IDENTIFICATION OF CRYODAMAGE ON PLASMA MEMBRANE INTEGRITY IN BULL SPERMATOZOA AND ITS RELATIONSHIP WITH FIELD FERTILITY

Identificación de daño criogénico sobre la integridad de la membrana plasmática del espermatozoide de toro y su relación con la fertilidad en campo

Armando Quintero-Moreno [1]*, *Jorge Rubio-Guillén* [1], *Decio González-Villalobos* [1], *Juan Carlos Gutiérrez* [2], *Ninoska Madrid-Bury* [1,3] *y José Julián Garde López-Brea* [4,5]

[1]*Laboratorio de Andrología/ Unidad de Investigación en Producción Animal.* [2]*Unidad de Investigación en Reproducción Animal. Facultad de Ciencias Veterinarias.* [3]*Departamento de Zootecnia. Facultad de Agronomía. Universidad del Zulia. Venezuela.* [4]*IDR, Sección de Recursos Cinegéticos y Ganaderos. Campus Universitario, 02071. Albacete, Spain.* [5]*IREC (UCLM-SSIC-JCCM). *arturo93@cantv.net*

ABSTRACT

The aim of this study was to investigate the effect of cryopreservation on the membrane integrity (MI) in the bull sperm, and determine the relationship between intact cryopreserved sperm and field fertility (FF). The eosin nigrosin exclusion and the hypo-osmotic swelling test were combined in a single test (HOS-EN test) to identify the spermatozoa with four types of MI. After cryopreservation, there was a marked decline (21.3%) in the percentage of spermatozoa with head membrane intact/ tail membrane intact (Type IV MI), and a significant increase in those with head membrane damaged/ tail membrane damaged (Type I MI) (P<0.001), whereas type II MI (head membrane intact /tail membrane damage) and type III MI (head membrane damage/ tail membrane intact) ocurred sparingly. A significant correlation was observed between the percentage of Type IV MI and FF (r=0.49, P<0.014). Cryopreservation causes a important demage in the plasmatic membrane of the spermatozoa. Intact MI is closely related to FF.

Key words: Bull semen, HOST test, membrane integrity, field fertility.

RESUMEN

El objetivo de este estudio fue investigar el efecto de la criopreservación sobre la integridad de la membrana plasmática (MI) del espermatozoide bovino, además de determinar la relación entre los espermatozoides intactos y la fertilidad en campo (FF). La evaluación de la vitalidad por eosina- nigrosina y el test hipo-osmótico, fueron combinados en un único test (HOS-EN test) para identificar los espermatozoides dentro de cuatro tipos de MI. Después de la criopreservación hubo una marcada disminución (21,3%) en el porcentaje de los espermatozoides con membrana de la cabeza intacta/membrana de la cola intacta (MI tipo IV), y un significativo incremento en aquellos con membrana de la cabeza dañada/membrana de la cola dañada (MI tipo I) (P<0,001), mientras que la MI tipo II (membrana de la cabeza intacta/membrana de la cola dañada) y la MI tipo III (membrana de la cabeza dañada/membrana de la cola intacta) no se apreciaron diferencias. Fue observada una correlación significativa entre el porcentaje del MI tipo IV y la FF (r=0,49, P<0,014). La criopreservación causa una alteración significativa en la membrana plasmática del espermatozoide. La MI está íntimamente asociada con la FF.

Palabras clave: Semen de toro, HOST test, integridad de membrana, fertilidad en campo.

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INTRODUCTION

Laboratory assessment of sperm quality is an essential procedure in many aspects of reproduction in domestic species [21]. Standard diagnostic evaluation of sperm is primarily based on physiological parameters such as progressive motility, sperm morphology and sperm concentration, however, these parameters are insufficient to predict fertility [12, 28]. Due to the great importance of sperm membrane in fertiliza-

tion, considerable attention is given to membrane integrity in sperm evaluation. To evaluate plasma membrane integrity, two simple basic test are used: supravital staining (eosin/nigrosin) and hypo-osmotic sweelling test (HOST/HOS) assay [10].

The HOS test for investigating the functional integrity of the mammalian sperm membrane was introduced as an useful assay in the diagnosis of human infertility [18]. The principle of the HOS assay is based on fluid transport across the sperm tail membrane under hypo-osmotic conditions until equilibrium is reached. Due to this influx of fluid, the tail expands and bulges and expresses a characteristic pattern, considered as a hyposmotic response, which can readily be identified with phase-contrast microscope [5] or by using supravital staining [6]. Such spermatozoa are denoted as swollen or HOS reactive (HOS +), signifying functionally intact membranes. Spermatozoa with functionally defected membrane do not swell (HOS -) and their tails do not invaginate [30]. The osmolarity of the solution should be sufficient to provoke the best effect without lysing the sperm membrane [24]. Fertilization of oocytes will not occur if the sperm membrane is biochemically inactive, even if it remains structurally intact. Supravital staining is based on the fact that the membrane of dead spermatozoa permits the passage of the red stain (eosin) into the cytoplasm, but the membrane of live spermatozoa does not permit that. This means that all dead spermatozoa in ejaculate will be coloured, while live spermatozoa will remain colourless [15]. The HOS test has been known to be a better indicator of fertilization potential than supravital staining [26]. however, the combination of sperm viability and HOS test have demonstrated to have advantages in comparison with the two methods evaluated individually on cryopreserved sperm [30]. These researches integrated the two methods into a single test to make the whole assay more convenient and introduced the HOS-EY test. In this trial, the HOS test was used together with eosin-nigrosin staining, and introduced the HOS-EN test [30].

The sperm plasma membrane is the primary site at which lesions occur during freezing-thawing of semen [13, 17, 19]. However, only a few attempts were made to evaluate the effect of cryodamage on head and tail sperm membranes. Attempts have been made to correlate sperm plasma membrane integrity to fertility, but great variation is seen between studies and methods used [8, 23]. The World Health Organization [27] recommends the eosin Y (EY) exclusion and the hyposmotic swelling test (HOS) as integral parts of sperm assay.

The object of this study was to estimate the effect of cryopreservation on plasma membrane integrity in the head and tail regions of individual spermatozoa, and the relationship between intact cryopreserved spermatozoa and field fertility (FF).

MATERIALS AND METHODS

Semen collection and cryopreservation

Five ejaculates during succesive weeks from four fertile *Bos indicus-taurus* bulls (two Brahman, and two Brahman- Holstein) in regular service were obtained from the artificial insemination plant of VIATECA® at Machiques, Zulia State, Venezuela. The ejaculates were collected between 6:00 am and 8:00 am with artificial vagina. 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). After semen collection sperm concentration and subjective scores of motility (wave motion) were evaluated. In addition, ejaculates were diluted and used to assess individual sperm motility. The sperm suspension was also used to assess acrosome integrity and sperm viability by using eosin-nigrosin stain [2]. Only samples with an initial sperm motility and sperm with normal apical ridges (NAR) greater than 70-75% were used for freezing.

Ejaculates were diluted with a skim milk-egg yolk diluent (EYD), contained 1% EYD. In particular, the base solution (Solution A) containing skim milk (15%), EYD (1%) and antibiotics (Tilosin 0.56%, Linco-Espectin 0.56%, Gentamicin 0.74%). Sperm dilution was performed in a two-step procedure, first adding at 30°C the base extender up to two times the final desired sperm concentration and then a second extender (Solution B) at 5°C to achieve a final concentration of ~ $30x10^6$ sperm/straws. Sperm samples in solution A were slowly cooled to 5°C. Cooling down to 5°C lasted for about 2 h. The second extender differed from the base diluent in the replacement of water (14%, v/v) with the same volume of glycerol (final concentration = 7%). Then, extended samples were held for equilibration at 5°C for an additional time of 2 h. After equilibration, the extended sperm was loaded in straws of 0.5 mL each containing $30x10^6$ spermatozoa. Subsequently, straws were frozen immediately in nitrogen vapors, 4 cm above the surface of liquid nitrogen for 10 min and then plunged into liquid nitrogen. Frozen semen was thawed in a water bath (37°C) (Gemmy industrial corp., modelo YCW-03S, Taiwn) for 30 s and sperm was allowed to equilibrate for 5 min before evaluation. Five straws per bull were assessed to evaluate the individual motility and morphological examination.

The HOS-EN test

The HOS-EN test is a modification of HOS-EY test introduced by Zhu and Liu [30] whose designed a combination of the HOS test [18] and the EY method [11]. Zhu and Liu [30] stained the smear with eosin stain; whereas, in this trial were used eosin-nigrosin stain. A hypo-osmotic solution (150 mOsm/mL) consisting of sodium citrate (1.47 g/ 100 mL) and fructose (2.7 g/ 100 mL) as employed. The eosin-nigrosin solution was prepared by dissolving 1.67 g eosin and 10 g nigrosin in 100 mL.

The HOST-EN test was performed as follows: 0.1 mL of fresh and cryopreserved semen was mixed with 1.0 mL hyposmotic solution and incubated at 37°C for 30 min. After incubation, slides were prepared from each sample by placing 10 µl of semen and mixed with 10 µl of eosin-nigrosin stain mixture. The sperm head stained red (EY positive) or unstained (nega-

tive) was observed. The swelling response of sperm tail irrespective of the types of tail coiling, type b to g described by Jeyendran et al. [18] was determined. Two hundred spermatozoa per slide were evaluated under light microscope (x 1000) (Globe, LEM 1600, Germany) and classified into the four clusters described bellow according to morphological changes of membranes at both the head and the tail regions.

Type I	head-red (EN +)	and	Tail-non-swollen (HOS -)
Type II	head-white (EN -)	and	Tail-non-swollen (HOS -)
Type III	head-red (EN +)	and	Tail-swollen (HOS +)
Type IV	head-white (EN -)	and	Tail-swollen (HOS +)

Field fertility evaluation

Data from artificial insemination records with pregnancy diagnosis were analyzed to evaluate the relationship between HOS-EN test results and FF. One hundred and fifty crossbred heifers from three farms located in sub-humid tropical forest in Perijá, Zulia State, Venezuela were inseminated according to AM-PM rule and after natural estrus. Pregnancy diagnosis was verified by rectal palpation 45 days after service. Correlations between HOS-EN test and FF were studied evaluating the relationship between the MI in five cryopreserved semen straws by bull (n=20), and FF by each semen straw.

Statistical analysis

All data recorded were analyzed with Statistical Analysis System for Windows, software 8.2 (SAS Inst. Inc.; Carry, NC. USA). The Student´s t-test was used to compare fresh and frozen-thawed samples for HOS-EN test score. Correlation between HOS-EN test and FF was verified by Spearman rank test. For all statistical analysis, significance was established at P<0.05.

RESULTS AND DISCUSSION

The fresh semen showed high quality with normal sperm morphology (≥85%) and good motility (≥3, scale: 1-5). Sperm motility in frozen-thawed samples was > 30%.

The eosin nigrosin exclusion and the hyposmotic swelling test were combined to a form a single test (HOS-EN test) to identify four types of spermatozoa according to membrane integrity and function. TABLE I, shows that significant differences in HOS-EN test scores exist between fresh and cryopreserved semen samples (P<0.01). After cryopreservation, there was a marked decline (21.93%) in the percentage of spermatozoa with head membrane intact/ tail membrane intact (Type IV), and a significant increase (14.15%) in those with head membrane damaged/ tail membrane damaged (Type I) (P<0.001), whereas type II (head membrane intact /tail membrane damage) and type III (head membrane damage/ tail membrane intact) ocurred sparingly.

Field fertility ranged between 33.33 and 60 % (μ= 51.32%). The number of inseminated heifers for each bull and their FF results were 27 and 47.05%; 29 and 60%; 47 and 52.08% and 47 and 33.33%, respectively. A high correlation was observed between the percentage of Type IV integrity and FF (r=0.49, P<0.014).

In this trial, the combination of HOS test with EN method permitted identify four patterns of sperm membrane integrity in fresh and frozen-thawed bull semen. The HOS-EN test has the advantage to diffenciate if the sperm damage is in the sperm head, tail, or both. Using only the HOS test, the sperm are clustered in two cathegories: tails swollen and tails non swollen, meanwhile, a EN method alone can only identify live and dead spermatozoa according the staining characteristics. Additionally, the combined HOS-EN test could avoid the overstimation in the percentage of spermatozoa with intact membrane observed with the use of eosin-nigrosin or trypan blue [3].

After cryopreservation, the percentage of types I was significantly increased. This sperm subpopulation represents the group of non-viable sperm with membrane defects in head and tail regions. The sperm plasma membrane is the primary site where lesions occur during freezing-thawing of semen [13, 19] and disruption to sperm head and tail membrane affected the normal sperm function. Sperm Type II was represented by live sperm with tail membrane damaged. Type III membrane integrity showed in frozen-thawed semen that 2.25% spermatozoa were non-viable but still HOS reacted. This may be explained by the fact that prolonged hypo-osmotic stress may lead to irreversible effects on the cell membrane and cell death [25]. Alternatively, non-viable sperm may exibit spontaneous swelling be-

TABLE I
MEMBRANE INTEGRITY (TYPE I-IV) BEFORE AND AFTER CRYOPRESERVATION

Semen samples (40)	Types of membrane integrity (%)			
	I	II	III	IV
Fresh (20)	6.50 ± 4.4 [a]	22.10 ± 3.2 [a]	0.87 ± 0.1 [a]	70.53 ± 3.6 [b]
	(4.13-9.01)	(15.42-28.82)	(0.51-1.23)	(63.43-78.51)
Thawed (20)	20.65 ± 2.6 [b]	28.5 ± 2.3 [a]	2.25 ± 0.3 [a]	48.60 ± 4.0 [a]
	(14.93-26.06)	(23.43-33.21)	(1.56-2.93)	(40.19-56.95)
Thawed- fresh	+ 14.15	+6.4	+1.38	- 21.93

Results are expressed as means ± error standard (confidence interval) from semen samples analysed from 4 different bulls.
Values with different superscripts (a, b) in the same column were significantly different (P<0.01).

fore exposure to the HOS solution and therefore be counted as HOS reacted when in fact they were already non-viable [9]. After cryopreservation, the percentage of type IV integrity decreased by 22%, which indicated that the frozen-thawed procedure increased the damage in the sperm membrane (head and tail regions). Sperm with type IV membrane integrity is the group of viable spermatozoa with intact membrane.

The use of HOS ENY test in this trial is supported in prevoius investigations in humans [4, 6, 18, 22, 25] and bull sperm [1, 7, 8, 20] where significant correlation between HOS test and viability by using eosin-nigrosin stain were observed. Jeyendran et al. [18] found a good correlation between the percentage of human spermatozoa sweeling and spermatozoa alive ($r=0.52$, $P<0.01$), and Nagy et al. [20] found a hight correlation coefficient between the percentage of stained sperm tails and the percentage of sperm tail that remained straight under hypo-osmotic conditions in bull and ram semen (0.81 and 0.94, respectively; $P<0.05$). Correa and Zavos [7] determined the efficacy of HOS test for bull sperm together with supravital staining and emphatized that the HOS test is a simple, inexpensive and readily technique wich could prove useful in addition to the standard semen analysis as a means to evaluate fertility.

The present study showed a significant correlation between sperm with intact membrane (Type IV) and FF in cryopreserved sperm. Attemps have been made to correlate sperm plasma membrane integrity to fertility, however, great variation is seen among methods used [8, 17, 23] and positive correlation between membrane integrity assessed by fluorometric methods and fertility has been observed [16]. However, many researchers consider the evaluation of the functional status of sperm membrane by the HOS test as a better indicator of fertilization capacity [14, 29], but Bacinoglu et al. [1] did not observe a relationship between simple HOS test response and fertility. Therefore, the positive correlation observed in the present study suggest that hypo-osmotic swelling together with eosin-nigrosin test may be useful in assessing changes in the functional integrity of sperm membrane during freezing-thawing procedures and explain reproductive potential of bull.

CONCLUSION

Plasma membrane integrity in head and tail sperm was compromised during freezing and thawing process, and there was relationship between intact cryopreserved spermatozoa and field fertility. However, if this trial was designed with a larger set of bulls and larger number of inseminated heifers could be a practical tool to predict fertility. The combination of HOS test and eosin-nigrosin stain (HOS-EN test) is a tool to evaluate the effect of cryopreservation process on sperm membranes integrity. Additionally, the HOS-EN test has a practical value for laboratories that are not equipped with phase-contrast microscope and for those unable to carry out fluorometric measurements.

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