HEAD DIMENSIONS OF BRAHMAN AND THEIR CROSSBRED BULL SPERMATOZOA ARE AFFECTED BY CRYOPRESERVATION.

Las Dimensiones de la Cabeza del Espermatozoide de Toros Brahman y sus Mestizos son Afectadas por la Criopreservación.

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ABSTRACT

The objective of this study was to determine the effect of cryopreservation on morphometrics characteristic of Brahman and their crossbred bull sperm heads. Five ejaculates were collected from 4 bulls and diluted at 30°C in a skim milk-egg yolk extender. Two microscope slides were prepared from single extended sperm samples prior to freezing in nitrogen vapors, and another one after thawing, sperm smears were prepared as described above. All slides were air dried and stained with Hemacolor®. Sperm-head dimensions for a minimum of 150 sperm heads/samples were analysed from each sample by means of the Sperm-Class Analyser® (SCA), and the mean measurements recorded. A GLM procedure was performed to evaluate the effect of cryopreservation on sperm head morphometric dimensions. Bull sperm heads were significantly (P<0.001) smaller in frozen-thawed spermatozoa than in the extended samples for length (9.00 µm vs. 9.43 µm), width (4.82 µm vs. 5.13 µm), perimeter (32.46 µm vs. 33.69 µm) and area (36.20 µm^2 vs. 39.97 µm^2) for all bulls. Also, differences (P<0.001) were found within all bulls for whole morphometric parameters. The individual variability of sperm head measurements across all bulls ranged from 5.9% to 10.2% for fresh and thawed samples, respectively. In conclusion, the present study indicate that cryopreservation of bull semen did affect the morphometry to reduce the dimensions of Brahman and crossbred bull sperm heads. The differences among bulls may be indicative of the individual bull resistance to the cryopreservation process.

Key words: Cryopreservation, morphometry, sperm heads, Brahman bulls.

RESUMEN

El objetivo de este estudio fue determinar el efecto de la criopreservación sobre las características morfométricas de las cabezas de espermatozoides de toros Brahman y sus mestizos. Cinco eyaculados fueron colectados de 4 toros y diluidos a 30°C en una solución de leche descremada-yema de huevo. Por cada muestra se hicieron dos frotis: uno del semen diluido, antes de su congelación en vapores de nitrógeno líquido, y otro de semen descongelado una semana después de la congelación. Todos los frotis fueron secados al aire y coloreados con Hemacolor®. Se analizaron las dimensiones de la cabeza espermática para un mínimo de 150 espermatozoides por muestra mediante el *Sperm Class Analyser*® (SCA). El procedimiento GLM se realizó para evaluar el efecto de la criopreservación sobre las dimensiones morfométricas de las cabezas espermáticas. Las cabezas espermáticas de los toros fueron significativamente (P<0,001) menores en los espermatozoides criopreservados que en las muestras frescas para la longitud (9,00 µm vs. 9,43 µm), el ancho (4,82 µm vs. 5,13 µm), el perímetro (32,46 µm vs. 33,69 µm) y el área (36,20 µm^2 vs. 39,97

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μm^2) para todos los toros. Así mismo, se encontraron diferencias (P<0,001) de todos los parámetros morfométricos de los toros evaluados, encontrándose dimensiones de cabeza menores en las muestras descongeladas. La variabilidad individual (CV) de las medidas de cabeza espermática de los toros osciló entre el 5,9 y el 10,2% para las muestras frescas y descongeladas, respectivamente. En conclusión, este estudio indica que el proceso de criopreservación de semen de toro afecta la morfometría, al reducir las dimensiones de la cabeza espermática de toros Brahman y sus cruces. Las diferencias entre los toros evaluados puede ser indicativo de diferencias individuales al proceso de criopreservación.

Palabras clave: Criopreservación, morfometría, cabezas espermáticas, toros Brahman.

INTRODUCTION

Sperm analysis are currently considered as the most desirable approach to evaluate the reproductive ability of bulls [16].This analysis, including normal sperm morphology assessment, has long been employed to evaluate the effects of freezing-thawing procedures on sperm cryosurvival. Poor semen morphology is an important indicator of decreased fertility in men [22], stallions [20], bulls [26] and goats [6, 28]. In particular, the occurrence of abnormal sperm head morphology is associated with lower fertility in the bull [2], and although normal sperm morphology may be an indicator of the fertility potential of a given sample, correlations have been based on subjectively performed analyses. However, large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist [17], making accurate interpretation of the resulting data difficult. The need for accurate objective assessment of sperm morphology has led to the development of computer-assisted sperm head morphometry analysis, (ASMA), which facilities the rapid examination of a high number of mammalian [5, 8, 11, 12, 17-19, 21] and fish [24, 33] spermatozoa.

Previous works utilizing computer-assisted spermmorphometry analysis (ASMA) have also demons- trated that cryopreservation affect sperm head morphometry of bull [14], human [31], goat [13], stallion [1], dog [25], and red deer [9] cryopreserved spermatozoa. In these studies, sperm heads were significantly smaller in the cryopreserved samples than in the fresh-extended ones. The differences in morphometric dimensions between fresh and cryopreserved spermatozoa have been explained by several possible mechanisms including osmotic changes, acrosome damage and alterations in chromatin condensation [4, 14, 30].

Thus, the aim of the present study was to determine the effects of cryopreservation (extended versus frozen-thawed) on sperm head dimensions in bull semen (Brahman and their crosses) by ASMA analyses.

MATERIALS AND METHODS

Semen collection and processing

Five ejaculates from 4 fertile bulls (two Brahman and two Holstein x Brahman), 3-5 years old, in regular service were obtained from the artificial insemination plant of VI-ATECA at Machiques, Zulia State, Venezuela. These bulls produced high quality semen with normal sperm morphology and good fertility. The ejaculates were collected between 6:00 and 8:00 am. After semen collection by using an artificial vagina, 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 sperm samples were diluted at 30°C to 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 30°C and 2h later, the second diluter (B) at 5°C. Seminal samples in the “diluent A” were cooled down slowly up at 5°C. 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 5°C 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°C 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.

Morphometric analysis of sperm heads

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 Hemacolor® (standard kit from Merck, Darmstadt, Germany) and permanently mounted procedure, originally described for staining of ram [11] and alpaca [5] sperm heads. Stained sperm samples were permanently mounted to the slide with a coverslip and dibutyl phathalate xylene (DPX). The morphometric evaluation was done in the laboratory of Biology of Reproduction of “Universidad de Castilla La Mancha”, Spain. Stained slides were used to perform ASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer®, Microptic, Barcelona, Spain). The equipment consisted of a Nikon (Labophot-2, Tokyo, Japan) microscope with a x 60 bright-field objective and a Sony 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 digitiser 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 0.11 µm per pixel in the horizontal and vertical axes, respectively.

Analysis of sperm midpiece and tail was not performed. The system only detected the boundary of sperm heads and their outlines were displayed as white overlays superimposed on the video image (FIG. 1). Computer software allowed four basic measurements of sperm heads to be obtained (area in μm^2, perimeter in µm, length in µm, width in µm) for 200 images. Acquiring 200 images assures that a minimum of 150 properly measured sperm heads are analysed after improperly measured sperm heads are deleted from the analysis. The sperm cells were randomly selected for the morphometric analysis. 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.

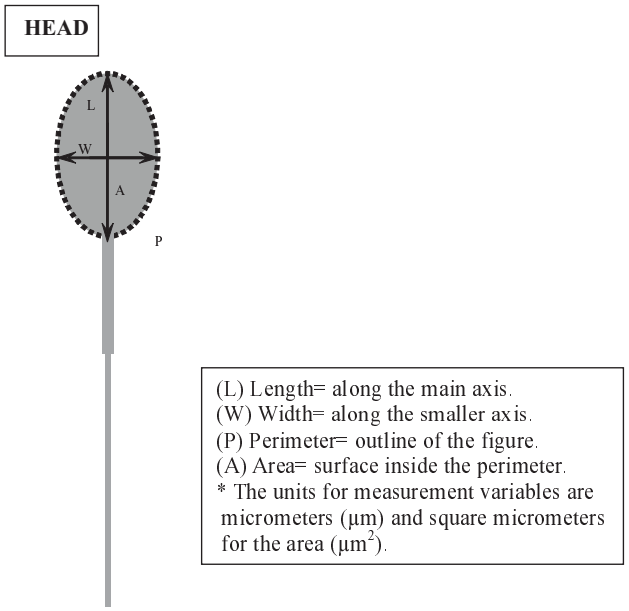

FIGURE 1. MORPHOMETRIC PARAMETERS EXAMINED IN THIS STUDY/ PARÁMETROS MORFOMÉTRICOS VALORADOS EN ESTE ESTUDIO.

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 effects of the cryopreservation procedure on the mean morphometric dimensions (extended or frozen-thawed) for Area (A), Perimeter (P), Length (L) and Width (W) for all sperm heads were analysed within and across bulls by general lineal model analyses of variance (GLM procedure) whereas the LSMEANS procedure was used to list these mean differences.

RESULTS AND DISCUSSION

In the present study, 2247 spermatozoa from fresh and frozen-thawed sperm samples were analysed using an ASMA system (Sperm-Class Analyzer®, SCA), in an attempt to quantify the morphometric dimensions of sperm heads from bulls. All samples maintained postthaw sperm motility >30%.

The results of the comparison of the morphometric dimensions of sperm heads from extended and thawed samples are summarised in TABLE I. A total of 550 property digitised spermatozoa for extended samples, and 1697 for cryopreserved samples were analysed. There were no differences in the number of properly analysed sperm heads between extended and cryopreserved samples (data not shown). The values for all measures of sperm heads dimensions were determined to be normally distributed by Kolmogorov-Smirnov normality test. In the present work is heteroscedasticity, however, the assumption that there is homoscedasticity does not invalidable; the errors obtained (data no shown) with the discriminant function were small, demonstrating that the differences sperm of *B. taurus* and taurus-indicus crossbred is considerable.

Analysis of variance showed a significant effect of treatment ($P<0.01$) on sperm head morphometry. Bull sperm heads were significantly ($P<0.001$) smaller in the cryopreserved samples than in the companion extended ones for length (9.00 µm vs. 9.43 µm), width (4.82 µm vs. 5.13 µm), perimeter (32.46 µm vs. 33.69 µm) and area (36.20 μm^2 vs. 39.97 μm^2) for all bulls. There are few studies on bull sperm head computerized morphometry [3, 10, 14, 15]. Comparing with these studies the morphometric values of fresh obtained samples were somewhat different. As a general rule sperm head size was larger in this study, this difference may be attributable to many different factors as fixation technique, staining procedure, individual variation or ASMA system employed. Conversely, the results are very similar to those obtain by Foote [10], using various methods to estimate bull sperm head area when spermatozoa were stained with India ink. He found overall sperm head area

means of 41.5 μm^2 and 41.6 μm^2 for unfixed and fixed samples, respectively. These values are very similar to those found in the present study (39.97 μm^2). However, when this author used the Feulgen procedure for morphometric spermatozoa evaluation, as expected, the Feulgen-stained portion of the sperm head (26.2 μm^2) was much smaller than the total head area; thus, the percentage of the total sperm head occupied by the DNA was on average 63% [10]. Taken together, all these results indicate that the main factor responsible of the differences in measured sperm size and morphometry between studies may be the staining procedure employed. In this sense, the accurate interpretation of the results should be different when nuclear or cellular stains were used.

The coefficients of variation (CV) of the mean measurements across all bulls ranged from 4.58% (L) to 10.16% (A) for fresh samples, and from 5.56% (L) to 10.00% (A) for cryopreserved samples (TABLE I). These CVs indicate sperm head measurements from different bulls are heterogeneous, at least as heterogeneous as the population within an ejaculate of a given bull. Therefore, it is possible that ASMA may be a sensitive and objective means of determining the freezeability of individual bull.

TABLE II shows the morphometric dimensions of the sperm heads of the four bulls from extended and thawed samples. The results of the GLM procedure revealed that sperm head measurements were significantly (P<0.001) affected by the interaction between individual factor (bull) and cryopreservation step for sperm head A, L, W and P. This was evidenced a decrease of all morphometric dimensions in frozen-thawed samples. Only morphometric parameter that did not present a marked diminution after the cryopreservation it was the P. Two of the evaluated bulls (1 and 4) did not show significant differences for the treatment effect for this variable. In general, the measures for extender and frozen- thawed semen related to size, such as area, perimeter, width, length were smaller for Brahman sperm cell than Holstein-Brahman sperm cell. This difference could be intrinsic to *B. taurus* specie. The morphometric differences observed between the *B. taurus* and *B. taurus- indicus* sperm cell indicate that the geometrical characterization of bull sperm through image analysis shoulb take into account morphological peculiarities that are specific to each subspecies, in order to avoid such morphological variation to be mistaken abnormalities [3].

The differences in morphometric dimensions between fresh and cryopreserved spermatozoa have been explained by several possible mechanisms, including osmotic changes, acrosome damage and alterations in chromatin condensation [4, 8, 14, 30].

One possible hypothesis for the reduction of sperm head dimensions spermatozoa of all bulls may be an increase in the number of spermatozoa in which acrosomal damage or loss

TABLE I

SPERM HEAD DIMENSIONS (LENGTH, WIDTH, AREA AND PERIMETER) FOR EXTENDED AND FROZEN-THAWED BULL SEMEN SAMPLES (Mean ± standard error)/ DIMENSIONES DE LA CABEZA ESPERMÁTICA (LONGITUD, ANCHO, ÁREA Y PERÍMETRO) PARA MUESTRAS SEMINALES FRESCAS Y DESCONGELADAS DE TOROS (Media ± error estándar).

Treatment	Nº	Length in μm	Width in μm	Area in μm^2	Perimeter in μm
Extended	550	9.43±0.02 ^{a} (5.94)	5.13±0.01 ^{a} (7.18)	39.97±0.17 ^{a} (9.02)	33.69±0.11 ^{a} (6.90)
Thawed	1697	9.00±0.01 ^{b} (6.56)	4.82±0.009 ^{b} (8.26)	36.20±0.09 ^{b} (10.80)	32.46±0.06 ^{b} (9.35)
Difference	-	0.43	0.31	3.77	1.23

Coefficients of variation (% CV) between bulls are shown in parentheses. Values with different superscripts (a, b) in the same column were significantly different (P<0.001).

TABLE II

EFFECTS OF CRYOPRESERVATION ON THE MORPHOMETRIC DIMENSIONS OF BULL SPERM HEAD/ EFECTOS DE LA CRIOPRESERVACION SOBRE LAS DIMENSIONES MORFOMÉTRICAS DE LA CABEZA DEL ESPERMATOZOIDE DE TORO.

Bulls	Morphometric parameters			
	Length in μm F/T	Width in μm F/T	Area in μm^2 F/T	Perimeter in μm F/T
1. Brahman (536)	9.02/ 8.72*	5.19/ 4.93*	38.63/ 35.80*	32.27/ 32.54
2. Brahman (444)	9.32/ 8.86*	4.88/ 4.70*	37.35/ 35.52*	34.35/ 30.90*
3. Holstein-Brahman (553)	9.89/ 9.42*	5.24/ 4.78*	42.91/ 37.57*	35.27/ 32.66*
4. Holstein-Brahman (714)	9.51/ 8.99*	5.22/ 4.86*	41.00/ 35.92*	32.85/ 33.75

The total number of analyzed sperms was 2247.
Numbers between parentheses represent the numbers of sperms analyzed by each bull.
F/T= samples extended/ thawed, *= significantly different for effect of the treatment (P<0.01).

occurred during cryopreservation and thawing [30]. The proportion of acrosome reacted spermatozoa is significantly increased after freezing and thawing [7]. Cryopreservation is also responsible for membrane damage of bovine [32] and human [23] spermatozoa. The decrease in normal sperm morphology resulting from cryopreservation may be also associated with changes in the sperm chromatin structure [14]. Studies investigating chromatin condensation and morphology of spermatozoa suggest that abnormal chromatin condensation morphological abnormalities. A variety of spermatozoal injury, such as heat stress and cryopreservation can induce changes in sperm chromatin structure, resulting in acid-induced denaturation [4]. Because the present research did not touch on the morphology of the nucleus and acrosome separately, it cannot provide evidence of the contribution of these two sperm compartments to the reduction of sperm head dimensions in the bull evaluated through the detrimental effect of cryopreservation procedure. However, the entire bull cryopreservation process normally leads to an increase in the number of dead sperm with loss of the acrosomal and plasma membranes during cell death [9]. Therefore, it appears that the decreasing of the dimensions of sperm cells within current work could be explained, at least partially, by the damage or loss of the acrosomes, as cited by Gravance et al [14]. The increasing of the percentage of acrosomes reacted, might be the responsible of the lower overall means for all sperm head dimensions found in this study, for cryopreserved samples.

The application of computerized image analysis for sperm head morphological characterization allows the identification of minor morphological alterations and minute differences between bull sperm cells, which would otherwise be unnoticed by traditional visual inspection. However, little is known about the influence of such small morphological alterations as related to male fertility [29, 34]. The morphological differences observed between the Brahman and Holstein-Brahman crossbred bulls identified in the current work clearly indicate that the morphometrical characterization of bull sperm through image analysis should take into account morphological peculiarities that are specific to each bulls, in order to avoid such morphological variations to be mistaken as abnormalities.

The detection of freeze/ thaw effects within four bulls may be also an indication that some individuals may be more sensitive to cryopreservation. Individual differences in freezability based on postthaw fertility have been previously established [27].

CONCLUSIONS

The results of the current study indicate that cryopreservation of bull semen did affect the morphometry to reduce the dimensions of Brahman and crossbred bull sperm heads. The statistical analysis showed considerable differences among males, which may be indicative of the individual bull resistance to the cryopreservation process.

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