COMPARISON OF TWO HISTOLOGIC STAINS IN THE EVALUATION OF SPERM HEAD MORPHOMETRIC MEASUREMENTS IN FROZEN-THAWED BULL SEMEN

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ABSTRACT
This study was designed to compare the performance of the kit Hemacolor (HC) in two protocols (A, B) and Toluidine blue stain (TB) for staining the bull sperm head in samples of frozen-thawed semen. Automated Sperm Morphology Analysis (ASMA) was performed to determine the sperm measurements: head size (length, width, area and perimeter). TB was found to be the best procedure for staining the frozen-thawed bull sperm ($p<0.0001$). The use of this method rendered the highest number of cells correctly analyzed (88.29%) and the lowest coefficient of variation on the image processing (4.54) and morphometric measurements. TB provided good colour intensity and optimum contrast of the sperm head with the surrounding background that allows efficient boundary detection and reduces the number of stained foreign particles. The staining methods affected significantly the sperm head dimensions ($p<0.0001$) with increased values from HC (protocol A) than HC (protocol B) and TB, respectively (HC>TB). HC provide more intense grey-level values, resulting in enlarged cells, which influence the size morphometric parameters. Based on these findings, we recommend TB for its accurate and reproducible morphometric results.

Keywords: Sperm head, morphometric analysis, toluidine blue stain, bull.

COMPARACIÓN DE DOS TINCIONES HISTOLÓGICAS PARA LA EVALUACIÓN DE LAS DIMENSIONES MORFOMÉTRICAS DE LA CABEZA ESPERMÁTICA EN SEMEN CRIOPRESERVADO DE TORO

RESUMEN
Este estudio se diseño con la finalidad de comparar el desempeño de dos protocolos (A, B) de tinción del kit Hemacolor (HC) versus la tinción azul de toluidina (TB) en el análisis morfométrico de la cabeza de espermatozoides en muestras de semen congelado-descongelado. Las mediciones espermáticas se realizaron con el Análisis Computarizado de la Morfología Espermática (ASMA) para determinar las dimensiones de la cabeza espermática (longitud, ancho, área y perímetro). TB fue la mejor técnica para la tinción de los espermatozoides ($p<0.0001$). El uso de este método rindió el mayor porcentaje de células correctamente analizadas (88,29%) y el menor coeficiente de variación en el procesamiento de las imágenes (4,54) así como también en el análisis morfométrico. La tinción TB proporcionó una buena intensidad del color y un contraste optimo de la cabeza espermática con el fondo circundante lo que conllevo a una detección eficiente del borde de la imagen y redujo el número de partículas extrañas teñidas. El método de tinción afectó significativamente las dimensiones de la cabeza espermática ($p<0,0001$), siendo estas mayores con el protocolo A de HM, seguido por protocolo B de HM y finalmente las dimensiones menores pertenecieron a la tinción TB (HM > TB). La tinción HC proporciona niveles de gris más intensos, lo que resulta en células mas alargadas, lo cual influye sobre las dimensiones de la cabeza espermática. Basándose en estos resultados, se recomienda la tinción TB por arrojar los resultados mas uniformes y reproducibles.

Palabras clave: cabeza espermática, análisis morfométrico, Azul de toluidina, toro.

INTRODUCTION

In order to identify and select bulls with acceptable reproductive efficiency; the semen analysis, especially sperm morphology, has received considerable attention in relation to the ability of the spermatozoa to achieved normal fertility [5]. In the last years, digital image processing is used for computer-aided or automated

sperm morphology analysis (ASMA) system to estimate its fertility by means of sperm head biometry and shape.

To reduce the subjetivity of the morphologic examamination, ASMA system have been developed and succesfully aplied to bull sperm analysis [4, 5, 7, 10, 11, 18, 19]. In human andrology, the sperm head dimensions have been defined and widely accepted by andrologic laboratories [22]. However, is necessary the application of optimal methods, concerning the sample preparation and staining procedures to improve the efficence of sperm morphology analyses. It is particularly important in frozen-thawed semen samples due to the presence of particles from the freezing extenders which could affect the accuracy of the image processing. Several studies have been conduced to determine the influence of staining and sampling procedure by using the morphometric analysis of mamalian spermatozoa [1, 5, 12, 13, 14, 21]. Most of these trials established the comparison among Diff-Quick®, Hemacolor® (HC) and Harris'Haematoxylin® and the results varied depending species; however, the researchers recommend the use of Diff-Quick or Harris'Haematoxylin. In our case, by using frozen-thawed semen and stain with HC, the efficiency obtained was low because the freezing diluent conteining egg-yolk, milk and glicerol, which produce interference in the stain, affecting the image quality by increassing the colour density in the background, propitiating head sperm images incorrectly analysed [18]. Toluidine blue stain (TB) is a nuclear dye used to evaluate sperm chromatin integrity in several species [16], and has recently been implemented as a viable alternative to assess the size of the sperm head in frozen-thawed bull semen [17], however, its efficacy has not been compared with others scientifically proven staining.

The aim of the present study was to evaluate the effect of two different stainning procedures on the correctly analyzed sperm head and the accuracy sperm morphometry parameters using frozen-thawed bull semen samples and the ASMA.

MATERIALS AND METHODS

Semen collection and processing

Ejaculates from 11 fertile bulls (six Brahman and five Holstein x Brahman), 3-5 years old, in regular service were obtained from the artificial insemination centre, located in Zulia State, Venezuela. The ejaculates were obtained by using an artificial vagina. After semen collection, an aliquot of the ejaculate was used to assess the sperm concentration and subjective scores of motility (wave motion) were performed. The sperm concentration of each sample was determined by photometer (SpermaCue, Minitub®, Germany). In addition, ejaculates were diluted and used to assess individual sperm motility. The rest of the sperm samples were diluted at 30ºC to a final sperm concentration ~$40x10^6$ sperm/mL with a skim milk-egg yolk medium (EY), containing 15 % of skim milk, 1% of EY, 7% glycerol, besides TRIS, fructose and antibiotics (Tilosin 0.56%, Linco-Espectin 0.56%, Gentamicin 0.74%) in a final solution with adjusted pH at 6.8. The dilution was carried out in two stages. The first diluter (A) was added at 30ºC and 2h later, the second diluter (B) at 5°C. Seminal samples in the "diluent A" were cooled down slowly up at 5 ºC. This cooling up at 5 °C lasted 2 h approximately. The second diluter differed of the first one in the substitution of water (14%, v/v) with the same glycerol volume (final concentration = 7% glycerol). At this point, sub-samples were taken for sperm head morphometric dimensions evaluation. Then, the diluted sperm suspensions were refrigerated slowly at 5ºC for 2 h, equilibrated at temperature for 2 h and loaded into 0.5 mL straws. The straws were frozen in nitrogen vapours, 4 cm above the surface of the liquid nitrogen, for 10 min and then plunged into liquid nitrogen. One week after, thawing was carried out by placing the straws in a water bath at 37 ºC for 20 s. and the sperm was allowed to equilibrate for 5 min before evaluation. After thawing, sub-samples were also taken for sperm head morphometric evaluation.

Staining procedures

After thawing, each ejaculate was individually diluted with distilled water, inducing that all the sperm dead and not be reactive osmotically when spread on the slide. To eliminate osmotic effects, the sperm not show shrinkage or swelling of live cells [7, 15].

Slides were prepared from each sample by placing 5 µl of semen on the clear end of a frosted slide and dragging the drop across the slide. Semen smears were air dried and stained with each of the two following stained (Table 1): HC (Merck, Darmstad, Germany, Cat. Nº 1.11957.2500) and TB [2, 3, 6]. The HC was used in two protocols (A and B).

Morphometric analysis of sperm heads

The morphometric evaluation was done in the laboratory of Andrology of "*Universidad del Zulia*", Venezuela. Stained slides were used to perform ASMA using the morphometry module of a commercially available system (SCA: Sperm-Class Analyzer®, Microptic, Barcelona, Spain, 2002). The equipment consisted of a microscope (Olympus BX41, Tokyo, Japan) equiped with a x 60 bright-field objective and a Basler video camera (CCD AVC-D7CE, Sony Corporation, Tokyo, Japan) connected to a Pentium 950 MHz processor. The illumination source was centred and the intensity of the bulb and the gain and offset of the camera were standardized for all samples. The configuration of the computer system included a PIP-1024 B video digitizer board (Matrox Electronic Systems Ltd., Quebec, Canada), the sperm image analysis software and a high-resolution assistant monitor Sony Triniton PVM-1443MD (Sony Corporation, Tokyo, Japan). The array size of the video frame recorder was 512 x 512 x 8 bits, digitised images were made up of 262.144 pixels (picture elements) and 256 grey levels. Resolution of images was 0.15 and 0.14 µm per pixel in the horizontal and vertical axes, respectively.

Table 1. Staining protocols used in this trial.

Staining procedure	Step	Reagent	Time
HM stain protocol A	1	Fixative solution	5 x 1s
	2	Stain solution A	6 x 1s
	3	Gently rinsing in buffer solution pH 7.2	1 x 10s
	4	Stain Solution B	7 x 1s
	5	Gently rinsing in buffer solution pH 7.2	45s
HM stain protocol B	1	Fixative solution	2 min
	2	Stain solution A	2 min
	3	Gently rinsing in buffer solution pH 7.2	1 x 10s
	4	Stain Solution B	2 min.
	5	Gently rinsing in buffer solution pH 7.2	45s
TB stain	1	Fixative solution, 96% ethanol: acetic acid (3:1)	1 min
	2	Ethanol (70%)	3 min
	3	Hydrolisis in 0.4 mol/l of clorhidric acid (HCL)	20 min
	4	Gently rinsing in distilled water	3 x 1s
	5	Air dried	30 min
	6	One droplet (10 µl) of TB in Mcllvaine buffer pH 4 or 5 over each smear and then coverslipped	

HM: Hemacolor®, TB: Toluidine blue.

Analysis of sperm midpiece and tail was not performed. The system detected the boundary of sperm heads and their outlines were displayed as white overlays superimposed on the video image. At least 300 sperm

cells were randomly selected for the morphometric analysis assures that a minimum of 150 properly measured sperm heads were analyzed after improperly measured sperm heads were deleted from the analysis. Computer software allowed four basic measurements of sperm head size (area in μm^2, perimeter in µm, length in µm, width in µm). The measurements of each individual sperm head from each bull were saved in an Excel® (Microsoft® Corporation, Redmond, Washington, USA)-compatible database by the software for further analysis.

Experimental design

Effect of the staining technique on the accuracy of image proceesing:

In order to determine the accuracy of the three staining technique to capture and subsequent digitisation of images, al least 300 spermatozoa from each slide were captured and subsequently analysed (n= 5783 spermatozoa over the entire set of semen sample). The sperm heads with had been converted into correct binary images were determined visually by checking if the boundary assigned by the SCA to the spermatozoa matched its microscopic image profile and correctly delineated the sperm head. When it was not possible to achieve a correct boundary, the cells were eliminated from the calculations of the mean morphometric measurements. However, the number of sperm heads incorrectly analysed and eliminated were recorded, and the subsequent percentage of properly analysed cells was assessed for each staining technique. This percentage of properly analysed sperm cells was used to determine the accurancy of the image processing, and the results were compared for the different staining techniques.

Effect of the staining technique on the sperm morphometric measurements.

At least 150 spermatozoa were analysed per slide using each of the three staining techniques over the entire set of semen samples. The morphometric parameters obtained with each method were then compared.

Statistical analysis

All data recorded were analysed by SAS/Statistical Analysis System for Windows, software 8.2 (*SAS Inst. Inc*.; Carry, NC. USA). Normality was assessed by the Kolmogorov-Smirnov normality test, included in the UNIVARIATE procedure. Also, was done the canonical discriminant analysis to assume that all bull have equal covariance matrix; in these case called the homoscedastic model. The differences among staining technique on the mean morphometric dimensions for area (A), perimeter (P), length (L), width (W), ellipticity (E), rugosity (RU), elongation (E) and regularity (RE) for all sperm heads were analysed by General Lineal Model Analyses of Variance (GLM procedure) whereas the LSMEANS procedure was used to list these mean differences.

RESULTS AND DISCUSSION

Frozed-thawed sperm head stained with Toluidine were more susceptible ($p<0.0001$) to morphometric analysis in that measurements could be made in 88.29% of the sperm head compared to 63.69% for HC (protocol A) and 50.77% HC (protocol B) (Table 2). Hemacolor (protocol A and B) were unable to stain all frozen-thawed cells with a similar intensity. The insufficient contrast allowed no recognition for digitization of several sperm head. In contrast, sperm head stained with Toluidine blue showed a good intensity of staining in the mayority of digitized images. Coefficients of variation revealed that sperm head size varies among the smears for protocols of HC staining technique; indicanding the handling procedure affected the final results. In contrast, the coefficients of variation obtained with TB were considerably lowest (Table 2).

Table 2. Summary statistic of correctly digitized (n= 5783) sperm head stained with two protocols of Hemacolor (HM®) and Toluidine blue (TB) stains in frozen-thawed bull semen (n= 33).

Staining procedure	Correctly digitized sperm head (%)	
	Mean±Error standart	Coefficient of variation (CV)
HC stain, protocol A	50.77±3,8 [c]	23.69
HC stain, protocol B	63.69± 4.5 [b]	20.06
TB stain	88.29±1.2[a]	4.54

HM: Hemacolor®, TB: Toluidine blue.

The superscript letters (a, b, c) indicate significant differences between mean values ($p<0.0001$).

Significant differences ($P<0.0001$) were observed in all dimensions of the sperm head according to the staining method. Sperm head displayed the biggest size when stained with HC (protocol A), followed by HC (protocol B) and TB ($p<0.0001$). The TB gave rise to the lowest values in the mayority of the variables; however, the shape of the head appeared slightly less rugocited (Table 3).

The accuracy of image processing using three staining techniques was compared. The results showed that all three staining procedures permited the digitalization of frozen-thawed bull spermatozoa, although some differences were found in the recognition and digitization errors to not obtain contrastation between sperm head and preparation background, finding some particles that interfere in the delineation of the sperm cells (Fig. 1). In our seminal contrast comparison study, the visual observation of the sperm head in frozen diluent with skim milk-egg yolk medium offered the best definition with TB technique, since it offered the best sperm head contrast. Toluidine blue was shown to be the most accurate methods an also the lowest variation coefficient. The spermatozoa stained obtain intense grey-level values, thus enhancing the contrast of images. In visual observation of the cell, this stain showed the best definition and sperm head background contrast and reduce the number of stained foreing particles and the boundaries correctly delineated the original microscopic image (Fig. 1).

Table 3. Size and shape morphometric values of sperm head in frozen-thawed bull semen (n= 33) associate with each staining technique.

Head sperm parameters	Staining technique ***		
	Hemacolor, protocol A	Hemacolor, protocol B	Toluidine blue
N	1652	1608	2523
Lenght (μm)	9.14±0.01	8.87 ± 0.01	8.43±0.007
Width (μm)	4.59±0.005	4.56 ± 0.005	4.43±0.004
Area (μm^2)	35.29 ± 0.05	34.17 ± 0.05	32.67±0.03
Perimeter (μm)	24.14 ± 0.02	23.53 ± 0.02	22.6 ± 0.01

Values are mean ± standard error.

Significant differences were found, *** $P< 0.0001$

N= number of spermatozoa analysed

TB is a classic nuclear (cationic) dye used for external metachromatic (purple) and orthocromatic (blue) stainning of chromatin. These features have been shown to be sensitive structural probe for DNA secondary structure and packaging in situ in human [6] and bull sperm [16]. However, TB is a very good alternative to use in morphometric analyse, because produces ligh background on which the sperm stand out as dark colored objects, since it offered the best visualize sperm.

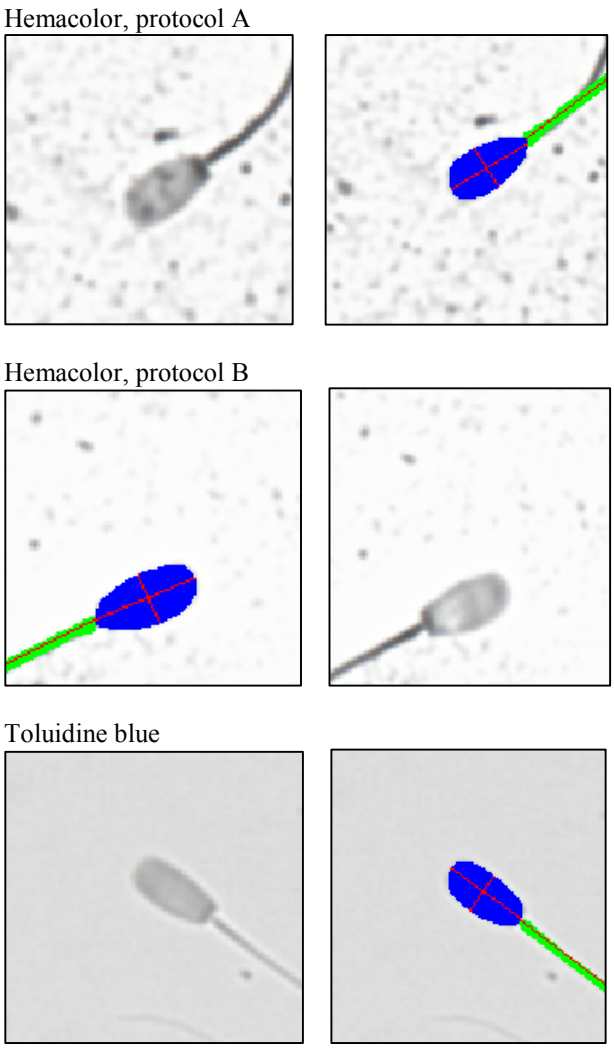

Fig. 1. Comparison of sperm head contrast among three staining techniques in frozen-thawed bull semen: Differences were found in the contrastation between sperm head and preparation background, finding some particles that interfere in the delineation of the sperm cells (Hemacolor, protocol A > protocol B). Sperm head stained with Toluidine blue showed a good intensity of staining in the mayority of digitized images.

The spermatozoa stained with HC, also obtained good grey-level values, however, the same or major intensity contrast ocurred with others particles found in the background stain. In this end case, the elimination a large number of cells was necessary, consequently slowing down the process. Also, the coefficients of variation calculated for the percentage of correctly analysed sperm head were highest. To compare the protocols done to apply HC, was evident that the best contrast and image definition was obtained by protocol B (prolonged stained). The protocol A only had the advantage to be more rapid than Protocol B and TB. These results were quite surprising because HC stain is one of the standard methods for morphometric examination of mamalian spermatozoa.

Acceptable values (60-80%) of correctly digitalized sperm head in mamalian species by staining fresh and diluted semen samples with Hemacolor have been publicated [13, 14, 21]. However, in frozen-thawed semen, is necessary to wash the sample to obtained good results. In previous studies done in bull semen and others species, the most suitable strategy to obtain good results is the use of washed semen samples. Staining like Trypan blue/ Giemsa, Papanicolaou, Spermac are not suitable for ASMA system as they result in poorly stained cells, which do not permit the correct digitalization [9]. However, the haematoxylin or the Papanicolaou with prolonged staining times provided acceptable results [4]. In this experiment, the use of TB in frozen-thawed semen permitted to evaluate the stain without wash the semen samples.

Morphometric values obtained with the SCA system were affected by the staining method. In general, the sperm staining protocols affect subtly the sperm head size by osmotic changes and dehydrating steps. The significant impact of several staining techniques on sperm head morphometry in mammalian using ASMA has also been shown in previous papers [4, 10, 12, 13, 14, 21]. In other hand, TB has been used to estimate the bull sperm dimensions [2, 3], however, the comparison with other tecniques only has been established in human [1].

In the present study, the protocols of Hemacolor have been found to provide more intense grey-level values, resulting in enlarged cells, which influence the size morphometric parameters such as length, width, area and perimeter. In general terms, the relationship between the three staining techniques for the sperm dimensions can be described as follows: HC (protocol A) > HC (protocol

B) > TB. These morphometric results obtained with HC are in accordance with other authors whose have found increased dimensions in semen samples stained with HC Protocol B to compare with Protocol A; that study demonstrated that increasing the HC staining time improve the intensity and contrast of sperm head images, but consequently increased the sperm dimensions [8, 13]. The size of the sample is also an important factor to take into consideration, and these results indicate that 100 properly digitised sperm cells were sufficient for the morphometric characterization of a frozen-thawed bull semen sample under these conditions, as the analysis of larger numbers of spermatozoa (150 or 200) produced similar measurements. The analysis of 100 spermatozoa obtained accurate measurements and greatly reduces the time required to perform an analysis, which was in agreement with results obtained in rams [20], goats [14] and stallions [13]. This number of spermatozoa is enough to represent the complete sperm population in samples obtained from species with a high constancy in sperm dimensions and shape such as small ruminants [14, 20]. The high precision and reproducibility of the method supported the fact that it is sufficient to analyse only 100 sperm heads to obtain reliable results [13, 14].

CONCLUSION

The morphometric analysis of the frozen-thawed spermatozoa was influenced by the staining procedure. TB could be considered a good staining method to stain frozen-thawed sperm to analyse with ASMA system, based on the greater percentage of analysable cells and the lower variability of results obtained.

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