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ARTÍCULO ORIGINAL

Post-thaw acrosomal viability and reaction in sperm obtained from equine epididymis tail

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ABSTRACT: In order to evaluate the post-thaw effect on acrosomal viability and reaction of sperm collected from equine epididymis tail, 62 testes were collected and transported at 4°C. Epididymis was dissected to perform the retrograde flushing of the epididymis tail , using 10 mL of a diluent based on skim milk (INRA 82). The diluent obtained with the spermatic content was recovered in graduated collecting tubes and 1mL of each sample was taken to be evaluated. For evaluations, the samples having a minimum of 30% of progressive motility were selected. The evaluations were on: percentage of live sperm, normal morphology, sperm concentration, percentage of live sperm without acrosomal reaction (VsRA) and live with acrosomal reaction (VcRA), evaluating this indicator with FITC-PNA staining and propidium iodide (PI). Averages of 53.2%, 72.7%, 82.9%, 507.3X10^6, 65.3% and 6.0% were respectively obtained. Subsequently, samples were packed into 0.5 ml straws at a 100x10^6 concentration, frozen in liquid nitrogen and stored for 8 days. Thawing was performed at 38°C for 37 seconds, resulting for sperm motility, percentage of live sperm, normal morphology and VcRA VsRA the following values: 26.3%, 60.9%, 73.8%, 43.4% and 14.2%, respectively. Although all studied sperm quality values were affected by cryopreservation ($P < 0.05$), a 43.4% of cells was recovered with characteristics making them suitable for use in biotechnological applications of assisted reproduction.

Key words: cryopreservation, viability, acrosomal reaction, FITC-PNA/IP, sperm, epididymis, equine.

Viabilidad y reacción acrosomal postcongelación en espermatozoides obtenidos de cola de epidídimo de equino

RESUMEN: Con el objetivo de evaluar el efecto de la congelación sobre la viabilidad y reacción acrosomal de espermatozoides obtenidos de cola de epidídimo de equinos se recolectaron 62 testículos los que fueron transportados a 4°C., se diseccionó el epidídimo, para realizar el lavado retrógrado de la cola de epidídimo, utilizando 10 mL de un diluyente a base de leche descremada (INRA 82). El diluyente obtenido con el contenido espermático fue recuperado en tubos colectores graduados, se tomó 1 mL de cada muestra para ser evaluadas. Para las evaluaciones se seleccionaron las que presentaran como mínimo 30.0% de motilidad progresiva, a éstas se les evaluó: porcentaje de espermatozoides vivos, morfología normal, concentración espermática y porcentaje de espermatozoides vivos sin reacción acrosomal (VsRA) y vivos con reacción acrosomal (VcRA) evaluando este indicador con la tinción FITC-PNA y Ioduro de propidio (IP); obteniendo promedios de 53.2%, 72.7%, 82.9%, 507.3X10^6, 65.3% y 6.0%, respectivamente. Posteriormente las muestras se envasaron en pajillas de 0.5 mL en concentración de 100X10^6 y se congelaron en nitrógeno líquido, almacenándose durante 8 días. La descongelación se realizó a 38°C., por 37 segundos, obteniendo como resultado para motilidad espermática, porcentaje de espermatozoides vivos, morfología normal, VsRA y VcRA los siguientes valores: 26.3%, 60.9%, 73.8%, 43.4% y 14.2%, respectivamente. Aunque todos los valores de calidad espermática estudiados fueron afectados por la criopreservación ($P<0,05$), se recuperaron un 43.4% de células con características que las hacen aptas para su empleo en aplicaciones biotecnológicas de reproducción asistida.

Palabras clave: criopreservación, viabilidad, reacción acrosomal, FITC-PNA/IP, espermatozoides, epidídimo, equino.

INTRODUCTION

The ability of sperm collection and preservation of a stallion epididymis after castration either by choice, a traumatic injury, serious illness or unexpected death on several occasions has allowed the genetics preservation, thanks to continuing advances in assisted reproductive techniques (1,2). Studies have shown that sperm retrieved from epididymis is highly efficient, and even when it is kept at room temperature, it remains viable up to 24 hours after performing orchiectomy, and could be subsequently used for artificial insemination (AI) (3).However, it has been seen that when testes are stored at 4-5°C., the sperm has a higher viability percentage (2).

Retrograde flushing is considered a fast and efficient procedure for the recovery of epididymis tail sperm in the equine species. With this procedure, a recovery of sperm similar to that obtained in ejaculates collected through artificial vagina is reported (4,3).

The success of epididymal sperm collections with resulting pregnancies has been reported in different animal species, including man. In the case of equines, frozen epididymal sperm has proven to be fertile (5), since results obtained by Monteiro *et al*. (4), show that sperm collected from epididymis tail, immediately or after 24 hours performing orchiectomy, remains fertility similar to that of ejaculated sperm. Morris *et al*. (6) reported pregnancies in mares by AI by hysteroscopy with thawed sperm collected from epididymis.

Sperm cryopreservation contributes to the expansion of diverse reproductive techniques such as AI, *in vitro* fertilization (IVF) and intracytoplasmic injection (ICSI). It has been seen that AI with frozen semen is an essential part in the breeding and selection programs, thereby contributing to increase the production of domestic species (7). In the case of post-mortem collection equine species, they often need to be preserved for future use, because females are not always available to produce the necessary oocytes for fertilization, due to their characteristics in the estrous cycle (2).

Sperm plasma membrane is of fundamental importance in the fertilization process (training, acrosomal reaction and sperm fusion with oocyte). During semen cryopreservation, alterations of the membrane are produced causing a decrease in sperm viability (8). There are reports indicating that equine sperm is severely damaged during freezing and no more than the 50.0% will survive to cryopreservation. Besides, that sperm that still alive but having an acrosomal reaction (AR) is not functional to penetrate the oocyte and loses its fertilizing capacity (9).

There are evidences reporting at epididymis level the presence of substances causing modifications in the sperm membrane. One of these substances is inmovilina, which is a high molecular weight glycoprotein that increases epididymal fluid viscosity and thus reduces sperm cell motility. It also raises an inhibition of the phosphodiesterase activity and changes in cAMP levels, which together help to provide greater resistance to cold shock, compared with ejaculated sperm, thus proving more resistant to cooling and freezing procedures that epididymal sperm (4, 3).

A generally accepted criterion for using post-thaw ejaculated semen of horses is that this must present a motility between 30.0-35.0% to be considered as acceptable (10). While Shulman *et al*. (11) claim that it is permitted the use of epididymis post-thawed sperm with a progressive motility within a range of 29.0-40.0%, for being used in AI programs, reporting that using a dose of $400X10^6$ sperm, a pregnancy rate of 69.2% can be registered (12). Considering the above, and due to there are not reports in Mexico in which parameters of potential viability in equine epididymal sperm are registered, the objective of this study was to evaluate the characteristics of equine sperm taken from the epididymis tail, fresh and cryopreserved for possible use in assisted reproduction procedures.

MATERIALS AND METHODS

Sixty two pairs of testes of adult horses were obtained in an abbatoir of the State of Mexico.They were collected immediately after slaughter and were linked to the pampiniform plexus level and placed in polyethylene bags containing 10 mL of physiological saline solution at 0.9% with antibiotic (100 IU/mL penicillin and 100 mg/ml streptomycin). The samples were transported to the Reproduction Laboratory of Handling UAM-Xochimilco at a temperature of 4°C. (2).

Once in the laboratory, the samples were handled in a cold room at a temperature of 4°C. To obtain sperm, epididymis tail was carefully dissected, including deferens vessel; and later a retrograde flushing was made from the deferens duct by introducing 18-gauge needle inserted at the entrance of the deferens duct. Gently washing was performed (5,13) with 10 ml of diluent INRA 82 previously prepared with: Glucose 200 g, Lactose 300 g, Raffinose 500 g, Trisodium citrate 60 g, Potassium citrate 82 mg, Penicillin 10 IU/ml, Gentamicin 10 IU/ml, 50 ml distilled water and 30 ml skim milk (14).Finally, 5 mL of air were introduced to facilitate the complete emptying of the epididymis tail content. The sperm sample was deposited into sterile collectior tubes of 15 mL.

Once the sample is retrieved, it proceeded to take an aliquot of 1 mL and placed in a water bath at 37°C., during 5 minutes for microscopic evaluation. Progressive motility was directly assessed by placing a drop of the sample on a slide covered with a slide tempered under a 10X objective of a microscope (SMZ645, Nikon) equipped with a platen (Tokai Hit, Nikon) at 37°C. Only those samples showing a progressive motility $\geq$ 30.0% were processed.

Viability and morphology estimate was carried out by staining with eosin-nigrosin and the evaluation with of 40X and 100X objectives, respectively. Either in fresh semen or post-thaw semen for each sample, 100 spermatozoa were counted (13,15,16). The concentration of the samples was evaluated by counting in a Neubauer chamber (2).

The viability and acrosomal status of sperm obtained from equine epididymis tail were determined in fresh and post-thaw stage by using the double staining technique of fluorescein isothiocyanate with Arachis hypogea (FITC-PNA) and propidium iodide (PI), according to the procedure of Garcia-Rosello *et al*. (17), to which 100 µl were taken from each sample, adding 5 µl of FITC-PNA and 5 µl of IP, incubated at 38.5°C., for 5 minutes. From this latter suspension, 10 µl were taken and 10 µl of paraformaldehyde were added for smear, observed with a fluorescence microscope (Eclipse E600, Nikon) at 400 magnification. The functional status of 100 spermatozoa per sample was evaluated and classified according to the following staining patterns: live sperm without RA (sperm without staining of FITC-PNA and PI) live sperm with RA (sperm with acrosomal staining), dead sperm without RA (sperm with nuclear and acrosome staining) (18). In order to determine the acrosomal stage, the filter-grade B-2A with excitation degree of 450-490 nm was used; and to evaluate sperm viability, the G-2A filter with excitation degree of 510-560 nm was used.

The samples considered acceptable for cryopreservation were left in the cold room for 2 hours, to later add the second diluent of freezing INRA 82. This was supplemented with 5% glycerol, and with it, the concentration was adjusted to $200X10^6$ sperm/ml. Then an equilibration period of 2 hours was given. Finally semen was packaged in 0.5 ml straws and sealed with polyvinyl alcohol, placed in liquid nitrogen vapor at 4 cm of nitrogen level for 10 minutes After the time, straws were immersed in nitrogen for preservation until analysis (4).

For post-thaw analysis, straws were immersed for 30 seconds at 37°C., (5,19) and it proceeded to carry out the evaluation of progressive motility, viability, normal morphology and acrosomal reaction, in the same way that those evaluated in fresh sperm.

Statistical analysis. Data were analyzed with SPSS 13.0 statistical package for the comparison of the sperm characteristics evaluated, as well as ANOVA and «t» of Student statistical tests, assessing the significance degree of $p < 0.05$ (20).

RESULTS AND DISCUSSION

As a result of the freezing process, there is an affectation of the indicators measured suggested by Neild *et al*. (9) and Zhang *et al*. (8) (Table 1).

The progressive motility of equine epididymis sperm in fresh samples obtained in this study was of 53.2%, a percentage similar to that obtained working the same type of samples by James (2), who found 57.0% progressive motility, and a value found within the range given by Heise *et al*. (13) who found a motility between

TABLE 1. Sperm characteristics obtained from 45 equine epididymis tail in fresh and post-thawed / *Características espermáticas obtenidas de 45 colas de epidídimos de equinos, evaluados en fresco y postdescongelado*

	% Prog Mot..	% Viabil.	% Norm. Morfol.	Concentr. $X10^6$/ml	Viability and acrosomal status	
					VsRA	VcRA
Fresh	53.2 ±13.5[a]	72.7 ±9.6[a]	82.9 ±6.7[a]	507.3 ±282.9	65.3±8.3[a]	6.0±3.4[a]
Post-thaw	26.3 ±11.8[b]	60.9 ±10.7[b]	73.8 ±9.6[b]	---	43.2±10.8[b]	14.2±5.6[b]

[a, b] Literal different columns indicate statistically significant difference ($p < 0.05$).
Prog Mot..= Progressive motility, Viabil.= Viability, Norm Morfol.= Normal morphology, AR= Acrosomal reaction, VsAR= Live without AR, VcAR= Live with AR
[a, b] *Literal diferente en columna indica diferencia estadística significativa ($p<0.05$).*
Mot. Prog.= Motilidad progresiva, Viabil.= Viabilidad, Morfol Norm.= Morfología Normal, RA= Reacción acrosomal, VsRA= Vivos sin RA, VcRA= Vivos con RA.

10.0-75.0% when evaluating 4 purebred stallions. At thaw, a motility of 26.3% was found, which was higher than that found by Heise *et al*. (13) who reported a progressive motility of 5.0-10.0% and Papa *et al*. (3), 19.2%, but lesser than that obtained by James (2) and Monteiro *et al*. (4) who observed 46.0 and 36.2% motility, respectively. This value in the progressive motility may result from the freezing protocol used in the assay since the diluent with glycerol was added in one step at 2 hours of cooling, unlike James (2) who added glycerol in fractions of 0.125% of the total volume in 30 minutes, up to the appropriate volume in 2 hours.

On the other hand, Monteiro *et al*. (4) used Botu-Crio®, which is a commercial diluent added to the sample and can be packaged immediately. This diluent only requires 20 minutes to be cooled at 5°C., to subsequently place samples at 6 cm of nitrogen for 20 minutes, and be completely submerged totally in nitrogen, a different technique to that employed in this study.

The most widely used parameter as fertility predictor for AI in horses is the individual motility of post-thaw spermatozoa, existing reports in which there is an acceptable motility of at least 30.0% in ejaculated sperm (21). In this research, an average motility of 26.3% was obtained, an acceptable value and found near the range provided by Schulman *et al*. (11): 29.0-40.0%, for use in AI programs.

The viability percentage assessed by eosin-nigrosin staining 72.7% of live sperm was found in fresh semen, a low percentage, considering that reported by Heise *et al*. (13) who found 91.0% of viability after regaining epididymal sample by washing. This difference may be due to two causes: 1) recovery time elapsed between animal death and epididymal washing process, since the samples worked in this study were transported from abbatoir to the laboratory within a period of 2 to 3 hours, and 2) the age and breed of animals, since samples were obtained from horses aged in most cases over 15 years, while Heise *et al*. (13) in their study used young stallions 4-5 years old, purebred, and samples were immediately recovered after castration. Nevertheless, this author reports having observed thawing of viable sperm 60.0%, a value similar to that found in this study (60.9%).

The percentage of normal morphology found in the samples before and after freezing was 82.9 and 73.8% respectively, which is higher than that reported by Heise *et al*. (13), who observed a 35.4 and 29.9% of normal sperm in fresh and post-thaw in samples tested with the same technique eosin-nigrosin, respectively. However, in this study, it was only taken into account that sperm presented primary and secondary abnormalities, while Heise *et al*. (13) took into account features in more detail: sperm with nuclear, acrosomal and flagellum defects, reporting significantly lower percentages.

In any assisted reproductive procedure, the main objective is the use of viable sperm having a high potential for fertilization, and in the case of choosing spermatozoa, the best option is to select those that are alive and without RA. This study found a 65.3% in recovered fresh sperm, a smaller percentage than that reported by Heise *et al*. (13), Monteiro *et al*. (4) and James (2), who obtained a 97.7, 88.6 and 76.7% of sperm without RA, respectively. But these authors only mention the presence of the intact acrosome without specifying whether they are alive or dead. Regarding thawed samples, there is a 43.2% of live sperm and without RA, a value similar to that reported by Monteiro *et al*. (4) who found 41.4% of intact acrosome but lesser than that assessed by Heise *et al*. (13) and Papa *et al*. (3), who reported 94.2 and 79.0% of sperm without RA, regardless cell viability and using CASA program for evaluation. Likewise Papa *et al*. (3) used Botu-Cryo® for the cryopreservation process, which is a commercial diluent that has shown to increase the sperm quality after thawing, because of the content of several amino acids responsible for the activation, protection and membrane integrity of post-thawed sperm. On the other hand, this value is also comparable to data from ejaculated sperm, since Graham (22) reported a range of 28.0-67.0% of live sperm without RA, values like those obtained in this research with 43.2%.

Based on the results obtained in this study, the potentiality of sperm obtained from equine post freezing epididymis can be considered to be used in biotechnological applications of assisted reproduction.

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