DISTRIBUTION OF SPERM WITH DAMAGED CHROMATIN AMONG MORPHOMETRICALLY DISTINCT SUBPOPULATIONS IN CRYOPRESERVED SEMEN FROM BRAHMAN BULLS

Distribución de Espermatozoides con Cromatina Dañada en Subpoblaciones Morfométricamente Distintas en Semen Criopreservado de Toros Brahman

Héctor Nava-Trujillo [1,2] y Armando Quintero-Moreno [2]

*[1]Producción Animal, Instituto de Investigaciones Agropecuarias, Facultad de Ciencias Forestales y Ambientales, Universidad de los Andes, Mérida, Mérida, Venezuela. [2] Laboratorio de Andrología, Unidad de Investigación en Producción Animal, Facultad de Ciencias Veterinarias, Universidad del Zulia, Maracaibo, Estado Zulia, Venezuela. *Corresponding author: hectornava00@gmail.com*

ABSTRACT

The aims of this study were to identify and characterize the morphometrically distinct sperm subpopulations into cryopreserved bull semen and to determine how sperm with damaged chromatin are distributed in each subpopulation. To this, sperm of fifteen cryopreserved ejaculates from Brahman bulls were stained with toluidine blue and chromatin integrity status was determined to each sperm, and simultaneously sperm head morphometry was determined using a CASMA software. Posteriorly, CLUSTER analysis to determine the number of sperm morphometrically distinct subpopulations according to morphometric parameters (length, width, area and perimeter) was carried-out, and the distribution of sperm with damaged chromatin in each sperm subpopulation was determined. A total of 2,273 sperm were analyzed, 70.35% with normal chromatin and 29.65% with damaged chromatin. CLUSTER analysis identified three sperm subpopulations, SP1 (smallest sperm-head) with 143 sperm (6.29%), SP2 (intermediate sperm-head) with 1,376 sperm (60.54%) and SP3 (biggest sperm-head) with 754 sperm (33.17%). Percentage of sperm with damaged chromatin was lower in SP2 (20.58%; P<0.05) in comparison with SP1 (53.15%) and SP3 (42.31%). No differences were observed between SP1 and SP3 (P>0.05). In conclusion, Cluster analysis identified three morphometrically distinct subpopulations in cryopreserved bull semen, and sperm with damaged chromatin were presented in all these subpopulations, with intermediate sperm-head subpopulation having the lowest percentage of sperm with damaged chromatin, suggesting that this sperm subpopulation has more stable chromatin.

Key words: Sperm chromatin**;** sperm head; toluidine blue; morphometry; subpopulations.

RESUMEN

Los objetivos de este estudio fueron identificar y caracterizar subpoblaciones espermáticas morfométricamente distintas en semen criopreservado de toros y determinar cómo los espermatozoides con cromatina dañada se distribuyen en cada subpoblación. Para esto, espermatozoides criopreservados de toros Brahman fueron teñidos con azul de toluidina para determinar la integridad de la cromatina y simultáneamente se determinó la morfometría de cada cabeza espermática usando un software para AMAC (Análisis Morfométrico Asistido por Computadora). Posteriormente, se realizó un análisis CLUSTER para determinar el número de subpoblaciones morfométricamente distintas de acuerdo a los parámetros morfométricos (largo, ancho, área y perímetro), y la distribución de los espermatozoides con cromatina dañada en cada subpoblación fue determinada. Un total de 2.273 espermatozoides fueron analizados, 70,35% con cromatina normal y 29,65% con cromatina dañada. El análisis CLUSTER identificó tres subpoblaciones, SP1 (cabezas pequeñas) con 143 espermatozoides (6,29%), SP2 (cabezas intermedias) con 1.376 espermatozoides (60,54%) y SP3 (cabezas grandes) con 754 espermatozoides (33,17%). El porcentaje de espermatozoides con daño en la cromatina en la SP2 (20,58%, P <0.05) fue menor que en SP1 (53,15%) y SP3 (42,31%). No se observaron diferencias entre SP1 y SP3 (P>0,05). En conclusión, se identificaron tres subpoblaciones espermáticas morfométricamente distintas en el semen criopreservado de toros Brahman y los espermatozoides con cromatina dañada estuvieron presentes en las tres subpoblaciones, con la subpoblación de cabezas intermedias (SP2) teniendo el porcentaje más bajo de espermatozoides con daño en la cromatina, sugiriendo que estos espermatozoides tienen una cromatina más estable.

Palabras clave: Cromatina espermática; cabeza espermática; azul de toluidina; morfometría; subpoblaciones.

Recibido: 26/01/2017 Aceptado: 08/06/2017

INTRODUCTION

Classical sperm quality evaluation includes the measure of integrity and functionality of sperm membrane, acrosome integrity, motility, morphology and capacitation and several studies have reported a relationship between these parameters and bull (*Bos taurus*/*Bos indicus*) fertility [10, 13, 27]. Currently, other parameters have become relevant. This is the case of chromatin integrity and sperm head morphometry. Damaged chromatin has a negative correlation with other parameters of sperm quality, embryo development and pregnancy rate [9, 15, 16, 18, 22, 29, 31, 48]. Few studies about relationship between sperm head morphometry and another sperm quality parameters or fertility has been published. In bull semen, pre-freeze morphometry parameters were related with fertility [11]. In Iberian red deer (*Cervus elaphus hispanicus*) semen, changes in the frequency distribution of sperm within some subpopulations after cryopreservation were related with post-thaw sperm quality [8], while in boar (*Sus scrofa domesticus*), sperm head morphometric parameters in fresh semen were related with post-thaw membrane integrity and motility [36].

Because sperm head is composed by chromatin (DNA condensed by protamine proteins), the chromatin integrity, the shape sperm head and the sperm head morphometry should be related, and several studies have been published. Variation of sperm head morphology is a good indicator of chromatin integrity [17] and sperm with damaged chromatin have more morphoanomalies than sperm with normal chromatin [2, 7, 30]. However, the relationship between chromatin integrity and sperm head morphometry is more controversial. Sailer et al. [44] observed a positive correlation between morphometric parameters with sperm susceptibility. To DNA denaturation measured with Sperm Chromatin Structure Assay (SCSA), but Beletti et al. [6] observed that sperm head with less packed chromatin have lower area than sperm head with normal chromatin. While, Nava-Trujillo *et al*. [32] observed than bull sperm with damaged chromatin had more width and area and less ellipticity than sperm with normal chromatin. Since both canine (*Canis familiaris*) and bull (*Bos taurus; Bos indicus*) sperm contain only protamine 1 [21, 23], and in canine, sperm head with fragmented chromatin were more elliptical [20] and a relationship between morphometric sperm subpopulations and damaged chromatin integrity has been observed [33], a similar situation can be expected in bull sperm.

The association between Computer-assisted Sperm Morphometry Analysis (CASMA) and statistical software have allowed cluster the spermatozoa according to morphometric parameters in morphometrically distinct subpopulations [8, 25, 36, 43]. Recently, toluidine blue, a nuclear stain used to evaluate chromatin integrity [4, 26, 31] has been used successfully to morphometric analysis by CASMA [4, 34, 38], and this combination has opened the possibility to evaluate simultaneously the sperm head morphometry and chromatin integrity as well as their relationship and could be a powerful tool to improve the results of spermiogram [33]. Currently, there are no studies about another characteristics different to morphometric parameters, like chromatin integrity, of sperm into each morphometrical subpopulation in bull semen, therefore, the purpose of this study was to identify and characterize the morphometrically distinct subpopulations of cryopreserved bull sperm and to determine how sperm with damaged chromatin are distributed in each subpopulation, using a combination of toluidine blue stain and CASMA.

MATERIALS AND METHODS

Semen collection and processing

Three ejaculates from five fertile Brahman bulls, 5-8 years old, in regular service were obtained from the artificial insemination center of VIATECA at Machiques, Zulia State, Venezuela. 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, containing 15% of skim milk, 1% of egg yolk, 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 extender (A) was added at 30°C and two (h) later, the second extender (B) at 5°C. Seminal samples in the “extender A” were cooled down slowly up at 5°C. This cooling up at 5°C lasted 2 h approximately. The second extender differed of the first one in the substitution of water (14%, v/v) with the same glycerol volume (final concentration = 7%). Then, the diluted sperm was 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 centimeter (cm) above the surface of the liquid nitrogen, for 10 minutes (min) and then plunged into liquid nitrogen (MVE® Millenium 2000, XC20, Minnesota, USA). One week after, thawing was carried out by placing the straws in a water bath (Gemmy® modelo YCW-03S, Taipei, Taiwán) at 37 °C for 20 seconds (s). and the sperm was allowed to equilibrate for 5 min before evaluation. After thawing, samples were taken for sperm head morphometric and chromatin evaluation.

Chromatin integrity

Chromatin integrity was evaluated with toluidine blue stain [26]; with this procedure, sperm with normal chromatin look light blue or green, whereas dark blue or violet sperm are considered as having damaged chromatin. Sperm slides were prepared and stained as described by Beletti et al. [4]. Five µL of semen were placed on the clear end of a frosted slide and dragging the drop across the slide, air dried and fixed in ethanol–acetic

acid (3:1, v/v) for 1 min and 70% ethanol for 3 min; then, smears were hydrolyzed for 25 min in 4 N of chloridric acid, rinsed in distilled water and air-dried. One droplet of 0.025% toluidine blue in McIlvaine buffer (sodium citrate-phosphate) pH 4.0 was placed over each smear and then coverslipped.

Morphometric analysis of sperm heads

Toluidine blue stained sperm heads were evaluated 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 ax 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 centered 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, digitized 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.

The system only detected the boundary of sperm heads and their outlines were displayed as white overlays superimposed on the video image and four measurements of sperm heads were obtained (length in µm, width in µm, area in μm^2, perimeter in µm). The sperm heads, regardless of morphology, were selected for the morphometric analysis and simultaneously were classified according chromatin integrity. The measurements of each individual sperm head and their chromatin integrity status (normal or damaged) were saved in an Excel® (Microsoft® Corporation, Redmond, Washington, USA)-compatible database by the software for further analysis.

Statistical analysis

Data analysis was performed using SPSS 20 for windows computer program (SPSS Inc, Chicago, IL, USA). All sperm head measurements were clustered by length, width, area, perimeter using iterative *k*-means cluster analysis to classify spermatozoa into a reduced number of subpopulation according to their head dimensions and then a step-wise discriminant analysis of the clusters was obtained [36]. The effects of cluster on the measurements of length, width, area and perimeter were analyzed by generalized linear model analysis of variance, and the effect of chromatin integrity (normal or damaged) on the relative frequency of spermatozoa into each morphometric subpopulation was analyzed by Mantel-Haenszel Chi-square tests.

RESULTS AND DISCUSSION

A total of 2,273 sperm head stained with toluidine blue were evaluated simultaneously to morphometry with CASMA system (SCA®) and chromatin integrity, 70.35% of sperm were with normal chromatin and 29.65% with damaged chromatin. After cluster analysis, according to morphometric parameters (length, width, area and perimeter) three morphometric distinct subpopulations were identified. Head dimensions to each subpopulation are summarized in TABLE I. The subpopulation 1 (SP1) was composed by the 6.29% of sperm and this included the smallest sperm head dimensions. Subpopulation 2 (SP2) characterized by spermatozoa with intermediate sperm head dimensions was integrated by the 60.54% of sperm; while subpopulation 3 (SP3) corresponding to the biggest sperm head was represented by the 33.17% of sperm.

Proportion of spermatozoa with damaged chromatin was significantly lower in the subpopulation with intermediate sperm head (SP2) with 20.28%, in comparison with the 53.15% and 42.31% in SP1 and SP3, respectively (TABLE II). No differences in the proportion of sperm with damaged chromatin between SP1 and SP3 were observed.

TABLE I
STATISTICAL DESCRIPTORS AND PERCENTAGE OF SPERM HEADS WITHIN EACH SUBPOPULATION FOR THAWED BULL SPERMATOZOA

	Sperm Subpopulation		
Morphometric Parameters	1	2	3
Length (µm)	6.97 ± 0.54[c]	7.77 ± 0.007[b]	8.30 ± 0.01[a]
Width (µm)	3.86 ± 0.31[c]	4.18 ± 0.004[b]	4.44 ± 0.008[a]
Area (μm^2)	23.69 ± 0.24[b]	28.51 ± 0.02[b]	32.10 ± 0.07[a]
Perimeter (µm)	19.03 ± 0.11[c]	21.05 ± 0.01[b]	22.57 ± 0.06[a]
Relative frequency %, (n)[*]	6.29 (143)[c]	60.54 (1376)[a]	33.17 (754)[b]

[a,b,c] values with different superscripts in the same row were significantly different (P < 0.0001). Results are presented as means ± standard error. The total number of spermatozoa analyzed was 2273.
[*]Relative frequency, values of chi-square test and Mantel-Haentsel chi-square revealed significant differences in the percentage of spermatozoa to each cluster (P < 0.0001).

TABLE II
PROPORTIONAL DISTRIBUTION OF SPERM ACCORDING TO CHROMATIN INTEGRITY STATUS IN EACH SUBPOPULATION

Sperm Subpopulation	Chromatin Integrity	
	Normal	Damaged
1	46.85[b]	53.15[a]
2	79.72[a]	20.28[b]
3	57.69[b]	42.31[a]

[a,b] Values with different superscripts in the same column were significantly different (P< 0.0001). The total number of spermatozoa analyzed was 2,273.

Mammalian ejaculate is not composed by a homogenous population of spermatozoa. Unlike, semen of different species have different sperm subpopulations according to motility [1, 24, 37, 39], morphometry [8, 25, 36, 43, 46], chromatin integrity [33] or morphology and chromatin integrity [7, 30]. In this study, three morphometrically distinct subpopulations were identified by cluster analysis, smallest (SP1), intermediate, (SP2) and biggest (SP3) and different proportions of sperm to each one were observed, with SP2 having the highest frequency and this is in agree with Rubio-Guillén *et al*. [43]. In Iberian red deer (*Cervus elaphus hispanicus*), subpopulation with more spermatozoa was those composed by the biggest sperm with 42.94% [8]. Sperm distribution within each subpopulation is affected by bull and cryopreservation process [43]. Cryopreservation reduce sperm head size [11, 12, 42]. Sperm head dimensions to each subpopulation observed in the present study were lower than those reported to thawed semen from Brahman bulls [43], and this difference could be due to the stain used (Hemacolor®), while in the present study it was used toluidine blue and this stain has been reported by reduce the sperm head size in comparison with Hemacolor [38]. In addition, it has been observed than sperm head from *Bos indicus* bulls are smaller than those form *Bos taurus* bulls or their crossbred [5, 34, 42].

In the present study, SP1, comprised by the smallest sperm head, had more than 50% of sperm with damaged chromatin. Sperm with less packed chromatin had a lower head area than sperm with normal chromatin [6] and a negative correlation between chromatin decondensation with sperm head area and perimeter has been observed [4], although this situation was not observed in sperm after Percoll centrifugation [34].

In SP3 (biggest sperm head), 42.31% of sperm had damaged chromatin. Probably an extensive loss of protamine could cause a more extensive decondensation of chromatin resulting in an increase of sperm head size. In human, percentage of sperm stained positively with Chromomycin A3, an indication of deprotamination, had a positively correlation with macrocephaly sperm [14]. Additionally, in bull, sperm head area after a decondensation treatment was positively correlated with retention of histones [35]. Diploidy has been related with an increase of sperm head area and alteration in chromatin condensation in bulls [41], although in stallion sperm, nuclear area was similar between haploid and diploid sperm, but a significant difference was observed among these two subpopulations and polyploid sperm [45]. Additionally, in bull it was observed than dead sperm have higher sperm head area than live sperm [40].

Abnormal chromatin in bull (*Bos taurus; Bos indicus*) sperm is heterogeneous and affects various regions of sperm head [3, 4] and its impact on sperm head morphometry (reduction, no effect, or increase) could depend of the alteration in the DNA-protamine link or another structure like matrix or annulus of sperm nucleus [4]. This could explain why there are sperms with damaged chromatin into the three sperm subpopulations determined in the present study. The present study was carried-out with cryopreserved sperm, and cryopreservation affects both morphometry [11, 12, 42, 43] and chromatin [19, 28, 47]. Now, what factors could explain why SP2 presented the lowest percentage of sperm with damaged chromatin, is an unanswered question. Spermatozoa in SP2 could represent a subpopulation resulting from cryopreservation process with more resistant chromatin. Cryopreservation affect the distribution of sperm into each subpopulation, and it has been observed that percentage of sperm into the subpopulation of intermediate sperm head increased from 39.24% in fresh semen to 50.45% in thawed semen, while percentage of sperm into the biggest sperm head subpopulation decreased from 52.06% in fresh semen to 15.51% in thawed semen and percentage of sperm into the smallest sperm head subpopulation increased from 8.70% in fresh semen to 34.04% in thawed semen [43], according to these results, intermediate sperm head subpopulation in thawed semen, is in part composed by sperm from the biggest sperm head subpopulation in fresh semen, to which the cryopreservation reduced the head size and also in few cases affected the chromatin integrity.

Although these reasons are purely speculative, the results of this study have important implications to the way that sperm quality is evaluated, to the design of cryopreservation protocols and to the evaluation of cryopreservation effects on sperm quality and the estimation of bull fertility and further research should be granted. Probably the use of fresh semen could offer clearer results, since the effect of cryopreservation process on head morphometry and chromatin integrity would avoid.

CONCLUSIONS

In conclusion, three morphometrically distinct subpopulations in thawed bull semen were identified, with most of the sperm into the subpopulation represented by sperm head with intermediate dimensions and within this subpopulation presenting the lowest percentage of sperm with damaged chromatin, suggesting that this sperm group has a more stable chromatin or than morphometric characteristics of these sperm could provide more protection or resistance to the chromatin. Additionally, toluidine blue is an appropriate stain to the simultaneously measure of morphometry

and chromatin integrity in bull sperm, which could improve the routine sperm quality evaluation and fertility estimation.

ACKNOWLEDGEMENT

This work has been supported by Council of Scientific and Humanistic Development of Zulia University (CONDES-LUZ, grant CC-0056-13) and Fundo La Rosita (El Guayabo, Zulia, Venezuela).

BIBLIOGRAPHIC REFERENCES

[1] ABAIGAR, T.; HOLT, W.V.; HARRISON, R.A.P; DEL BARRIO, G. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr)* semen as revealed by pattern analysis of computer assisted motility assessments. **Biol. Reprod.** 60: 32-41. 1999.

[2] ACEVEDO, N. Effects of scrotal insulation on spermatozoal morphology and chromatin stability to acid denaturation in the bovine. Virginia State University. Grade Thesis. 84 pp. 2001.

[3] BELETTI, M.E. A computational approach to characterization of bovine sperm chromatin alterations. **Biotech. Histochem.** 70:17-23. 2004.

[4] BELETTI, M.E.; COSTA, L.; MENDES, M. Morphometric features and chromatin condensation abnormalities evaluated by toluidine blue staining in bull spermatozoa. **Braz. J. Mophol. Sci.** 22:85-90. 2005a.

[5] BELETTI, M.E.; COSTA, L.; VIANA, M.P. A comparison of morphometric characteristics of sperm from fertile *Bos taurus* and *Bos indicus* bulls in Brazil. **Anim. Reprod. Sci.** 85:105-116. 2005b.

[6] BELETTI, M.E.; LEMOS, M.S.; GAMA-CAMPOS, M.N.; PINTO, D.A.; MORAES, A.S. Less packed states of chromatin leads to volume changes of heads of bull sperm. **Proceedings of 15th Meeting of the Brazilian Society for Cell Biology**. São Paulo, Brazil. Abstract 57. 2010.

[7] ENCISO, M.; CISALE, H.; JOHNSTON, S.D.; SARASA. J.; FERNANDEZ, J.L.; GOSÁLVEZ, J. Major morphological sperm abnormalities in the bull are related to sperm DNA damage. **Theriogenol.** 76:23-32. 2011.

[8] ESTESO, M.C.; FERNÁNDEZ-SANTOS, M.R.; SOLER, A.J.; MONTORO, V.; MARTÍNEZ-PASTOR, F.; GARDE, J.J. Identification of sperm-head morphometric subpopulations in iberian red deer epididymal sperm samples. **Reprod. Domest. Anim.** 44:206-211. 2009.

[9] GARCÍA-MACÍAS, V.; DE PAZ, P.; MARTÍNEZ-PASTOR, F.; ÁLVAREZ, M.; GOMES-ALVES, S.; BERNARDO, J.; ANEL, E.; ANEL, L. DNA fragmentation assessment by flow cytometry and Sperm Bos–Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. **Int. J. Androl.** 30:88-98. 2007.

[10] GRAHAM, J.K. Assessment of sperm quality: a flow cytometric approach. **Anim. Reprod. Sci.** 68:239-247. 2001

[11] GRAVANCE, C.G.; CASEY, M.E.; CASEY, P.J. Pre-freeze bull sperm head morphometry related to post-thaw fertility**. Anim. Reprod. Sci** 114:81-88. 2009.

[12] GRAVANCE, C.G.; VISHWANATH, R.; PITT, C.; GARNER, D.L.; CASEY, P.J. Effects of cryopreservation on bulls perm head morphometry. **J. Androl.** 19:704-709. 1998

[13] GUILLAN, L.; KROETSCH, T.; MAXWELL, W.M.; EVANS, G. Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. **Anim. Reprod. Sci.** 103:201-214. 2008.

[14] IRANPOUR, F.G.; NASR-ESFAHANI, M.H.; VALOJERDI, M.R.; AL-TARAIHI, T.M. Chromomycin A3 staining as a useful tool for evaluation of male fertility. **J. Assist. Reprod. Genet.** 1:60-66. 2000.

[15] JANUSKAUSKAS, A.; JOHANNISSON, A.; RODRÍGUEZ-MARTÍNEZ, H. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. **Theriogenol.** 55:947-961. 2001.

[16] JANUSKAUSKAS, A.; JOHANNISSON, A.; RODRÍGUEZ-MARTÍNEZ, H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability; chromatin structure; and field fertility. **Theriogenol.** 60:743-758. 2003.

[17] KARABINUS, D.S.; VOGLER, C.J.; SAACKE, R.G.; EVENSON, D.P. Chromatin structural changes in sperm after scrotal insulation of Holstein Bulls. **J. Androl.** 18:549-555. 1997.

[18] KAROUI, S.; DÍAZ, C.; GONZÁLEZ-MARÍN, C.; AMENABAR, M.E.; SERRANO, M.; UGARTE, E.; GOSÁLVEZ, J.; ROY, R.; LÓPEZ-FERNÁNDEZ, C.; CARABAÑO, M.J. Is sperm DNA fragmentation a good marker for field AI bull fertility?. **J. Anim. Sci.** 90:2437-2448. 2012.

[19] KHALIFA, T.A.A.; REKKAS, C.A.; LYMBEROPOULOS, A.G.; SIOGA, A.; DIMITRIADIS, I.; PAPANIKOLAOU, T. Factors affecting chromatin stability of bovine spermatozoa. **Anim. Reprod. Sci.** 104:143-163. 2008.

[20] LANGE-CONSIGLIO, A.; ANTONUCCI.N.; MANES, S.; CORRADETTI, B.; CREMONESI, F.; BIZZARO, D. Morphometric characteristics and chromatin integrity of spermatozoa in three Italian dog breeds. **J. Small Anim. Pract.** 51:624-627. 2010.

[21] LEE, C.H.; CHO, Y.H. Aspects of mammalian spermatogenesis electrophoretical analysis of protamines in mammalian species. **Molecules and Cells.** 9:556–559. 1999.

[22] LYMBERPOULOS, A.G.; KHALIFA, T.A. Sperm chromatin stability during in vitro manipulation of beef bull semen. **Reprod. Domest. Anim.** 45:307-314. 2008.

[23] MAIER, W.M.; NUSSBAUM, G.; DOMENJOUD, L.; KLEMM, U.; ENGEL, W. The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene. **Nucleic Acids Res.** 18(5):1249-1254. 1990.

[24] MARTINEZ-PASTOR, F.; TIZADO, E.J.; GARDE, J.J.; ANEL, L.; DE PAZ, P. Statistical series: opportunities and challenges of sperm motility subpopulations analysis. **Theriogenol.** 75:783-795. 2011.

[25] MAROTO-MORALES, A.; RAMÓN, M.; GARCÍA-ÁLVAREZ, O.; SOLER, A.J.; FERNÁNDEZ-SANTOS, M.R.; ROLDAN, E.R.; GOMENDIO, M.; PÉREZ-GUZMÁN, M.D.; GARDE, J.J. Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in Ram ejaculates: intra- and interindividual variation. **Theriogenol.** 77:1529-1539. 2012.

[26] MELLO, M.S.L. Induced metachromasia in bull spermatozoa. **Histochem.** 74:387-392. 1982.

[27] MOCÉ, E.; GRAHAM, J.K. In vitro evaluation of sperm quality. **Anim. Reprod. Sci.** 105:104-118. 2008.

[28] MUKHOPADHYAY, C.S.; GUPTA, A.K.; YADAV, B.R.; CHAUHAN, I.S.; GUPTA, A.; MOHANTY, T.K.; RAINA, V.S. Effect of cryopreservation on sperm chromatin integrity and fertilizing potential in bovine semen. **Livest. Sci**. 136:114-121. 2011.

[29] NAVA-TRUJILLO, H.; QUINTERO-MORENO, A. Importancia de la evaluación de la cromatina espermática en rumiantes. **J. Vet. Androl.** 2(1):8-22. 2017.

[30] NAVA-TRUJILLO, H.; QUINTERO-MORENO, A. Integridad de la cromatina y forma de la cabeza del espermatozoide de toro: evaluación simultánea con la tinción de azul de toluidina. **Rev. Científ. FCV-LUZ** XXII(3):211-216. 2012.

[31] NAVA-TRUJILLO, H.; QUINTERO-MORENO, A.; FINOL-PARRA, G.; OSORIO-MELÉNDEZ, C.; RUBIO-GUILLÉN, J.; VALERIS-CHACÍN, R. Relationship among damaged chromatin; motility and viability in cryopreserved spermatozoa from Brahman bulls. **Rev. Colomb. Cien. Pec.** 24:116-122. 2011.

[32] NAVA-TRUJILLO, H.; QUINTERO-MORENO, A.; HERNÁNDEZ-FERNÁNDEZ, A.; VÍLCHEZ-SIU, V.; OSORIO-MELÉNDEZ, C.; RUBIO-GUILLÉN, J.; FINOL-PARRA, G. Efecto de la integridad de la cromatina sobre la morfometría de la cabeza del espermatozoide de toro. **Rev. Científ. FCV-LUZ** XXIII(1):67-72. 2013.

[33] NÚÑEZ-MARTÍNEZ, I.; MORAN, J.M.; PEÑA, F.J. Identification of sperm morphometric subpopulations in the canine ejaculate: do they reflect different subpopulations in sperm chromatin integrity?. **Zygote** 15:257-266. 2007.

[34] OLIVEIRA, L.Z.; DOS SANTOS, R.M.; HOSEEPIAN DE L, V.F.M.; ARRUDA, R.P.; ANDRADE, A.F.C.; JUNIOR, V.G.; BELETTI, M.E. Chromatin and morphometry alterations of bovine sperm head after Percoll™ density gradient centrifugation. **Livest. Sci.** 141:267-271. 2011.

[35] OLIVEIRA, R.V.; DOGAN S.; BELSER, L.E.; KAYA, A.; TOPPER, E.; MOURA A.; THIBAUDEAU, G.; MEMILI, E. Molecular morphology and function of bull sperm linked to histones and associated with fertility. **Reprod.** 146:263-272. 2013.

[36] PEÑA, F.J.; SARAVIA, F.; GARCÍA-HERREROS, M.; NÚÑEZ-MARTÍNEZ, I.; TAPIA, J.A.; JOHANNISSON, A.; WALLGREN, M.; RODRÍGUEZ-MARTÍNEZ, H. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to post-thaw quality. **J. Androl.** 26:716-723. 2005.

[37] QUINTERO-MORENO, A.; MIRÓ, J.; RIGAU, T.; RODRÍGUEZ-GIL, J.E. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. **Theriogenol.** 59:1973-1990. 2003.

[38] QUINTERO-MORENO, A.; RAMIREZ, M.L.; NAVA-TRUJILLO, H.; HIDALGO, M. Comparison of two histologic stains in the evaluation of sperm head morphometric measurements in frozen-thawed bull semen. **Acta Microscop.** 24(2):103-110. 2015.

[39] QUINTERO-MORENO, A.; RIGAU, T.; RODRÍGUEZ-GIL, J.E. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. **Reprod. Domest. Anim.** 42:312-319. 2007.

[40] RÉVAY, T.; NAGY, S.; KOVÁCS, A.; EDVI, M.E.; HIDAS, A.; RENS, W.; GUSTAVSSON, I. Head measurements of dead; live; X- and Y-bearing bovine spermatozoa. **Reprod. Fertil. Dev.** 16:681–687. 2004.

[41] RÉVAY, T.; NAGY, S.; KOPP, C.; FLYCKT, A.; RENS, W.; RATH, D.; HIDAS, A.; KOVÁCS, A.; JOHANNISSON, A.; RODRÍGUEZ-MARTÍNEZ, H.; ANDERSSON, M. Macrocephaly in bull spermatozoa is associated with nuclear vacuoles; diploidy and alteration of chromatin condensation. **Cytogenet. Genome Res.** 126:202-209. 2009

[42] RUBIO-GUILLÉN, J.; GARDE, J.J.; ESTESO, M.C.; FERNÁNDEZ-SANTOS, M.; GONZÁLEZ, D.; PALOMARES-NAVEDA, R.; VELARDE, J.; MADRID-BURY, N.; SOTO-BELLOSO, E.; QUINTERO-MORENO, A. Head dimensions of Brahman and their crossbred bull spermatozoa are affected by cryopreservation. **Rev. Científ. FCV-LUZ** XVII (5):508-513. 2007

[43] RUBIO-GUILLÉN, J.; GONZÁLEZ, D.; GARDE, J.J.; ESTESO, M.C.; FERNÁNDEZ-SANTOS, M.R.; RODRÍGUEZ-GÍL, J.E.; MADRID-BURY, N.; QUINTERO-

MORENO, A. Effects of cryopreservation on bull spermatozoa distribution in morphometrically distinct subpopulations. **Reprod. Domest. Anim.** 42:354-357. 2007.

[44] SAILER, B.L.; JOST, L.K.; EVENSON, D.P. Bull sperm head morphometry related to abnormal chromatin structure and fertility. **Cytometry** 24:167-173. 1996.

[45] SPIRITO, S.; CAMPI, S.; BOQUET, M.; FERNÁNDEZ, H.; FERRARI, M. Equine sperm nuclei with different ploidy levels: relationship between the nuclear DNA content and the nuclear area. **Androl.** 3:248-253. 2011.

[46] THURSTON, L.M.; WATSON, P.F.; HOLT, W.V. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. **J. Reprod.Fertil.** 117:271-280. 1999.

[47] WATERHOUSE, K.E.; GJELDNES, A.; TVERDAL, A.; DE ANGELIS, P.M.; FARSTAD, W.; HÅÅRD, M.; KOMMISRUD, E. Alterations of sperm DNA integrity during cryopreservation procedure and in vitro incubation of bull semen. **Anim. Reprod. Sci.** 117:34-42. 2010

[48] WATERHOUSE, K.E.; HAUGAN, T.; KOMMISRUD, E.; TVERDAL, A.; FLATBERG, G.; FARSTAD, W.; EVENSON, D.P.; DE ANGELIS, P.M. Sperm DNA damage is related to field fertility of semen from young Norwegian red bulls. **Reprod. Fertil. Dev.** 18:781-788. 2006