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THE USE OF DIFFERENT STAINING METHODS IN THE EVALUATION OF FROZEN-THAWED CAUDA EPIDIDYMAL RAM SPERM MORPHOLOGY

Uso de diferentes métodos de tinción en la evaluación de la morfología del espermática de la cola del epidídimo congelado en carnero

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ABSTRACT

Sperm morphology evaluation is an important parameter for determining the quality of semen and predicting fertility in rams. Different staining methods have been developed to detect the morphological status of sperm, but there is no optimized protocol, especially for animals yet. This study was designed to compare the results using SpermBlue®, Diff-Quick, and Coomassie Blue stain in the morphological evaluation of epididymal ram semen. In the study, samples collected from a Bafra ram (epididymal sperm/ram) known to have a good breeding history were diluted with Tris-based diluent and frozen. After thawing for each straw, three semen smears were made and stained with SpermBlue®, Diff-Quick, and Coomassie Blue. All morphological parameters were evaluated using a light microscope. 100 spermatozoa were examined randomly and classified according to their characteristics for each slide. In identifying morphological abnormalities, the staining protocols have compared amongst themselves, and no significant difference between Diff-Quick and SpermBlue® staining methods was observed. However, significant differences were observed in midpiece abnormalities when SpermBlue® and Coomassie-Blue staining methods were compared, while significant difference was found in total abnormality in SpermBlue® and Diff-Quick staining comparison ($P < 0.05$). As a result, all staining methods evaluated can be easily optimized for laboratory conditions and used in the morphological analysis of ram semen.

Keywords: Epididymal Semen, Morphology, Ram, Staining Procedures

RESUMEN

La evaluación de la morfología espermática es un parámetro importante para determinar la calidad del semen y predecir la fertilidad en los carneros. Se han desarrollado diferentes métodos de tinción para detectar el estado morfológico de los espermatozoides, pero aún no existe un protocolo optimizado, especialmente para animales. Este estudio fue diseñado para comparar los resultados usando SpermBlue®, Diff-Quick y Coomassie Blue en la evaluación morfológica de los espermatozoides del epidídimo de carnero. En el estudio, las muestras recolectadas de un carnero Bafra (esperma del epidídimo/carnero) conocido por tener un buen historial reproductivo se diluyeron con diluyente a base de Tris y se congelaron. Después de descongelar cada pajuela, se hicieron tres frotis de semen y se tiñeron con SpermBlue®, Diff-Quick y Coomassie Blue. Todos los parámetros morfológicos se evaluaron utilizando un microscopio óptico. Se examinaron aleatoriamente 100 espermatozoides y se clasificaron según sus características para cada lámina. Al identificar anomalías morfológicas, los protocolos de tinción se compararon entre sí y no se observaron diferencias significativas entre los métodos de tinción Diff-Quick y SpermBlue®. Sin embargo, se observaron diferencias significativas en las anomalías de la pieza intermedia cuando se compararon los métodos de tinción SpermBlue® y Coomassie-Blue, mientras que se encontraron diferencias significativas en la anomalía total en la comparación de tinción SpermBlue® y Diff-Quick ($P < 0,05$). Como resultado, todos los métodos de tinción evaluados pueden optimizarse fácilmente para las condiciones de laboratorio y utilizarse en el análisis morfológico del semen de carnero.

Palabras clave: Semen del epidídimo, Morfología, Carnero, Procedimientos de tinción

INTRODUCTION

Sperm morphology is a crucial determinant of a sperm sample's quality and ability to fertilize (Cerdeira et al., 2020). Mammalian spermatozoa have a unique morphological structure that enables them to transfer genetic information during the fertilization of egg cells. Evaluation of sperm morphology allows determination of sperm structure regularity and revealing morphological abnormalities to diagnose male fertility status (Kondracki et al., 2017). It has been shown that there is a significant relationship between morphologically normal sperm percentage and fertility in male animals (such as stallions, bulls, goats, and rams) (Yaniz et al., 2012).

The morphometry of sperm is affected by cryopreservation (Cerdeira et al., 2020). Mammalian sperm are exposed to temperature variations during the freeze-thawing process that causes physical and chemical stress, changes in plasma membrane lipid composition, and externalization of phosphatidylserine residues (Andreeva et al., 2019). Especially ram spermatozoa have a low membrane cholesterol-to-phospholipid ratio compared to other species, and because of this feature, ram semen is more sensitive to temperature changes (Rizkallah et al., 2022).

The subjective sperm morphology evaluations result in significant discrepancies among laboratories and workers. Moreover, sperm evaluation procedures are not standardized today. Because of these differences, morphologic examination as a quality predictor of sperm for reproduction has been limited (Yaniz et al., 2012). In evaluating sperm morphology, different staining methods are used to determine the percentage of sperm with normal morphology. In addition, staining methods consist of many different protocols. For this reason, reasons such as collecting sperm with appropriate methods fixing and staining sperm affect the accuracy of sperm morphology evaluation (García-Herreros et al., 2006). The Hancock solution, Papanicolaou stain, Diff-Quick stain, Spermac, eosin-nigrosin, and Giemsa stain are the most used methods for human and animal sperm (Van der Horst et al., 2009). Studies on human sperm found that the staining method might significantly impact the results of morphometric measurements (Maree et al., 2010). Also, staining procedure effects on sperm morphometry have been found in sperm of bulls, stallions, and rams (Freneau et al., 2010; Łącka et al., 2016).

This study aims to compare the results using Spermblue®, Diff-Quik, and Coomassie Blue dyes in the morphological evaluation of epididymal semen after freezing and thawing in rams. This study will also allow for the standardization of the staining methods used in ram epididymal semen and the interlaboratory comparability of the data.

MATERIAL AND METHODS

Obtaining cauda epididymal ram semen

The testicles used for this study were collected as early as possible from the slaughtered one ram of 2 years of age from one abattoir in Samsun city. The testicles were transported to the laboratory in an ice chest at refrigeration temperature (4.9–6 °C). To limit contamination, epididymis samples were thoroughly dissected free of blood clots and other tissues. It was taken great care not to cut any blood vessels. The cauda epididymis was placed in a 100 mm petri dish and was sliced with a lancet. For the release of epididymal spermatozoa, 3-5 ml of PBS was added to the petri dish, and the released spermatozoa were collected and then centrifuged at 700 g for 6 min (Merati and Farshad, 2020). Consequently, the resulting supernatant was discarded, and the isolated spermatozoa were diluted to 120 × 10^6 spermatozoa/ml by a prepared Tris-based extender at 37 °C.

Semen freezing and thawing

Immediately following sperm evaluation, each sperm sample was diluted to 120 × million/ml with tris base extender (3.63 g of tris-(hydroxymethyl)-aminomethane, 1.99 g of citric acid, 0.5 g of glucose) with 20% egg yolk, and cooled at 4°C for 2 hr. The sperm was drawn into 0.25 mL plastic straws and sealed with polyvinyl alcohol (PVA). After the equilibrated ram semen straws were frozen in liquid nitrogen vapor for 15 minutes at -90, -110 °C, the frozen semen straws were dipped directly into liquid nitrogen (-196 °C). After at least a week, samples were thawed in a water bath (37 °C for 30 s) for microscopic semen evaluation immediately after thawing. The sperm motility and progressive motility parameters were determined using the computer-assisted sperm motility analysis (CASA: SCA®, Microptic, Barcelona, Spain).

Evaluation of sperm morphology

10 straws from the same animal were evaluated for each staining procedure.

-SpermBlue® staining

It is a sperm stain applied to all species for sperm morphological evaluation with SpermBlue®. SpermBlue® is also suitable for clearly identifying the main components of sperm. Sperm morphology evaluation requires different staining of each region of the sperm; in this way, the boundaries of the acrosome, head, midpiece, and tail are revealed (Van der Horst and Maree, 2010). The sperm morphology was evaluated under the phase-contrast microscope (Nikon, Eclipse E200, Japan) by staining with SpermBlue® (Microptic S.L., Spain), performed according to the manufacturer's instructions, and observed under a magnification of 40 x.

-Coomassie Blue Staining

The semen sample smeared and dried preparations were prepared with 0.22% Coomassie Blue G-250 (Fisher Scientific, Fair Lawn, NJ), 50% Methanol (Merck-1.06008.2500), 10% acetic acid, and 40% distilled water stained for 2 minutes. Distilled water was used to remove any undesired dyes from the preparations. It dried again after being put through xylene (Larson and Miller, 1999). The sperm morphology was evaluated under a phase-contrast microscope (Nikon, Eclipse E200, Japan) and observed under a magnification of 40 x.

-Diff-Quick Staining

The Diff-Quick (Bio-Diff Kit BioGnost®, Zagreb, Croatia) staining method was applied following the producer's kit recommendations. To prepare the sperm sample, a thin and homogeneous smear was created by placing 15 µL of fresh sperm sample on one side of the glass slide. Then, at least 10 minutes were waited for the smear to dry. The slides were dipped into the Bio-Diff 1,2, and 3 reagents for 5 x 1-second, respectively. Then the excess reagent remaining on the slide was filtered onto filter paper, and this step was done after

each immersion step. After the last reagent, the slides were rinsed off in pH 7.2 buffer solution for 1 min, and the slides were left to dry (Anonymous, 2022). Then, 100 spermatozoa were examined randomly at 100 x magnification under oil immersion. According to their morphological characteristics, spermatozoa were classified into four categories: abnormal head, abnormal midpiece, abnormal tail, and total abnormal spermatozoa.

Statistical analysis

Mann Whitney U was used for mean comparisons. The SPSS software (Version 21, SPSS, IBM) was used for all statistical analyses, and differences were considered significant at the P <0.05 level. The results were shown as the Mean ± SD.

RESULTS

When a comparison between fresh and cryopreserved ram semen was performed, motility and progressive motility parameters were adversely affected by cryopreservation. These spermatological parameters significantly reduced (P <0.05) in cryopreserved sperm compared to fresh sperm (Table 1).

Table 1. Effect of cryopreservation process on the fresh and frozen-thawed epididymal sperm motility and progressive motility.

	Fresh semen (n=1)	Frozen-Thawed semen (n=10)
Motility (%)	98.62	60.32 ± 8.24
Progressive motility (%)	65.64	11.26 ± 2.92
VCL (µm/s)	98.26	45.37 ± 6.71
VAP (µm/s)	70.15	28.86 ± 3.77
VSL (µm/s)	46.78	19.51 ± 3.00
STR (%)	59.64	60.14 ± 8.80
LIN (%)	43.25	39.54 ± 7.82
WOB (µm/s)	69.31	61.48 ± 9.76
ALH (µm)	3.55	1.51 ± 0.22
BCF (Hz)	6.86	5.95 ± 1.57

All samples were stained immediately after thawing of ram semen. Abnormal morphology obtained by different staining methods was categorized as head, mid-piece, tail, and total abnormality. In the statistical analysis of the staining methods in pairwise comparisons, no statistically significant difference was found between the Coomassie Blue-Diff-Quick staining methods. However, when SpermBlue® and Coomassie Blue staining methods were compared, a statistical difference was found in terms of mid-piece abnormalities (P <0.05). Again, when SpermBlue® and Diff-Quick staining methods were compared, a statistically significant difference was found in the rate of total abnormal spermatozoa (P <0.05). Abnormal morphology values obtained with different staining methods in ram epididymal spermatozoa are presented in Table 2. Figures 1,2 and 3 show the staining images of SpermBlue®, Coomassie Blue, and Diff-Quick, respectively.

Table 2. Mean ± S.D., minimum and maximum values (%) of ram epididymal spermatozoa with different staining methods.

		Head	Mid-piece	Tail	Total
Sperm Blue (n=10)	Min. (%)	1	0	5	9
	Max. (%)	2	4	11	13
	Mean ± S.D. (%)	1.5 ± 0.52	2.2 ± 1.22	7.4 ± 1.83	11.1 ± 1.37
Coomassie Blue (n=10)	Min. (%)	1	2	3	9
	Max. (%)	4	5	12	18
	Mean ± S.D. (%)	2.0 ± 0.94	3.4 ± 0.84	7.6 ± 3.02	13.0 ± 3.19
Diff-Quick (n=10)	Min. (%)	0	1	6	11
	Max. (%)	4	5	10	15
	Mean ± S.D. (%)	2.2 ± 1.13	2.6 ± 1.35	7.9 ± 1.1	12.7 ± 1.25

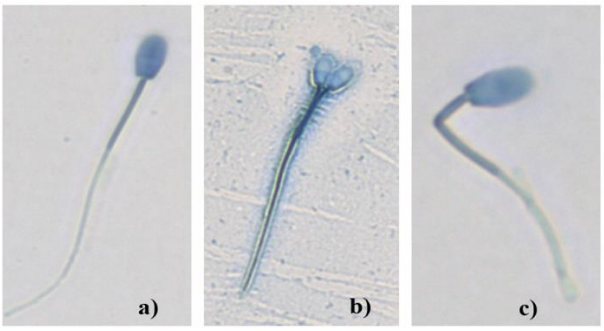

Fig 1. Spermatozoa stained with SpermBlue®. a) normal, b) double head, c) bent tail.

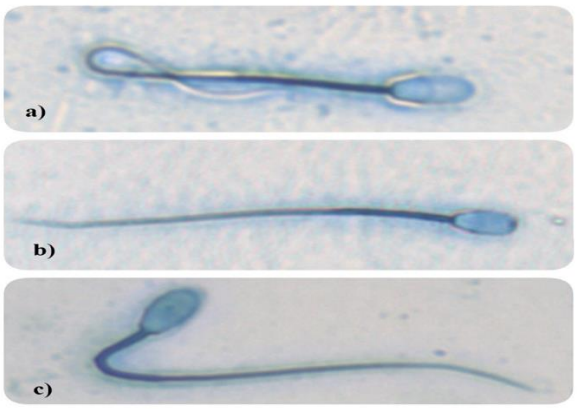

Fig 2. Spermatozoa stained with Coomassie Blue. a) coiled sperm tail, b) normal, c) bent tail.

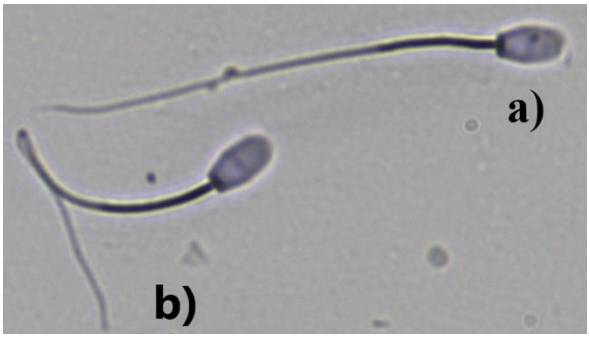

Fig 3. Spermatozoa stained with Diff-Quick®. a) normal, b) bent tail.

DISCUSSION

Collecting cauda epididymal semen from slaughtered animals is a fast and inexpensive approach, and for genetic stocks, epididymal semen delivers a sufficient number of viable spermatozoa (Ehling et al., 2006). Additionally, cryopreservation of obtained epididymal sperm is a safe and effective method and can provide high fertility (Holt, 2000). In the light of this information, we received good quality semen from testicles taken from one Bafra ram brought for slaughter in our current study. Ram spermatozoa have low membrane cholesterol-phospholipids, making them susceptible to freezing damage (Rizkallah et al., 2022). It is thought that the decrease in motility values obtained in our study after freezing-thawing is due to this. However, the positive correlation between sperm kinematic parameters (VCL, VSL and VAP) with sperm motility and morphological changes after freezing is consistent with previous data on fresh ram semen (Favareto et al., 2010). Adding various antioxidant substances and supplements to the diluent can minimize this decrease. Studies have demonstrated the cryoprotective effect of additives during the freezing of sperm from rams (Galarza et al., 2020; Sangeeta et al., 2015). Since this study aimed to determine the effect of the freezing procedure on the morphology of cauda epididymal ram semen, no special additive was added to the semen extender. In this way, we enabled the evaluation of sperm morphology without being affected by other factors with three different staining procedures.

The growing number of papers documenting research conducted worldwide and on numerous species attests to the importance of sperm morphometry (Andreeva et al., 2019; Cerdeira et al., 2020). But, as many writers have pointed out, some procedures work well for one species but not for another. The most accurate methods for examining sperm morphology and abnormalities are phase contrast and differential interference contrast microscopy (Pozor et al., 2012). However, veterinary practitioners do not typically use these methods due to the high price of these methods. In today's medical practice, sperm morphology is evaluated using staining techniques. The morphology of sperm is not homogeneous, even within the same ejaculate, according to microscopic analysis of ejaculates and staining methods, which complicates fertility diagnostics (Gago et al.,1998; Banaszewska et al., 2011). As a result, it is necessary to design and standardize sperm morphology assessment procedures appropriate for various species (Walczak-Jedrzejowska et al., 2013).

The purposes of our study were to compare the results of ram sperm morphology evaluation using three different staining methods to identify the most suitable one among them. The appropriate staining approach for sperm morphology assessment must interfere as little as possible with the spermatozoon's structure and size while still clearly exhibiting the borders of its head, midpiece, and tail (Czubaszek et al., 2019). Our present findings indicate differences in the midpiece morphology between Coomassie blue and Sperm Blue stain and differences in the total morphology between Sperm Blue and Diff Quick. The sample preparation, fixation procedure, staining method, microscopic equipment (optics and camera), and technician activity are the primary sources of variation in sperm morphometry. All of these factors may impact the analyses' repeatability and capacity to compare results across laboratories (Brito et al., 2011). In our study, applications were made by the same investigator whenever possible to limit potential variation while preparing the smear or staining.

In general terms, the staining with Coomassie blue is an easy, inexpensive, and applicable method. Similar results were obtained in a study comparing FITC-PNA/PI with Coomassie blue (Carretero et al., 2015). Considering the need for a fluorescent microscope, it is observed that it is more usable. Brum et al. (2006) suggested that the Coomassie blue stain would be useful in the routine evaluation of canine and equine spermatozoa, as well as for the assessment of the morphology of spermatozoa from these species after cryopreservation that disrupts the plasma and acrosomal membranes.

Diff-Quick is one of two staining methods recommended by the WHO for evaluating human sperm (Murcia-Robayo et al., 2018). Although it is not the only factor, the total abnormalities found with Coomassie Blue or SpermBlue® compared to Diff-Quick may be explained by low stain penetration or an unclear background.

Coomassie blue, Diff-Quick, Papanicolaou stain and a group of closely related rapid stains, as well as SpermBlue®, is the most widely used stains for sperm of humans and animals. SpermBlue® also is suitable for clearly defining the main components of sperm. In addition, SpermBlue® can eliminate the disadvantages of a subjective examination by doing appropriate readings in CASA (Computer Assisted Sperm Analysis) systems (Van der horst and Maree, 2010).

CONCLUSION

The evaluation of sperm morphometry with Coomassie Blue, SpermBlue® and Diff-Quick can be useable in rams and other animal semen in laboratory conditions. However, standardization of morphological examination and forward more studies involving interlaboratory comparisons are needed.

Conflicts of interest
The authors declare that there is no conflict of interest.

Author contribution
All authors designed and carried out the study.

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