

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