UNIVERSITI PUTRA MALAYSIA

COMPARISON OF DIFFERENT CYTOLOGIC STAINING TECHNIQUES ON BOER GOAT SPERMATOZOA MORPHOLOGY AND MORPHOMETRY

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COMPARISON OF DIFFERENT CYTOLOGIC STAINING TECHNIQUES ON BOER GOAT SPERMATOZOA MORPHOLOGY AND MORPHOMETRY

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Comparison of Different Cytologic Staining Techniques on Boer Goat Spermatozoa Morphology and Morphometry", by Suliza Bt Abd Wahab and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project

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DEDICATIONS

Praised to Allah S.W.T., I have completed this project and this project paper is dedicated to

My family,

Father

Mother

Brothers

Friends

And to all my teachers who have committed themselves towards the noble cause of education.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
^{0}C	Celcius
CASA	Computer-Assisted Sperm Analysis
cm	centimeter
DPX	dibutyl phathalate xylene
DQ	Diff-Quik
EN	Eosin-Nigrosin
et al.	and others
fig.	figure
g	gram
HE	Hematoxylin-eosin
hr	hour
mg	milligram
min	minute
ml	milliliter
µl	microliter
NS	normal saline
Obj.	objective
RD	Rapidiff
s	seconds

SE	standard error
SPB	Sorenson Phosphate Buffer
temp	temperature
µm	micrometer
kV	kilovolt
wt	weight

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 - Project

COMPARISON OF DIFFERENT CYTOLOGIC STAINING TECHNIQUES ON BOER GOAT SPERMATOZOA MORPHOLOGY AND MORPHOMETRY

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2016

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Sperm morphology is the most reliable parameter for predicting fertility whereas sperm morphometry value as an indicator of reproductive capacity in males. Visual observation has led to widely varying results due to numerous factors such as the use of different staining procedures and lack of standardization of staining techniques. Thus, this study was conducted to compare different cytology staining techniques and to identify the best staining methods for better semen evaluation. Seven semen samples were collected

using a standardized electro ejaculator from Boer bucks at TPU UPM (n = 4), and Labu farms (n = 3). Each sample was primarily assessed using routine semen evaluation protocols and processed accordingly. The semen smears were stained using different stains namely Eosin-Nigrosin (EN), Giemsa (G), Diff-Quik (DQ) and Hematoxylin-Eosin (HE). The slides were examined and morphometric measurements of 50 randomly selected sperm in each stained slides were performed and compared. Morphometric analysis revealed the smallest values recorded in HE for measurements of the head. Results showed there are significant difference ($p<0.05$) of all the morphometric parameters between EN and HE and; DQ and HE. Higher values of head width and length recorded in all stains except HE. It was concluded that there were alteration of measurement in buck sperm morphology with different staining method used.

Keywords: spermatozoa, staining, morphology, morphometry, Boer, buck

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

PERBANDINGAN TEKNIK PEWARNAAN SITOLOGI YANG BERBEZA KE ATAS MORFOLOGI DAN MORFOMETRI SPERMATOZOA KAMBING BOER

Oleh

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Morfologi sperma adalah parameter yang dipercayai bagi meramalkan kesuburan, manakala nilai morformetri sperma digunakan sebagai penunjuk kapasiti pembiakan haiwan jantan. Pemerhatian secara visual secara umumnya telah membawa kepada keputusan yang berbeza-beza kerana pelbagai faktor seperti penggunaan prosedur pewarnaan yang berlainan dan kekurangan penyeragaman teknik pewarnaan. Oleh itu, kajian ini dijalankan untuk membandingkan teknik pewarnaan sitologi yang berbeza dan untuk mengenal pasti kaedah pewarnaan yang terbaik bagi penilaian air mani yang lebih baik. Tujuh sampel air mani dikumpul menggunakan elektro ejakulator daripada kambing jantan Boer di TPU, UPM (n = 4), dan ladang Labu (n = 3). Umumnya, setiap

sampel dinilai menggunakan protokol rutin penilaian air mani dan seterusnya diproses. Smear air mani nipis disediakan dan diwarnakan menggunakan teknik pewarnaan yang berbeza yang melibatkan; Eosin-Nigrosin (EN), Giemsa (G), Diff-Quik (DQ) dan Hematoksilin-eosin (HE). Slaid diperiksa dan ukuran morfometri dijalankan ke atas 50 sperma yang dipilih secara rawak dalam setiap slaid berwarna dan dibandingkan. Analisis morfometri menunjukkan nilai terendah direkodkan pada HE bagi ukuran kepala. Hasil kajian menunjukkan terdapat perbezaan yang signifikan ($p < 0.05$) bagi semua parameter morfometri antara EN dan HE dan; DQ dan HE. Nilai lebih tinggi bagi lebar kepala dan panjang kepala telah dicatatkan pada semua pewarnaan kecuali HE. Kesimpulannya, terdapat perubahan dalam ukuran dalam morfologi sperma kambing dengan kaedah pewarnaan yang berbeza digunakan.

Kata Kunci: spermatozoa, pewarnaan, morfologi, morfometri, Boer, kambing jantan

1.0 Introduction

1.1 Semen Evaluation and Analysis

When choosing a male for breeding, it is important to assess its potential fertility by undertaking clinical and laboratory examinations. Reproduction of the male is closely related to semen quality and normal sperm structure. Therefore, semen analysis is crucial and it comprises of multi-staged process. The semen analysis routinely includes an immediate assessment of volume, appearance sperm concentration and motility, as well as later determination of sperm morphology and the presence of foreign cells. Once screened for normality, ejaculates are assessed for sperm concentration and sperm motility. The morphological evaluation, which is increasingly expanded to include detailed morphometry of the spermatozoon and is considered as a useful tool in the clinical diagnosis of sub fertile animals. Motility assessment can easily be evaluated with a computer-assisted sperm analysis (CASA) instrument. However, the CASA instruments are not in widespread commercial use due to its need for proper programming with species-specific settings, and also the validation requirement.

According to Villaverde *et al*. (2008), the goal for sperm staining technique is to ease the visualization of the cells and provide a better identification of the abnormalities through light microscopy. In the laboratory practice, there are varieties of slide-staining methods used during morphological evaluation of semen to predict male fertility. However, the common encountered problem during evaluation of the morphology and morphometry of sperm is the lack of standardization of staining techniques. Comparison among techniques is essential in order to identify the most suitable for the species function, because sperms are highly specialized cells operating

at a microscopic level in a complex environment (Gage, 1998). Therefore, the method of staining and evaluating semen can significantly affect the results of morphometric measurements.

1.2 Importance of Semen Quality Assessment

Fertility of sperm is the ultimate test of sperm quality. Even though fertility cannot be accurately predicted, the deviation of the sperm morphology from the norm is usually a good predictor of low fertility. According to Nuti (2013), the objective of semen quality evaluation is to identify those samples that have traits pointing to the probability of below normal fertility and therefore discard them, as an extension is to remove the males that continuously produce poor quality semen.

Thus, this study is conducted to compare the simple and practical staining techniques, which only using simple equipment, such as a bright field microscope. The main objectives of this study are:-

1. To compare different cytology staining methods on Boer goat spermatozoa morphology and morphometry.
2. To identify the best staining techniques for better semen evaluation.

The hypotheses of this study are different types of stain produces different dimension of goat sperms head and also the morphology and morphometry of the Boer goat spermatozoa.

2.0 Literature Review

2.1 Staining

Staining is technique used in microscopy to enhance contrast in the microscopic image. Different stains have different affinities for different organisms, or different parts of organisms. They are used to differentiate different types of organisms or to view specific parts of organism. Staining is an auxiliary technique used in microscopy. There are many staining methods to evaluate the morphology of spermatozoa (Uven *et al.*, 1998). According to Coetzee *et al*. (1997), the search for a staining technique which is both fast and easy and stains spermatozoa differentially for morphology analysis has been an on-going process. Several staining procedures have been used to assess sperm morphology in humans and animals (Coetzee *et al*., 1998).

Assessment of sperm morphology can be influenced by many factors such as fixation and staining technique like Diff-Quik or Eosin-Nigrosin, sperm preparation procedures, quality of the microscope and also the examiner's skills. Haematoxylin and eosin stain is probably the most widely used in histological and cytological stain according to Steven and Wilson (1996). A number of studies of sperm staining procedures used to assess sperm morphology for several animal species have reported that the same fixatives and stains have different reactions with the sperm of individual species (Van der Horst & Maree, 2010). Therefore, it is important to find the suitable staining technique for each species.

2.2 Sperm Morphology

Sperm morphology is the science of normal anatomical structure of spermatozoon. Morphology is one of the factors influencing the quality of sperm (Morrell & Rodriguez- Martinez, 2009). According to Kuster *et al*. (2004) morphology of spermatozoa is an important for a successful fertilization and early embryonic development in assisted reproductive techniques. Morphological abnormalities of sperm can have a detrimental impact upon fertilization and embryonic development (Saacke, 2008). The importance of normal sperm morphology in bulls, goats, stallions and men is well known. Morphologically, the sperm is divided in to three main parts- head, middle piece and tail. The head is capped by an acrosome which should ideally occupy 40-70% of the total head area. The acrosome contains enzymes required for penetration of the oocyte and plays a vital role during fertilization process. The midpiece is packed with mitochondria which provide the energy required for movement of the sperm. Poor semen morphology is also an important indicator of decreased fertility as reported in few study related to goats and few other species (Chandler *et al.,* 1988).

The male factor is considered a major influential factor to infertility. Sperm morphology has become an area of great interest to assess of male infertility, since observation of normal and abnormal morphological sperm forms in semen samples. Evaluation of sperm morphology displays a potential impact on male fertility and has been recognized to be the best predictor of outcome of natural fertilization, as well as artificial insemination.

2.3 Sperm Morphometry

Morphometric sperm traits, including the sperm length and midpiece volume are known to be influenced by sperm competition in a variety of taxa (Immler *et al*., 2007). Accurate assessment of sperm morphology depends on meticulous preparation, fixation, and staining of sperm cells, as this affects the morphometry of the sperm head and of the entire cell (Menkveld, 2007). The method used should interfere as little as possible with stained cells (Maree *et al*., 2010) and clearly show the boundaries of the sperm head and the remaining elements of the sperm cell. The relationship between sperm function and morphometry includes the size of the head, midpiece, and flagellum/tail, is empirically supported across different species (Immler *et al*. 2010). In bulls, the use of sperm head morphometric parameters has been considered a good indicator of semen quality (Phillips *et al.*, 2004), and it is recommended as a part of the spermiogram for domestic animals (Rodríguez- Martínez, 2007).

2.4 Boer goat

Boer goat (*Capra hircus*) is a famous meat type breed well known for its rapid growth, excellent meat quality and high fertility (Greyling, 2000; Malan, 2000). Over the past decades, Boer goats have gained worldwide recognition for excellent body conformation, fast growing rate and good carcass quality. Their desirable genetic trait for meat production of Boer goats had causes it to successfully improved productive performance of indigenous breeds through cross breeding (Lu, 2001). Cross breeding with Boer goats increased birth weight, growth rate, and mature weight in cashmere goats (Newman and Paterson, 1997).

3.0 Material and Methods

3.1 Semen collection in animals

This study used 7 adult Boer cross goats, with proven fertility, from Tok Seri Buak Agrofarm, Labu, Negeri Sembilan and Taman Pertanian Universiti, Universiti Putra Malaysia. Semen was collected from the goats following electro stimulation using electro ejaculator that was standardized for small ruminants. Collection of semen was done by restraining each goat in a crush or manually restrained. A gel lubricated probe was inserted into the rectum, parallel to the bladder; pressure was applied to the front of the probe, so that the electrodes remained near the upper portion of the ampullary region. The electrical stimulation was applied for 4–8s. This cycle was repeated until 0.5–2 mL of semen was collected with approximately 3–4 electro stimulations using 0.5- 3kV. Collected semen was placed in a warm water bath (37°C) and evaluated immediately for consistency, wave motion (scale of 0–5), and percentage of motile spermatozoa.

3.2 Semen evaluation

The wave pattern was observed using 1 drop of net semen on the slide under the light microscope with 4x magnification. Later, net semen was diluted with normal saline (NS) in a dilution of 1:100. If examination of wave-pattern indicates the concentration is not dense, the dilution can be between 1:25 or 1:50 depending on discretion mix and place a drop of diluted semen on a glass slide and place a coverslip. Examine under objective 10x or 20x and estimate the percentage of general motility for motile sperm. Examine under objective 20x or 40x and estimate percentage of individual motility with forward/progressive, backward, rotating and vibrating movement.

After initial assessment, the diluted semen with normal saline (1:100) was again diluted in formal saline with the ration of 1:10, and sperm concentration was determined by using hemocytometer or CASA machine. The hemocytometer has often been referred to as the "gold standard" for assessing sperm numbers (Prathalingam *et al.* 2006). The equipment is slow and multiple measurements of each sample are needed to obtain a precise result (Prathalingam *et al.* 2006).

3.3 Preparation of smears

After collection, three semen smears were prepared with 5 to 20µl of semen was applied to the slide using micropipette. Slides were prepared from each sample by placing 5µl of semen on the clear end of a frosted slide using micropipette and dragging the drop across the slide. Semen smears were air dried overnight. At least two smears should be made from the fresh semen sample for duplicate assessment and in case of problems with staining.

3.4 Staining procedure

3.4.1 Eosin- Nigrosin staining technique

The water bath was warmed at 35°C and Eosin-Nigrosin stain was placed. On a glass slide, drop about 3-4 drops of Eosin-Nigrosin (EN) stain and a drop of net semen was mix and left for 3 minutes. By placing 8 µl droplet of semen and EN stain mixture at the end of the glass slide, the smears were drawn and air dried.

3.4.2 Giemsa's staining technique

The staining procedure was followed the protocol of Watson, 1975. 3 µl of Sorenson Phosphate Buffer (SPB) was put into clean frosted slide, on that 30 µl of fresh semen was mixed and a smear was drawn and air dried. The slides were put into 5% formaldehyde solution for fixing at 37° C for 30 min (Campbell *et al.*, 1960). The slides were removed from the solution, washed in running tap water and air dried for further processing. The working solution of Giemsa was prepared by mixing 3 ml of Giemsa's stock, 2 ml of SPB and 45 ml of distilled water in a measuring cylinder and then filtered. The solution was transferred into a coffin jar and warmed at 37° C for 30 min. The smeared slides of spermatozoa was put into the working solution and kept at 37° C for 5 hrs. The slides were removed from the stain and washed in running tap water and finally air dried. Examine under a microscope for acrosomal reaction.

3.4.3 Diff- Quik (DQ) or Rapidiff (RD) staining technique

The staining was carried out using a commercially available kit, KY Rapidiff. The DQ staining kit was obtained from University Veterinary Hospital, UPM. The slides were placed horizontally on a staining tray and covered with stain solution 1 for about 10s. Immediately afterwards, without washing or drying the slides, stain solution 2 was applied to each slides for 15s. Care was taken to spread the stain equally across the smear surface. After the stain was removed by gently running it off, the slides were slowly rinse using tap water for 3 s. Then the slides were left in an upright position to air-dry.

3.4.3 Hematoxylin- Eosin (HE) staining technique

Smear slides were fixed in ethanol for 20 minutes and washed with running tap water for 1-5 minutes and let to dry. Immerse the slides into hematoxylin for 30 minutes and then wash with running tap water for 1-5 minutes. Dip in 1% acid alcohol twice. The slides were immersed into eosin for 5-10 minutes. The slides were rinse using tap water for 1-5 minutes. The slides were left in an upright position to air-dry.

3.4.4 Mounting

Following completion of the staining procedures, the morphology slides were mounted using DPX mounting glue and a cover-slip.

3.5 Morphology and morphometric evaluation

By staining with EN techniques, 200 cells were counted through light microscopy (1000x) in randomly selected fields for each smear. Morphologic defects were classified according to the region of the sperm, as head, midpiece including droplets or tail defects. For the purpose of this study to compare each stains staining efficiency, only the head, midpiece and tail variables were included in the evaluations. Morphometric variables such sperm head length, head width; midpiece length and tail length were assessed and calculated. For morphometric evaluation, 50 sperm cells from random fields were captured through light microscopy connected to image analyzer software (analySIS LS Research FIVE, Olympus Soft Imaging Solutions versions: 5.0). Sperm structures such as head, mid-piece, and tail were separately measured. The measurement data then was recorded as: (i) the length of the head, ii) the width of the head, (iii) the length of the midpiece, and (iv) the length of the tail.

3.6 Statistical Analysis

Statistical analysis was done using SPSS version 20. Analysis of the normal distribution of data was examined with Shapiro-Wilk Test. In subsets of data that appeared to have non-parametric data distributions, the Kruskal–Wallis one way ANOVA test was employed and further elaborated for individual differences using the Mann–Whitney test for independent samples. Data are represented as the mean $\pm$ standard error (SE) in the tables and $P < 0.05$ was considered significant.

4.0 Results

4.1 Morphology Analysis

The present study has been undertaken for comparative assessment of morphology of sperms by using Eosin Nigrosin, Giemsa, Diff-Quik and, Hematoxylin Eosin stains (Fig. 1- 4). Under EN stain, dead sperm were stained pink; meanwhile, live sperm are not stained. The morphology of head can be seen clearly. The boundary of the midpiece and the remainder of the tail were difficult to distinguish; both parts were pink.

The spermatozoa were stained dark blue purple with Giemsa (Fig. 2a, 2b). The acrosome part stained rose-pink whereas the post- acrosomal was stained dark blue purple and continues to the midpiece and tail. Head morphology and condensation could be seen very well but the middle piece and the tail cannot be differentiated clearly.

The head is stained as a dark red colour homogeneously under DQ (Fig. 3a, 3b). The acrosome stained red and the post-acrosome area stained dark red. The midpiece part and tail can be evaluated as very clear. Therefore, all the morphological defects of spermatozoa could be easily evaluated.

Under HE staining, the buck sperm heads shows a basophilic colour (Fig. 4a, 4b). The acrosomal area and cytoplasmic fragments is stained slightly pink and the post- acrosomal area is stained dark purple to bluish. The acrosomal part was lighter, gradually becoming darker towards post-acrosomal. The midpiece and tail were stained eosinophilic, and the end of the tail was difficult to distinguish and the boundary between the midpiece and the tail. The background of the smear was light and unstained and did not hinder the evaluation.

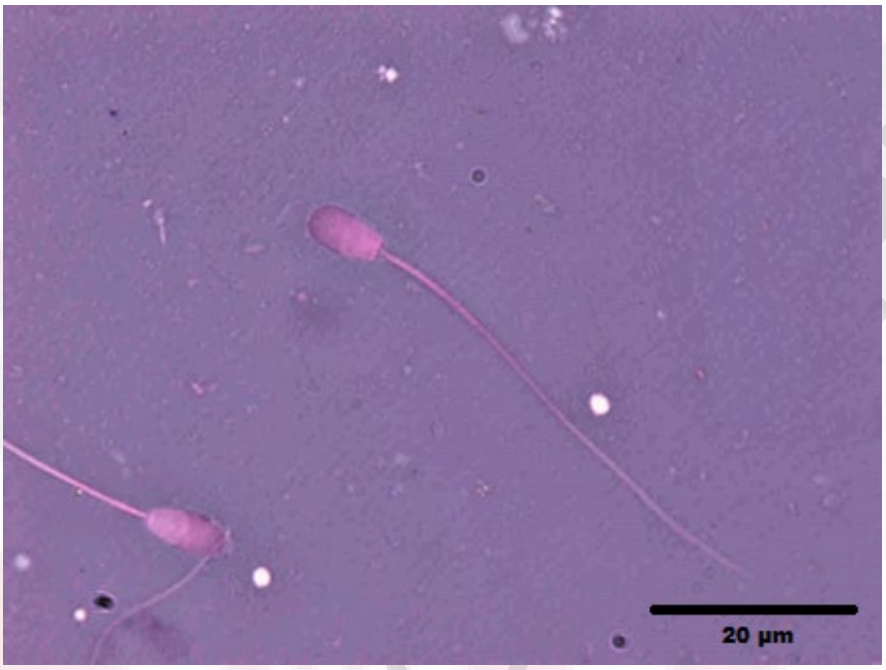

Figure 1(a). Sperm stained with Eosin-Nigrosin (x100 Obj.)

Note that different assays and microscope/camera settings can lead to different backgrounds.

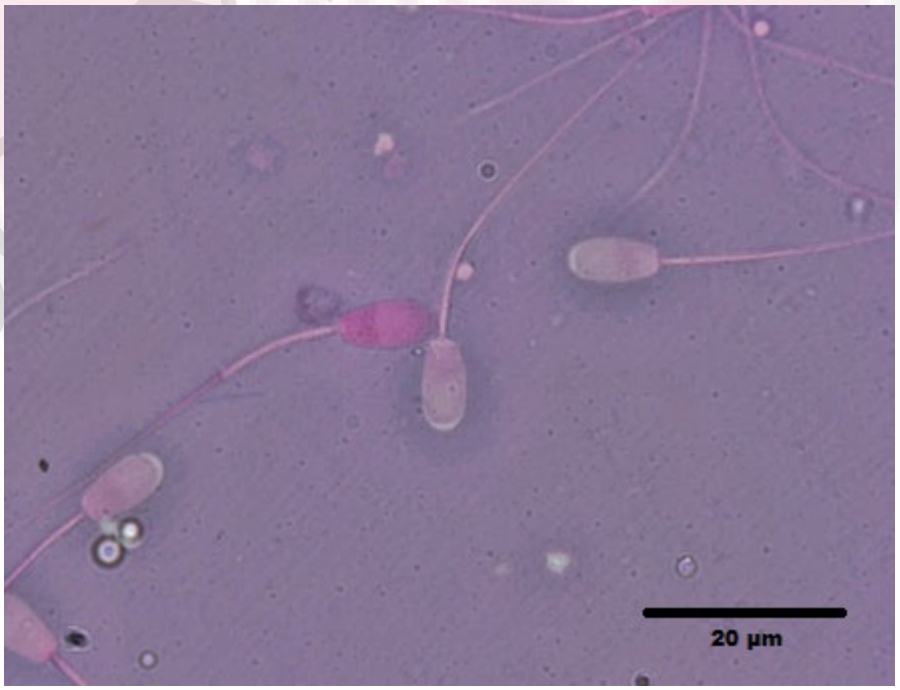

Figure 1(b). Sperm stained with Eosin-Nigrosin (x100 Obj.)

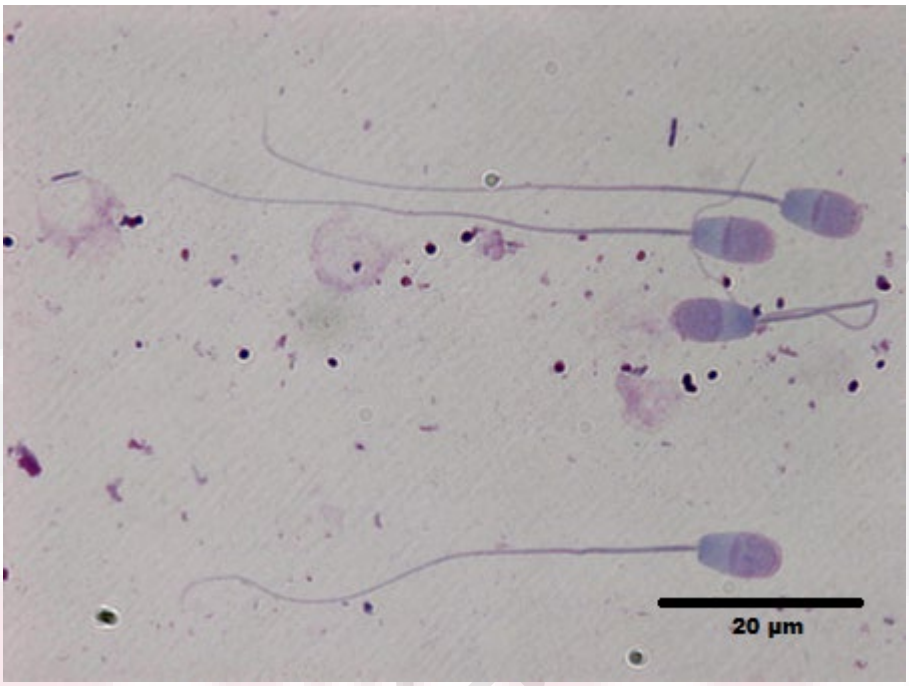

Figure 2(a). Sperm stained with Giemsa (x100 Obj.)

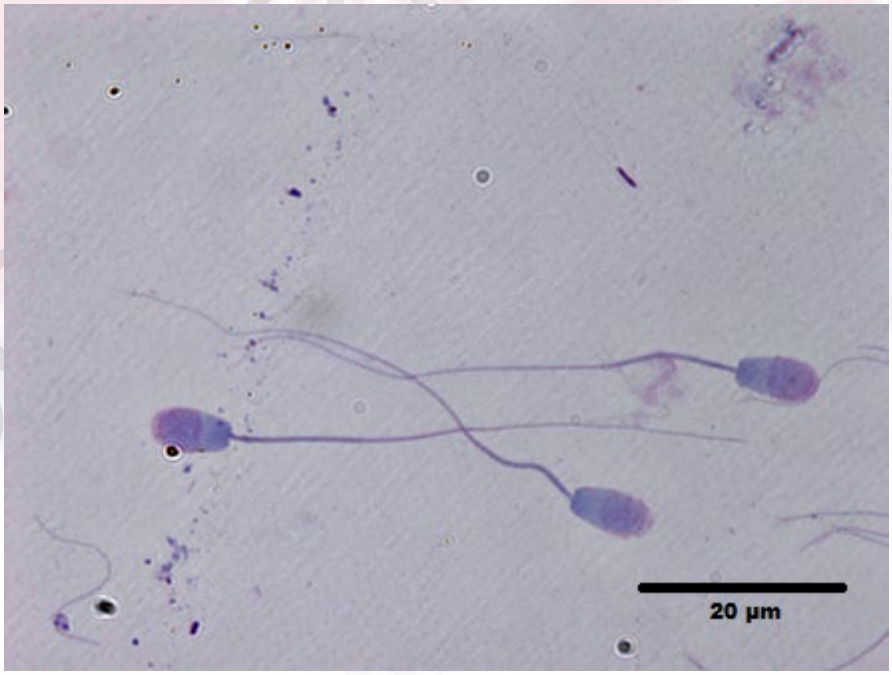

Figure 2(b). Sperm stained with Giemsa (x100 Obj.)

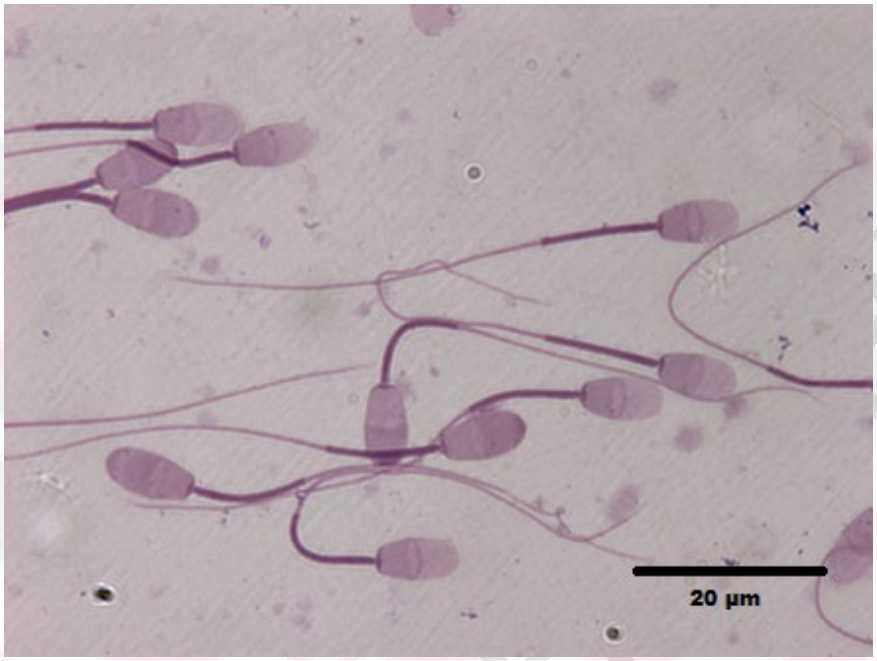

Figure 3(a). Sperm stained with Diff-Quik (x100 Obj.)

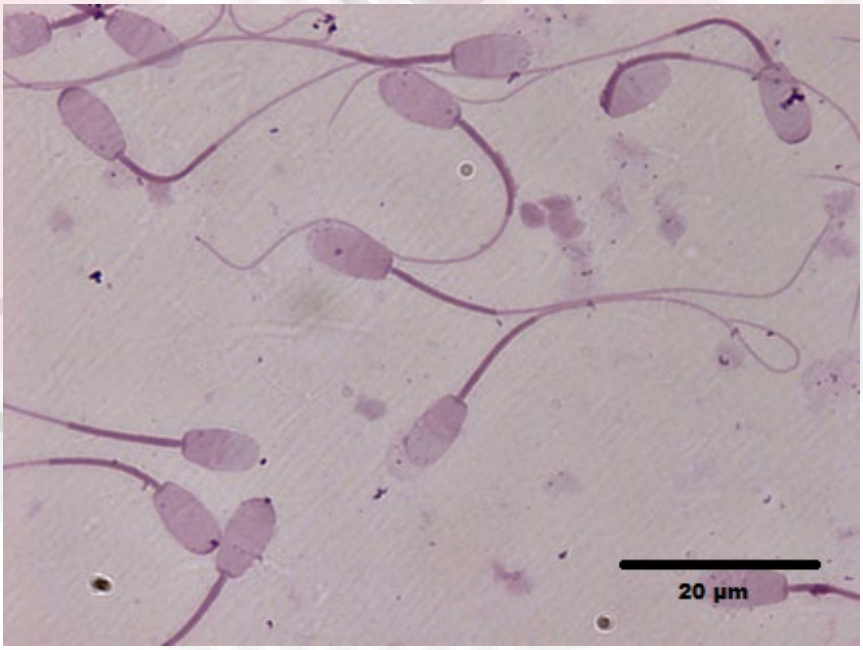

Figure 3(b). Sperm stained with Diff-Quik (x100 Obj.)

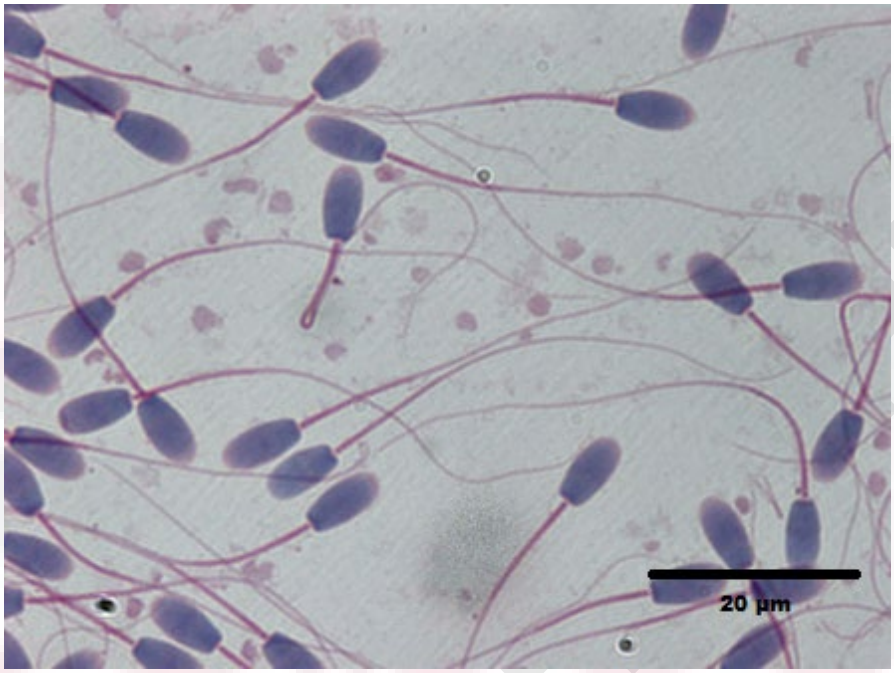

Figure 4(a). Sperm stained with Hematoxylin-Eosin (x100 Obj.)

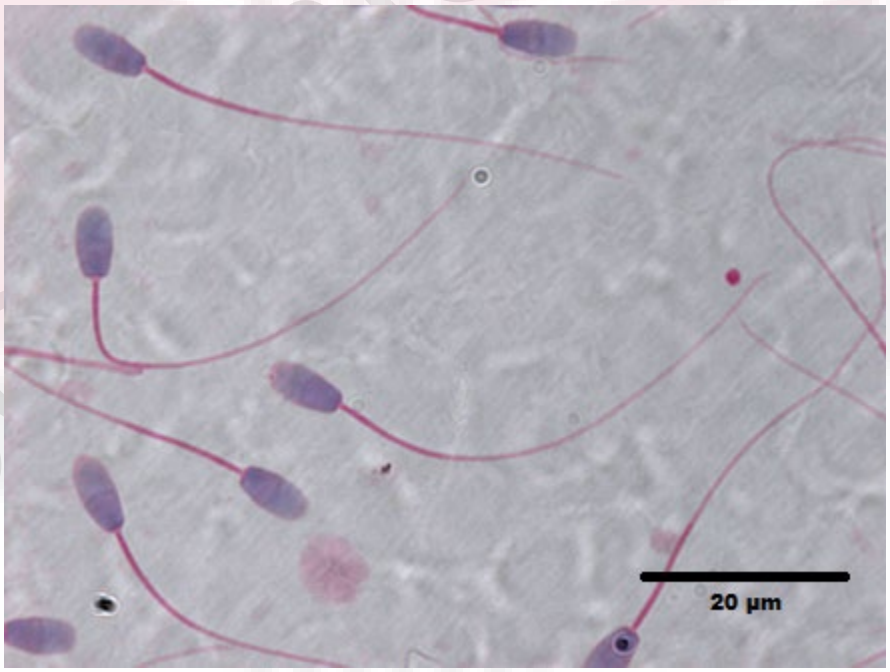

Figure 4(b). Sperm stained with Hematoxylin-Eosin (x100 Obj.)

Morphology	Head	Acrosome	Midpiece	Tail
Eosin Nigrosin	++	++	+	+
Giemsa	++	+++	+	+
Diff-Quik	+++	++	+++	+++
Hematoxylin Eosin	+++	+++	+	++

Table 1. Staining efficiency based on staining technique used (modified by Lingappa *et al*., 2015). Note that *Score 0- Pale/poorly stain; Score 1-Not clear; Score 2 –Clear; Score 3 –Very clear.* ***Observations were subjective and discrepancies and differences of opinions are possible owing to inter-observer variations.***

In this study, the efficiency staining technique was scored according to modified method of Lingappa *et al.,* 2015 as showed in Table 1. From the results, DQ stain produced most clear image of spermatozoa morphology viewed as compare to other stain.

4.2 Morphometric Analysis

The morphometric values of the various sperm variables according to the four staining procedures showed in Table 2. All these variables were significantly influenced by the staining procedure (p= 0.05). However, the differences between the four staining procedures revealed various trends. Our findings indicate that the staining procedure has effects on the morphometric parameters of the buck spermatozoa. Although Table 2 only includes the mean values, the same significant differences and trends were found among the four staining procedures for each buck.

Group	Head Length ±SE	Head Width ±SE	Midpiece Length ±SE	Tail Length ±SE
Eosin-Nigrosin	8.18± 0.03	4.11± 0.02	11.70± 0.07	39.11± 0.23
Giemsa	8.24± 0.03	4.12± 0.02	11.97± 0.05	38.36± 0.20

Diff-Quik	8.22± 0.03	4.11± 0.02	12.02± 0.05	38.69± 0.20
Hematoxylin-Eosin	7.55± 0.02	3.77± 0.02	12.13± 0.04	38.33± 0.18

Table 2. Measured values of head length and width, midpiece length and tail length in spermatozoa stained with EN, G, DQ and HE. Results represent the mean ± SE in terms of percentage of cells, from 50 sperm observed for each slides.

The result shows that HE had the smallest mean values of head length and head width (7.55± 0.02) and the largest belong to G (8.24± 0.03). The smallest mean value of midpiece length was EN stain group whereas the largest was HE. The smallest mean value for tail length was under HE, while the largest belong to EN group. There were significant differences for head length and head width, under HE stain when compared to other stains.

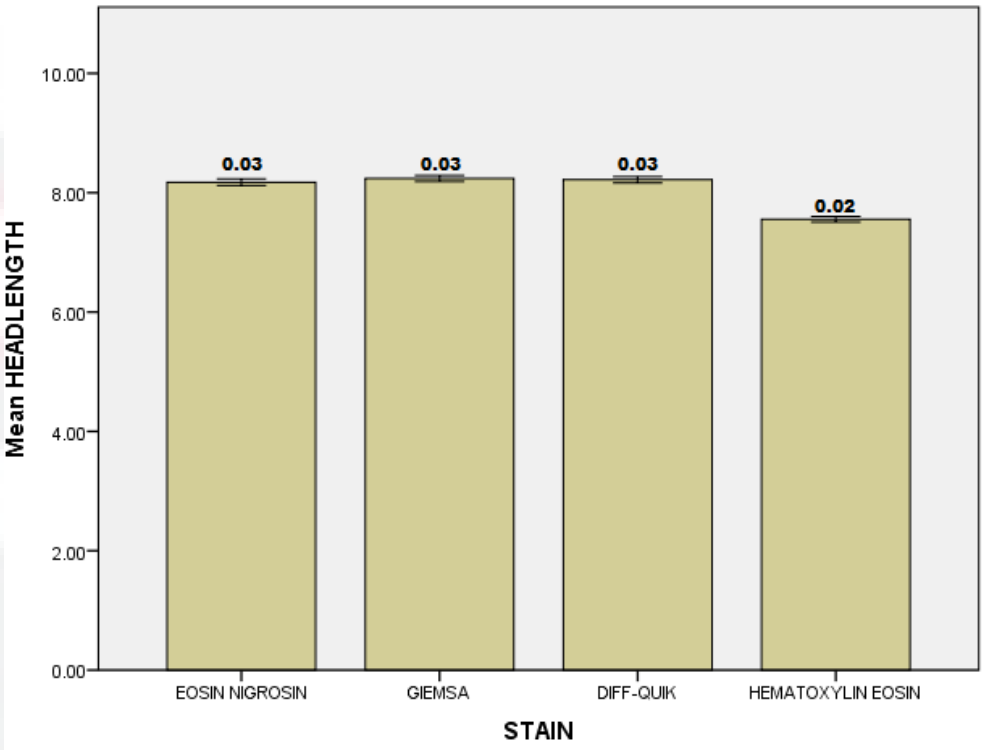

Figure 5. Effect of staining on the head length of the spermatozoa.

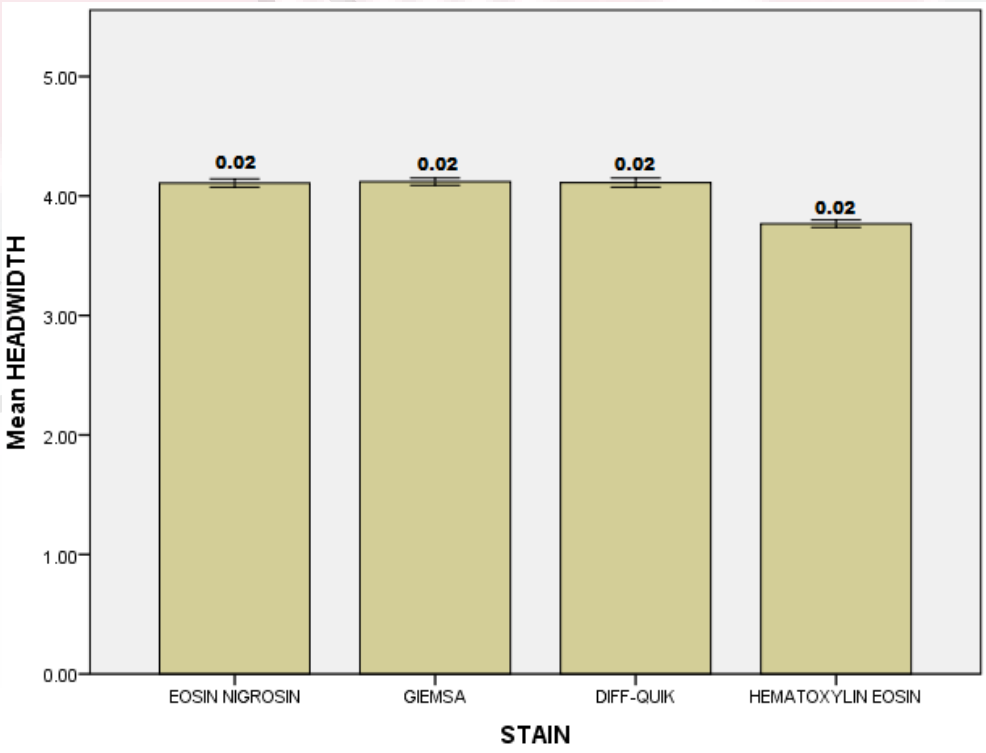

Figure 6. Effect of staining on the head width of the spermatozoa

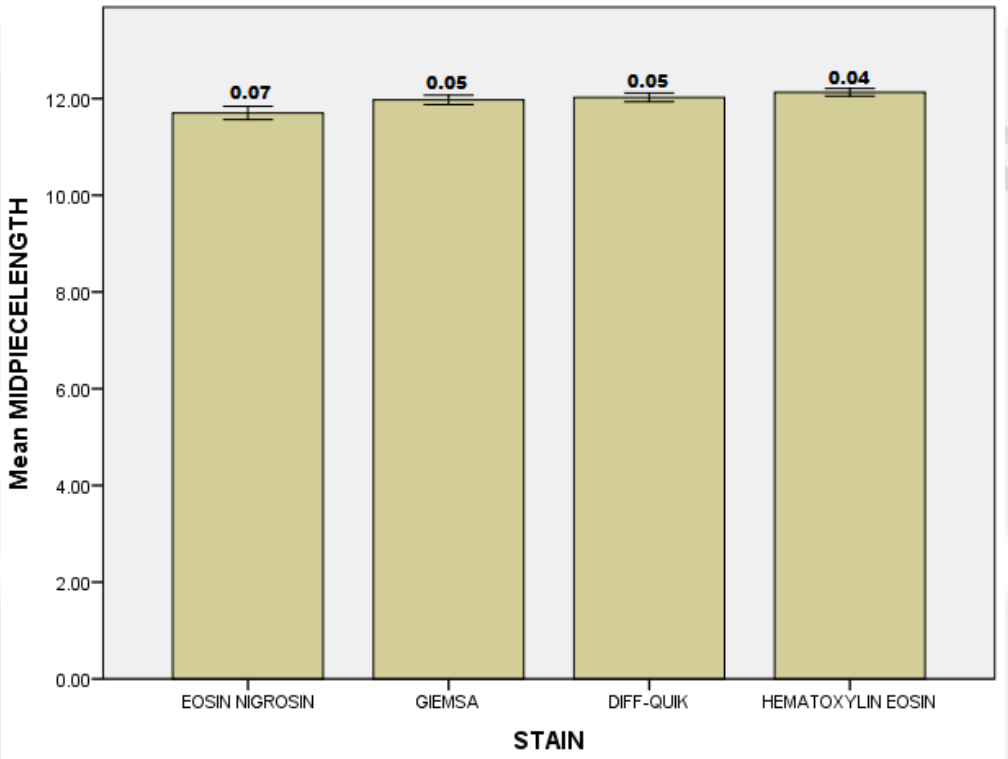

Figure 7. Effect of staining on the midpiece length of the spermatozoa

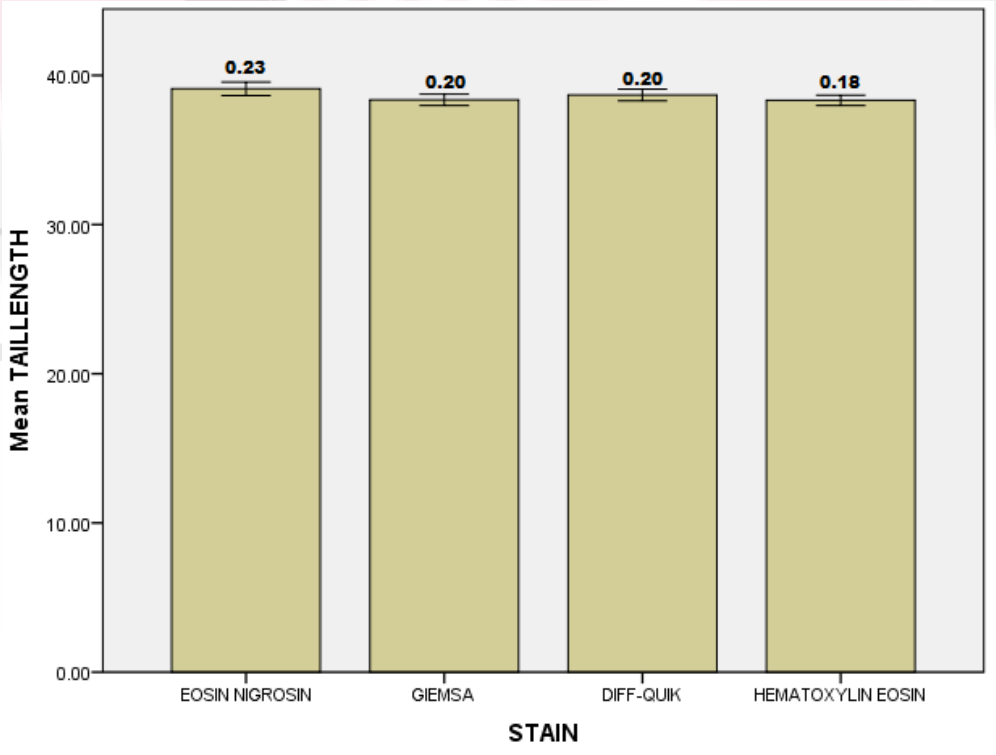

Figure 8. Effect of staining on the tail length of the spermatozoa

Stain	Head Length	Head Width	Midpiece Length	Tail Length
Eosin-Nigrosin * Giemsa	0.02	0.71	0.01	0.00
Eosin-Nigrosin * Diff-Quik	0.14	0.62	0.00	0.02
Eosin-Nigrosin * Hematoxylin-Eosin	0.00	0.00	0.00	0.00
Giemsa * Diff-Quik	0.32	0.35	0.62	0.55
Giemsa * Hematoxylin-Eosin	0.00	0.00	0.01	0.21
Diff-Quik * Hematoxylin-Eosin	0.00	0.00	0.01	0.04

Means with similar superscript within column did not differ significantly at p=0.05

Table 3. Non-parametric test using Mann-Whitney test.

From the statistical analysis using Kruskal- Wallis test, there was significant difference between the morphometric parameters and the staining techniques used. Based on the statistical analysis using Mann–Whitney for independent samples, there was no significant difference in head width between EN and Giemsa with p= 0.71 (p< 0.05). There was no significant difference in head length and head width between EN and DQ with p=0.14 and p=0.62 respectively (p< 0.05). There was a statistically significant difference in all morphometry parameter between EN and HE (p<0.05). There was no significant difference in all morphometry parameter between G and DQ (p< 0.05). There was no significant difference in tail length between Giemsa and HE with p= 0.21 (p< 0.05). There was a statistically significant difference in all morphometry parameter between DQ and HE with p=0.00, p=0.00, p=0.01 and p=0.04 respectively (p<0.05).

5.0 Discussion

5.1 Effect of different staining on the sperm morphology

The present study was carried out in order to analyse and compare spermatozoa morphologically and morphometrically following staining using different methods. EN is a supravital ("live-dead") stain and are the most widely used stains for sperm of humans and animals. This staining technique was developed for ‘‘live-dead’’ staining of sperm (Bjorndahl *et al*. 2003) and also has been used to assess sperm morphology for many animal species according to Van der Horst *et al*. (2009) also can be used as a measure of the integrity of the cellular membrane surrounding the sperm cell. However, according to Aksoy *et al*. (2009) this stain does not clearly differentiate the various components of the sperm. The idea of using eosin is to mark dead cells, which

take up eosin, while nigrosin stained the background, to increase the contrast between faintly stained cells and an otherwise bright background. Björndahl *et al*. (2003) in his report claimed that the simplified one-step technique, which exposed sperm to a mixture of eosin and nigrosin, was introduced on boar, bull and ram sperm (Campbell *et al*., 1956), on rabbit sperm (Beatty, 1957) and further evaluated for various mammalian sperm (Dott and Foster, 1972). This stain was considered as a rapid stain. It can be used to evaluate morphology, live-dead and membrane integrity at the same time. However, this staining technique can cause false high percentage of dead sperm by storage of stained and unmounted smears in a humid environment. It is suggested to mount the slides prior to examination to reduce the false dead percentage.

With the usage of Giemsa stain, the spermatozoa were stained dark blue purple. Head morphology and condensation could be seen very well but the middle piece and the tail cannot be seen clear (Aksoy *et al.*, 2012). The spermatozoa are classified as live spermatozoa which have been through acrosome reaction (light rose-coloured post-acrosomal areas and white acrosomal areas), dead spermatozoa with abnormal acrosome or, degenerative acrosomal reaction (white acrosomal area and blue post-acrosomal areas), live spermatozoa with healthy acrosome (light rose-coloured post-acrosomal area and pink acrosomal areas) and dead spermatozoa with healthy acrosome (blue post-acrosomal areas) as reported by Ferrari *et al*. We observed that even though the morphology of the sperm head was very clear by Giemsa, the midpiece and tail morphology were not clear, thus not compatible with other studies. The percentage of acrosomal coverage can be seen more clearly and this stain can be applied for better acrosomal assessment. This technique is much preferable for

assessment of cryopreserved semen. In addition, the background was also stained which permit a better differentiation of other cell types and bacteria presence in the semen smear.

Diff-Quik is classified as a simple and quick staining method. It gave moderate differentiation of structures, which the acrosome stained red and the post-acrosome area stained dark red. The use of the DQ, Hemacolor and Giemsa kits was optimized as previously described for cat sperm (Mota and Ramalho- Santos, 2006). A direct comparison of the DQ method with the other stains appears to result in higher percentages of morphologically normal spermatozoa (Hidalgo *et al*., 2006). The optimization of staining kit is mostly related with the incubation period needed in each staining solution that sequentially dependent of each observer's ability to discriminate sperm structures and may also vary with different Diff-Quik-like kits. In our study, we had identified DQ are the best stained to evaluate the sperm morphologically.

In the case of HE staining, the buck sperm heads took on a pale violet colour (Fig. 4a). The acrosomal area and cytoplasmic fragments is stained pink and the post-acrosomal area is stained dark purple. The acrosomal part was lighter, gradually becoming darker towards the tail, and the boundary of the acrosome was quite difficult to identify precisely. The stain was homogeneously distributed on the head and dark purple colour was seen, the outline of the head was sufficiently clear, smooth, and easy to identify. Head, midpiece and tails of spermatozoa were seen clearly with HE. The midpiece and tail were pale pink, the end of the tail was difficult to distinguish and the boundary between the midpiece and the tail. The background of the smear was light and unstained and did not hinder the evaluation. The middle piece and tail were seen

clearly and can be evaluated very well (Aksoy *et al.*, 2012). Garcia-Herreros *et al*. (2006) concluded that Haematoxylin is the best staining method for the evaluation of sperm heads. Our observations were concurrent with this study regarding to staining of sperm heads which HE are a good staining procedure to evaluate sperm morphologically.

5.2 Effect of different staining on the sperm morphometry

The normal spermatozoa measurement in this study was referred to the personal measurements by Campbell *et al.* included that they were made on air-dried Eosin-Nigrosin live/dead sperm smears (Campbell, Dott & Glover, 1956) using a calibrated projection microscope. Overall sperm dimension for head length, head width, midpiece length and tail length for this study, are somewhat smaller than reported by Woodall and Cummins which were 8.27µm, 4.25µm, 11.38µm and 39.75µm respectively. The differences may appear be due to usage of different staining techniques. Alternatively, the morphometric differences may be visible due to utilization of different breed. The breed utilized in Woodall and Cummins study is not clearly stated and may cause the difference for the observed measurement. The head length and head width measurement for DQ staining methods in this study are more less the same as compared to previous study done by Sundararaman & Edwin (2004) using which range from 8.22 to 8.34µm. According to Gravence *et. al*., the sperm head dimension for length and width were, 7.69µm, and 3.80µm respectively with the usage of HE staining and had no significant difference with the present study.

In many cases, the fixation of semen on the microscope slide itself can change the structure of the sperm cell. The staining procedure and reagents used can

significantly affect the morphometric parameters of the spermatozoa. Different staining techniques use different chemical reagents. Alcohol is commonly used and in various concentrations may cause dehydration and thus shrinkage of the sperm head, which may result in smaller dimensions of the sperm head in the case of HE staining. The results obtained in the present study confirm this hypothesis.

In the report by Banaszewska *et al.* (2015), it was mentioned that the use of stains with basic and acidic pH enabled more precise characterization of individual elements of sperm structure. For an example, silver nitrate in comparison with routine staining gave accurate differentiation of the sperm head and the tail, with a clearly visible midpiece. The use of stains with different pH or osmotic pressure, as well as the duration of the procedure, may influence the shape and size of the sperm, thus result on the morphological and morphometrical evaluation of the semen. The changes may affect not only the dimensions of the sperm cell, which may lead to an invalid evaluation, but also chromatin structure, which translates directly into reduced fertility or even infertility (Andraszek *et al*., 2014). Osmotic pressure was calculated for the components of RD and all the components were found to be hyperosmotic in relation to the semen. The differences between the osmotic pressure in the solution and the sperm head can lead to the detection of a substantial number of swollen heads during RD staining.

Research has shown that not only the size of the sperm affects fertilization capacity, but also the size of the tail and midpiece (Ciftci and Zülkadir, 2010). Sperm cells with longer tails have greater fertilization capacity due to their greater motility (Gomendio and Roldan, 2008). Sperm cells with longer heads and shorter midpiece

have also been found to swim faster than sperm cells with longer midpieces and shorter heads (Malom *et al*., 2006).

It is difficult to determine which staining method most faithfully reveals the dimensions and shape of the buck sperm. It is necessary to develop an evaluation procedure for sperm morphology and morphometry that will minimize the changes in the structure of the evaluated semen in relation to the native semen. In case of morphometric evaluation, from the present study, HE is considered not suitable as it cause significant difference from the sperm normal morphometric range.

6.0 Conclusion

In conclusion, staining techniques had effects on the sperm morphology. The choice of stain has the potential to cause small alterations to the sperm dimensions, in this context it may result in the evaluated semen to be classified as either normal or abnormal for sperm morphology. From this study, the best staining method was Diff-Quik, besides Eosin-Negrosin taking its advantage to evaluate live- dead of sperm and assessment of membrane integrity at the same time.

In general, the Diff-Quik staining technique showed by this study is a simple and rapid procedure, produces good results view which allows for more accurate morphology evaluation. Hematoxylin- Eosin (HE) is not recommended as tools for morphometric analysis as it causes significant differences compared to other staining techniques.

7.0 Recommendations

For future study, a larger size of semen samples is necessary to provide more accurate result. Diversity of animal selection either between breed and species can be done to evaluate any effects of staining on different species or breed. Ideally staining techniques should also be compared with fresh, unstained spermatozoa in order to evaluate the influence of fixation and staining on spermatozoa rather than referring to the literature.

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8.0 Appendices

Preparation of Eosin-Nigrosin stain:

Add approximately 85 ml of distilled water into a 250 ml bottle/beaker and add 10g Nigrosin. Drop a magnetic bar into the bottle/beaker and place it on a hot plate cum stirrer. Boil Nigrosin in distilled water (70-80°C) with continuous stirring until the granules dissolved. Cover the bottle/beaker with aluminium foil to prevent evaporation but not air tight. Add 1.67g Eosin and 2.9g of sodium citrate with continuous stirring until cool down. Filtering is not necessary but permitted upon cooling. Transfer the stain into a measuring cylinder and make up the volume to 100 ml with distilled water. Store the stain in a 100 ml bottle with a screw cap.

Preparation of Giemsa stock solution:

The stock Giemsa's stain was prepared as per manufacturers' protocol. Firstly, 0.77 gm Giemsa's powder (MERCK) was measured in weighing balance and saturated for long time (2-3 hrs) with methanol and glycerol mixture pre-warmed at 40^0C. The methanol-glycerol mixture was prepared by measuring 75 ml absolute alcohol and 25 ml of glycerol (98% pure). After proper mixing, the stain was filtered with 0.22 µm sterile Millex (Millipore). The solution was kept at 37 ^{0}C in an incubator for 7 days in

amber colour bottle for maturation with intermittent shaking. The stock solution was ready to assess the acrosomal structure of spermatozoa.

Semen evaluation

Animal	Buck 1	Buck 2	Buck 3	Buck 4	Buck 5	Buck 6	Buck 7
ID	**207**	**R18**	**NS101**	**No Tag**	**No Tag**	**12521**	**A4355**
PE findings	NSF	NSF	NSF	NSF	NSF	NSF	NSF
Scrotal circumference or area	83.6cm x26.3cm	89.4cm x25.8cm	101.6cm x28.1cm	90cm x24cm	140cm x27cm	29cm	30cm
Volume of ejaculate	0.5ml	1ml	0.8ml	0.3ml	2ml	1ml	1.9ml
Colour	Milky	dilute whitish	milky	dilute whitish	Creamy	thin milky	light yellow/ cloudy
Wave pattern (0/1/2/3/4/5)	1	2	2	1	4	4	2
General motility (%)	17	27	77	9	85.5	95	90
Forward motility (%)	7	25	70	15	92	90	80
Backward motility (%)	1	3	1	2	1	2	5
Rotating motility (%)	2	7		3	1	3	5
Vibrating motility (%)	90	65	25	80	6	5	10
Live (%)	31.5	42.7	71.5	45.2	89.5	78	67
Abnormal (%)	11	15.08	6	9.72	2.36	1.07	0.89

Concentration (per ml)	1660 $x10^6$	1176 $x10^6$	3450 $x10^6$	906 $x10^6$	3685 $x10^6$	5500 $x\ 10^6$	2800 $x10^6$