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ARTÍCULO ORIGINAL

Effect of cryopreservation of sheep semen related to its viability and acrosomal status

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ABSTRACT: In the present research, the effects of cryopreservation on the viability and acrosomal status of sheep sperms were analyzed. Forty five ejaculates were obtained by the artificial vagina method for analysis, evaluating in fresh semen the following characteristics: volume (Vol), progressive motility (PM), viability percentage (Viab), normal morphology (NM), spermatic concentration (Concentr); as well as its viability and acrosomal status, The last two parameters were evaluated staining with fluorescein isothiocyanate conjugated with *Arachis hypogaea* lectin and propidium iodide (FITC-PNA/IP), in which there were different patterns of staining: live sperms without acrosomal reaction (VsRA), live with acrosomal reaction (VcRA), dead without acrosomal reaction (MsRA) and dead with acrosomal reaction (McRA), obtaining averages of: 1.2±0.3 ml, 88.0±3.4%, 91.4±3.8%, 94.2±2.9%, 1768.1±5.5 x10^6/ml, 76.7±5.5%, 7.6±2.7%, 11.2±4.1% and 4.5±2.6% respectively. Semen was frozen in a commercial diluent (Triladyl®) and packaged in 0.5 ml straws at a concentration of 150x10^6/ml in liquid nitrogen for 8 days, thawed at 37°C for 60 seconds. In post-thawed semen evaluation, the following values were determined: PM= 37.4±5.3%, Viab= 67.5±4.7, NM= 79.5±5.7, VsRA= 26.9±7.3%, VcRA= 29.2±6.4%, MsRA= 27.7±7% y McRA= 15.9±6.2%, obtaining statistically significant differences ($p<0.05$) caused by cryopreservation effects. It is concluded that although viability and acrosomal status of sperm are affected by cryopreservation, live sperm without acrosomal reaction can be used in assisted reproduction techniques.

Key words: cryopreservation, viability, acrosomal reaction, FITC-PNA/IP, sheep semen.

Efecto de la criopreservación de semen de ovino en relación a su viabilidad y estado acrosomal

RESUMEN: En la presente investigación, se analizaron los efectos de la criopreservación sobre la viabilidad y el estado acrosomal de espermatozoides ovinos. Se obtuvieron 45 eyaculados por el método de vagina artificial para el análisis, evaluando en semen fresco las siguientes características: volumen (Vol), motilidad progresiva (MP), porcentaje de viabilidad (Viab), morfología normal (NM), concentración espermática (Concentr); así como su viabilidad y el estado acrosomal, Los dos últimos parámetros se evaluaron usando tinción con isotiocianato de fluoresceína conjugada con *Arachis hypogaea* lectina y yoduro de propidio (FITC-PNA/IP), en los que había diferentes patrones de tinción: espermatozoides vivos sin reacción acrosomal (VsRA), vivos con reacción acrosomal (VcRA), muertos sin reacción acrosomal (MsRA) y muertos con reacción acrosomal (McRA), obteniéndose un promedio de: 1,2 ± 0,3 mL 88,0 ± 3,4%, 91,4 ± 3,8%, 94,2 ± 2,9%, 1768,1 ± 5,5 x106/mL 76,7 ± 5,5%, 7,6 ± 2,7%, 11,2 ± 4,1% y 4,5 ± 2,6%, respectivamente. El semen se congeló en un diluyente comercial (Triladyl ®) y se empacó en pajuelas de 0,5 mL a una concentración de 150x10^6/ml en nitrógeno líquido durante 8 días, descongeladas a 37°C durante 60 segundos. En la post-evaluación del semen descongelado, se determinaron los valores siguientes: MP = 37,4 ± 5,3%, Viab = 67,5 ± 4,7, NM = 79,5 ± 5.7, VsRA = 26,9 ± 7,3%, VcRA = 29,2 ± 6,4%, MsRA = 27,7 ± 7% y McRA = 15,9 ± 6,2%, obteniéndose diferencias estadísticamente significativas ($p <0,05$) causadas por los efectos de la criopreservación. Se concluye que, a pesar de que la viabilidad y el estado acrosomal de los espermatozoides se ven afectado por la criopreservación. Los espermatozoides vivos sin reacción del acrosomal se pueden utilizar en las técnicas de reproducción asistida.

Palabras clave: criopreservación, viabilidad, reacción acrosomal, FITC-PNA/IP, semen ovino.

INTRODUCTION

The evaluation of acrosomal integrity is often used as an indicator to compare different methods for obtaining semen (1) and freezing in sheep (2).

It has been demonstrated that after freezing and thawing cycle, the number of non-viable sperm undergoing a false acrosome reaction (AR), as well as the percentage of acrosome reaction viable sperm, increased (3).

AR is the true fusion of the sperm plasma membrane with the outer acrosomal membrane, followed by extensive blistering of the anterior segment of the acrosome. The fusion and the acrosome vesiculation cause the release of acrosomal contents, allowing the action of hydrolytic enzymes such as hyaluronidase and acrosin, which dissolve the structure of the pellucide zone and allow penetration of the sperm to the perivitelline space (4).

In order to determine the acrosome structural damage, there has been a number of protocols such as observation under light microscope, that although it shows in detail the acrosome morphology, it presents disadvantages since fixation chemical treatments required in these procedures cause AR in sperm which would lead to an overestimation of the percentage of the acrosomes reacted (5). Another technique is the combination of dyes (blue trypan and giemsa, neutral red and Giemsa, Bismarck brown and Bengal red) which makes a more complicated implementation (6,7). Fluorescein isothiocyanate staining conjugated *Arachis hypogaea* lectin and propidium iodide (FITC-PNA/IP) has been currently used which indicates the viability and acrosomal status of sperm (2).

Arachis hypogaea lectin (PNA) is a plant from the peanut which binds galactose-β residues associated outer acrosomal membrane, cells with the intact acrosome remain unstained by the inability to penetrate the PNA (8). As to propidium iodide (IP), cells with intact plasma membrane prevent its entrance, while when membranes are permeable, it enters the cell, where it has the property of binding and cellular DNA staining, indicating they are damaged when IP enters the cell, it emits a red fluorescence in the sperm head (9).

After the thawing of ram semen, the percentage of alive and AR must be known, which is of interest for use in assisted reproduction such as artificial insemination (IA) in the uterus by laparoscopy, in vitro fertilization (FIV) and intracytoplasmic sperm injection (ICSI).

Based on the above indicated, the objetive of this research was to determine the effect of cryopreservation on the viability and acrosomal status of spermatozoa in sheep, by staining FITC-PNA/IP evaluation, for possible use in assisted reproduction techniques.

MATERIALS AND METHODS

Two sexually mature sire, breed Suffolk of two years of age were used with a diet based on alfalfa hay and concentrate feed and water *ad libitum*. The ejaculates were obtained by means of an artificial vagina (VA), moving the samples to the laboratory Manejo de la Reproducción of UAM-Xochimilco in a thermal box at 37°C and placed in a water bath at 37°C (10). The characteristics evaluated were: volume, motility, viability, morphology, concentration and acrosome reaction in fresh and post-thaw.

Volume. It was determined using a graduated collection tube (1).

Progressive motility. For evaluation, a drop of diluted semen was placed on a slide tempered at 37 °C., in warming plate, covered with a coverslip at the same temperature and immediately observed in a light microscope at 10X (Eclipse E600, Nikon) and evaluated on a scale from 0 to 100% (11).

Viability and sperm morphology. They were evaluated by eosin-nigrosin staning, evaluating 100 spermatozoa with a 40X objective of a light microscope, considering as those without viable sperm stained and dead who were stained (10).

Sperm morphology was classified as follows: normal and abnormal sperm, observing 100 spermatozoa from each sample (10).

Sperm count. It was determined with a Neubauer chamber after dilution of 1:200, using a light microscope at 40x (12).

Viability and acrosomal status. Acrosomal status was determined from sheep spermatozoa, fresh and post-thaw by lectin staining technique Peanut Aglutinin (PNA) coupled with fluorescence isothiocyanate (FITC) and viability by propidium iodide (IP). As shown in figure 1.

The functional status of 100 spermatozoa per sample was evaluated and classified according to the following staining patterns: live sperm without AR (sperm without FITC-PNA staining or IP) with AR live sperm (sperm acrosomal staining) dead sperm without AR (sperm with nuclear staining) with AR dead sperm (sperm with nuclear staining and acrosomal staining) (14). To determine the acrosomal status, a B-2A filter with a degree of excitation of 450-490 nm. was used determining sperm viability with G-2A filter with an excitation level of 510-560 nm.

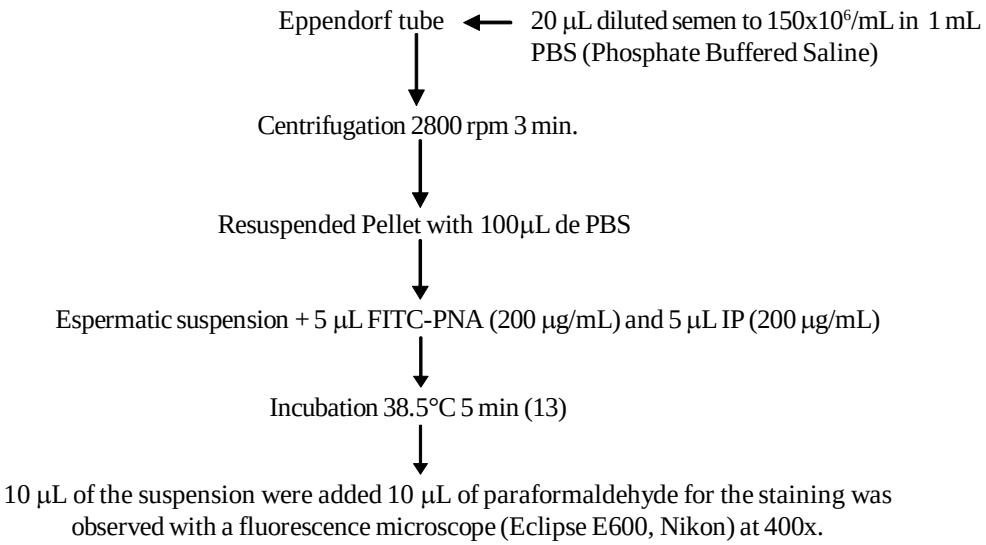

FIGURE 1. Method for assessment of viability and acrosomal status./ *Método para evaluación de viabilidad y estado acrosomal.*

Cryopreservation. For freezing sheep semen, a diluent of 250 ml Triladyl®, 250 ml egg yolk and 750 ml distilled water was prepared (15), packing the semen in straws of 0.5 ml at a concentration of 100 x 10^6/ml and stored in liquid nitrogen for one week (16), thawing at 37°C, for 60 seconds (12). Once the semen was thawed, the same characteristics were evaluated in fresh semen.

Statistical analysis. Data were analyzed with SPSS 13.0 to compare sperm characteristics using statistical tests of ANOVA and «t» of Student, assessing the significance degree of $p < 0.05$ (17).

RESULTS AND DISCUSSION

In fresh semen obtained through AV , a volume (1.2 ml), higher than that reported by Hernández *et al*. (18), of 0.78 ml and similar to that reported by Guerrero *et al.* (12) of 1.1 mL, was determined

Sperm characteristics of the 45 sheep fresh semen samples after freezing and expressed in % are shown in Table 1. It shows the freezing effect on these indicators.

The average motility found in fresh semen (88.0%) was similar to that reported by Cabrera and Pantoja (19) of 86.0%, and Guerrero et al., (12) with 87.0%, but higher than that reported by Anel *et al.* (2) that was 70.8%, whose assessment was performed using a computerized system.

In relation to the mean percentage of live spermatozoa (91.4%), this is similar to that found by Guerrero *et al.* (12) which was 90.2%, but higher than that reported by Hernandez *et al.* (18) with 65.8%. This is possibly because in the first two studies, the sample was obtained by AV while Hernandez *et al.* (19) semen obtained by electroejaculation (EE). It is reported that the semen collected by AV shows a higher percentage of live sperm and a higher concentration than that collected by EE (20).

TABLE 1. Characteristics of fresh semen and sperm post-thaw, obtained from 45 ejaculates./ *Características espermáticas de semen fresco y post-descongelado, obtenido de 45 eyaculados de ovino*

				Viability and acrosomal status			
	% Mot. Prog.	% Viabil.	% Morfol. Norm.	% VsRA	% VcRA	% MsRA	% McRA
Fresh	88.0±3.4[a]	91.4±3.8[a]	94.0±2.9[a]	76.7±5.5[a]	7.6±2.7[a]	11.2±4.1[a]	4.5±2.6[a]
Post-thawed	37.4±5.3[b]	67.5±4.7[b]	79.5±5.7[b]	26.9±7.3[b]	29.2±6.4[b]	27.7±7.0[b]	15.9±6.2[b]

[a, b] Literal different columns indicate statistically significant difference ($p<0.05$).
Mot. Prog.= Progressive motility, Viabil.= Viability, Morfol Norm.= Normal morphology, RA= Acrosomal reaction, VsRA= Live without RA, VcRA= Live with RA, MsRA = Dead without RA, McRA = Dead with RA.

Regarding the average of normal sperm , the result obtained in our assay 94.0% is considered more appropriate but less than that reported by Guerrero *et al.* (12) of 98.2% , which is similar to that reported by Cabrera and Pantoja (19), with 93.6%. According to Diaz and Arancibia raises (21) is classified as optimal ejaculates having less than 15% of abnormalities.

The average concentration per milliliter was obtained $1768.0X10^6$, lower than that reported by Guerrero *et al.* (12) who reported $3800.0X10^6$. It is noted that the latter researches worked only ejaculates having a sperm concentration greater than 2000 million sperm per mL. Mellisho and Gallegos (22), indicated that sperm concentration in sheep semen is 1000.0 to $3000.0x10^6$ sperm per mL.

As for the viability and acrosomal status evaluated FITC-PNA/IP staining was found in fresh sheep semen with the following values: 76.7%, 7.6%, 11.2% and 4.5% for VsRA, VcRA, MsRA and McRA, respectively. Most studies only report the percentage of sperm with or without AR, regardless whether sperm is alive or dead. The case in this research has 87.9% of sperm without RA, which is similar to that previously reported by Hernandez *et al.* (18) and Sandoval *et al.* (11) with 85.6% and 86.6% of sperm without RA, respectively, using Coomassie blue staining and double trypan blue and Giemsa techniques to determine these values.

The average percentage of post-thaw motility in this study was 37.4%, however higher motilities were reported by Sandoval *et al.* (11) and Brito *et al.* (23) with 69.2% and 40.2% respectively. Considering that post-thaw motility may be affected by the freezing method, the present study was performed with liquid nitrogen, while in Brito *et al.* (23) study, it was frozen by dry ice.

Freezing in liquid nitrogen vapor is performed by placing the straw containing the semen at a distance of 4-5 cm. from the surface of liquid nitrogen (24), while freezing in dry ice is accomplished by small holes in the surface thereof and depositing 0.1 ml of diluted semen (23), which causes direct contact with the medium of freezing providing a rapid freezing and tolerates a wide range of freezing (-79°C to -160°C), relative to the vapors of liquid nitrogen (-60°C to -86°C), which allows a greater recovery motile sperm at thawing (25).

Another reason may be the temperature at which glycerol is added to the semen, since in this research they were added at 37°C , while Sandoval *et al.* (11), added 5°C. There are reports that mention the best post-thaw motilities adding it to 5°C (24). In sheep semen, post-thaw motilities reported 40-60%, but only 20-30% are functional (26).

Both living and morphologically normal sperm are appropriate indicators after thawing. The percentage of living sperm decreased only 23.9%, from the difference values before and after freezing Table 1, which is considered favorable if one considers that expressed by Stornelli *et al.* (27), who pointed out that during the freeze-thaw process, about 50% of the initial population is lost.

Sperm percentage with normal morphology post-thaw was 79.5%, representing a decrease of 14.5% from the initial value. This result is within the range proposed by Diaz and Arancibia (21), who established classification parameters for sheep semen as indicating normal morphology and good values of 80-85%.

In this study, the values of viability and acrosomal status in post-thawed sperm were 26.9%, 29.2%, 27.7% and 15.9% of sperm VsRA, VcRA, MsRA and McRA, respectively. Thus there is a 26.9% of sperm fertilizing ability, as they are alive and have not implemented its AR. This is similar to that reported by Anel *et al.* (2) with 26.3%, but higher than that found by Marco-Jiménez *et al.* (1) with 21.2%, applying the same evaluation technique. Considering only the acrosomal status of sperm, there is a 54.6% of sperm without AR , which is similar to that reported by Joshi *et al.* (28) with 54.1%, assessed by a computerized system, but less than that reported by Sandoval *et al.* (11) with 63.1%, determined by double staining.

The cryopreservation process allows to retrieve enough sperm under adequate conditions (26.9 million sperm per straw) in order to carry out assisted reproductive techniques such as intrauterine insemination by laparoscopy in which from 20 to 25 million motile sperm is deposited (23, 29). *In vitro* fertilization, 1 million sperm is required (30) , and in ICSI, one sperm per oocyte is required (31).

It is concluded that although viability and acrosomal status of sheep spermatozoa are affected by cryopreservation, there is enough alive sperm without acrosome reaction potentially useful that can be used in assisted reproduction techniques.

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