

Effect of Two Protocols of Cryopreservation on Fertilizing Capacity of Stallion (*Equus caballus*) Semen

Efecto de Dos Protocolos de Criopreservación sobre la Capacidad Fecundante de Semen Equino (*Equus caballus*)

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Abstract. Semen cryopreservation is a fundamental process for the development of biotechnologies for assisted reproduction in horses. The use of cryopreservation techniques with changes in concentrations and the nature of the cryoprotectant, as well as, the different types of vials for storage of semen, have become an alternative to improve the protocols used. The objective of this work was to evaluate the effect of two protocols of cryopreservation (freezing and vitrification) on the fertilizing capacity of stallion semen. The study was conducted with horses of the Criollo Colombiano breed. For freezing was used a extender supplemented with egg yolk (4%) and dimethyl formamide (5%), and 0.5 mL straws as vials, whereas for vitrification, the extender was supplemented with egg yolk (8%) and dimethyl formamide (8%), and cryovials were used as carriers. As post thaw parameters were evaluated: progressive motility, vitality, normal morphology and integrity of the plasma membrane through the hypoosmotic swelling test (HOS). For statistical evaluation was fitted a generalized linear model (GLM) and means were compared by the Tukey test. Were found average percentages of progressive motility, vitality, normal morphology and HOS of 41.6 ± 11.8 and 37 ± 8.5 , 54.3 ± 10.2 and 52.3 ± 7.8 , 83.1 ± 5.4 and 83.6 ± 5.8 , 41.7 ± 9.8 and 38.9 ± 3.6 , for cryopreserved semen by freezing and vitrification, respectively. There were no statistically significant differences ($P \leq 0.05$) between treatments for any of the parameters evaluated. The fertilizing capacity of equine semen cryopreserved by vitrification is comparable to that obtained by conventional freezing.

Key words: Artificial insemination, seminal quality, freezing, vitrification.

Resumen. La criopreservación de semen es un proceso fundamental en el desarrollo de biotecnologías para la reproducción asistida en equinos. El uso de diferentes técnicas de criopreservación con cambios en las concentraciones y la naturaleza de los crioprotectores, así como en los diferentes tipos de soportes para el almacenamiento del semen, se ha constituido en una alternativa para mejorar los protocolos empleados. El objetivo de este trabajo fue evaluar el efecto de dos protocolos de criopreservación (congelación y vitrificación), sobre la capacidad fecundante del semen equino. El estudio se realizó con equinos de la raza Criollo Colombiano. Para la congelación se empleó un diluyente suplementado con de yema de huevo (4%) y dimetilformamida (5%), y pajillas de 0,5 mL como soportes; mientras que para la vitrificación, el diluyente fue suplementado con yema de huevo (8%) y dimetilformamida (8%) y se usaron crioviales como soportes. Post-descongelación, se evaluaron los parámetros: movilidad progresiva, vitalidad, morfología normal e integridad de la membrana plasmática (HOS). Para la evaluación estadística se ajustó un modelo lineal generalizado (GLM) y las medias se compararon por la prueba de Tukey. Se encontraron porcentajes promedio de movilidad progresiva, vitalidad, morfología normal y HOS de $41,6 \pm 11,8$ y $37,0 \pm 8,5$, $54,3 \pm 10,2$ y $52,3 \pm 7,8$, $83,1 \pm 5,4$ y $83,6 \pm 5,8$, $41,7 \pm 9,8$ y $38,9 \pm 3,6$, para el semen criopreservado por congelación y vitrificación, respectivamente. No se encontraron diferencias estadísticamente significativas ($P \leq 0,05$) entre los tratamientos para ninguno de los parámetros evaluados. La capacidad fecundante del semen equino criopreservado por vitrificación es equiparable a la obtenida por congelación convencional.

Palabras clave: Inseminación artificial, calidad seminal, congelación, vitrificación.

There are several methods for cryopreservation of semen, which mainly modify the temperature drop rates, the concentrations of cryoprotectants, and the storage vials used. Many cryoprotectants have been evaluated in the cryopreservation process, such as glycerol, dimethyl sulfoxide, ethylene glycol and dimethyl formamide, among others (Chenier *et al.* 1998; Mantovani *et al.*, 2002; Medeiros *et al.*, 2002). The methodology used mainly for conservation

of stallion semen, is possibly the refrigeration at 4 °C. This technique results in a reduction in the metabolic rate of sperm, allowing extending their survival. However, the sperm can only be stored for a few hours due to rapid reduction of fertility (Ponglowhapan *et al.*, 2004), which depends primarily on the resistance of the plasma membrane damage caused by temperature changes and thermal shock (Sánchez *et al.*, 2006).

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Freezing and storage in liquid nitrogen allow the preservation of semen for long periods of time, reaching post-thaw fertility rates between 60% and 70%, in species as bovine (Liu *et al.*, 1998) and canine (Alamo *et al.*, 2005). For the case of stallion semen is still limited the effective use of frozen semen, mainly by its association with the low fertility and the inconsistency in the results. As is known, the equine sperm are extremely sensitive to alterations generated by the freezing, by osmotic stress resulting from exposure to hypertonic media, and by the osmotic changes induced during the process (Ball, 2008). The osmotic stress has been associated with adverse effects on the motility, viability and mitochondrial membrane potential of equine sperm cells, which could be associated with oxidative stress (Ball and Vo, 2001; Pommer *et al.*, 2002). The osmotic stress in both hypotonic and hypertonic conditions can increase the production of superoxide anion in the equine sperm (Burnaugh *et al.*, 2007).

Equine sperm cryopreservation causes cellular changes associated with apoptosis, such as alterations in the percentage of active caspases, in the mitochondrial activity, in the plasma membrane permeability and in the total and progressive motility (Brum *et al.*, 2008). Hence it is considered that different markers of apoptosis can be used to predict the resistance for freezing of the equine sperm cells (Ortega *et al.*, 2009). Mitochondria suffer significant damage by cryopreservation, since in the post-thaw morphological evaluation has been observed moderate swelling of the mid piece, associated with distention of these organelles (Ball, 2008). Have also been reported in the cryopreserved equine spermatozoa, changes similar to those that occur in sperm capacitation, in a process called "cryo-capacitation" (Thomas *et al.*, 2006).

Rapid and ultra-rapid freezing can prevent the intracellular ice formation due to the cell dehydration and the cell exposure to high concentrations of permeable cryoprotectants (4 to 6 mol L⁻¹) and sugars. The rapid freezing involves the cell exposure to liquid nitrogen vapors (Albarracin, 2005). Vitrification corresponds to a cryopreservation technique which triggers the formation of a vitreous state similar to glass, without the presence of intracellular and extracellular ice crystal, thereby decreasing cell damage (Bailey *et al.*, 2000; Orief *et al.*, 2005).

The advantages of vitrification are large compared to the traditional methods of freezing, because of lower

equipment costs, simplicity and reduced exposure time at low temperatures. However, it has adverse effects of osmotic shock by the use of hypertonic solutions, toxic effects caused by the chemical nature and high concentrations of cryoprotectants, and cellular changes related to exposure to low temperatures (Lopera *et al.*, 2007). New techniques of vitrification of human spermatozoa without cryoprotectants, made by direct exposure of these cells to liquid nitrogen in special vials, have managed to preserve their ability to fertilize reaching motility rates 2.87 times higher than the vitrification processes with cryoprotectant (Isachenko *et al.*, 2004; Saki *et al.*, 2009). The attainment of the application of vitrification processes in equine semen can mean a breakthrough in the fertility rates of cryopreserved semen. The objective of this research was to evaluate the effect of two protocols of cryopreservation (freezing and vitrification) on equine sperm fertilizing capacity.

MATERIALS AND METHODS

Stallion semen collection. The study was conducted with three horses (*Equus caballus*) of the Criollo Colombiano breed, located at the municipality of Girardota (Antioquia, Colombia). Animals had standard conditions of feeding, body condition, environment and reproductive management. Were collected between 3 and 5 ejaculates per stallion, with a maximum rest period of 7 days. The procedure for collection of semen was performed using the method of artificial vagina, with a Missouri model vagina (Minitube®, Tiefenbach, Germany) lubricated with non-spermicidal gel. The gel fraction of the ejaculate was removed by filtration. A mare was used to enhance sexual stimulation of the stallion. The raw semen was kept at 37 °C and later was diluted in a 1:1 ratio in EquiPro® extender (Minitube®, Tiefenbach, Germany). Subsequently, the semen was transported in refrigeration at 4 °C to the Laboratory of Animal Biotechnology of the Politécnico Colombiano Jaime Isaza Cadavid, within eight hours of collection.

Evaluation of semen quality. In each ejaculate were performed classical evaluations to determine the seminal quality. Sperm motility was determined using a phase contrast microscope Eclipse E200 (Nikon® Inc., Melville, USA) by averaging the progressive motility of five different fields of view (400X). The concentration of the fresh semen was established through a SpermaCue® photometer (Minitube®, Tiefenbach, Germany). The assessment of sperm

viability and morphology was made using a eosin-nigrosine technique modified by Barth and Oko (1989). On a glass slide at 37 °C a drop of semen was mixed with a drop of eosin-nigrosin (Sigma-Aldrich® 861006 and N4763) for 30 seconds; moreover, was made a semen smear and then fixed with heat. Through a phase contrast microscope (1000X) the 200 spermatozoa morphology was assessed, being classified as abnormal those that presented primary or secondary anomalies. Stained spermatozoa were classified as dead, while those who did not incorporate the dye were classified as living. Finally, were established general rates of morphologically normal spermatozoa and live spermatozoa.

For the assessment for the integrity of cell membranes of cryopreserved spermatozoa the hypoosmotic swelling test (HOS) was used. 0.5 mL for thawed semen was diluted in 1 mL for sodium citrate (2.94%) (Aldrich W302600) and was centrifuged at 1500 rpm for 8 min. The pellet was resuspended in 500 µL of sodium citrate (2.94%). Then, 50 µL of this solution were added to a tube containing 500 µL of hypoosmotic solution (100 mOsmol L⁻¹) composed by 5.4% sucrose (Sigma S0389) and 2.94% sodium citrate in reagent grade water. This mixture was incubated at 38.5 °C for 30 min and then evaluated the spermatid swelling at least in 100 cells in five different fields (400X) using phase contrast microscopy. Finally, the total proportion of reacted sperm was determined. As selection criteria were processed only the ejaculates with minimum parameters of 60% of progressive motility, 100 million of sperm mL⁻¹ and 70% of normal spermatozoa.

Cryopreservation of stallion semen. Semen cryopreservation was performed using two protocols (rapid freezing and vitrification). For rapid freezing each ejaculate was centrifuged at 1500 rpm for 15 min, then the pellet was resuspended in 2 mL of EquiPro® (Minitube, Tiefenbach, Germany) supplemented with dimethyl formamide (DMF; 5%) and egg yolk (4%). Then, the semen was resuspended in the same medium in sufficient quantity for a final concentration of 100 x 10⁶ spermatozoa mL⁻¹. The extended semen was maintained in refrigeration at 4 °C for 30 min and was packaged in 0.5 mL straws. The straws were cooled again at 4 °C for 15 min and then were subjected to rapid cryopreservation in liquid nitrogen vapors, being placed horizontally at a distance of 4 cm from the surface of liquid nitrogen for 15 min. Finally, straws were immersed in a storage tank of

liquid nitrogen at -196 °C. After one week the straws was thawed at 37 °C for one minute in a water bath. Vitrification of semen was performed according to the procedure reported by Peirouvi *et al.* (2007), which was modified for use in equine semen. The semen was diluted with EquiPro® supplemented with egg yolk (8%) and DMF (8%) in sufficient volume to a sperm concentration of 100 x 10⁶ spermatozoa mL⁻¹. For packaging 1.8 mL cryovials were used, which was added in a volume of 500 µL. The cryovials were exposed to 4 cm nitrogen vapor for 10 min and then were stored in a liquid nitrogen tank. A week later, each cryovial was removed from the tank and the semen was devitrified in a water bath at 37 °C for 5 min.

Statistical design. For statistical evaluation was performed a completely randomized design and the data were analyzed using a generalized linear model (GLM) for each of the dependent variables (progressive motility, vitality, normal morphology and HOS). The means for each treatment were compared by the Tukey test.

The adjusted model was:

$$Y_{ijklm} = \mu + E_i + Tto_j + R_k + Cov_l + e_{ijklm}$$

where:

Y_{ijklm} : Progressive motility / Vitality / Normal morphology / HOS

μ : Media common to all treatments.

E_i : Fixed effect of the stallion (i). (i=1... 3).

Tto_j : Fixed effect of the treatment (j). (j= Rapid freezing, vitrification).

R_k : Fixed effect of repetition (k). (k=1... 12).

Cov_l : Fixed effect of the covariates (l). (l=0... 100).

e_{ijklm} : Random error.

According to the dependent variable for each fitted model were included as covariates the progressive motility, vitality, normal morphology and HOS. To determine the association between variables was developed a Pearson correlation analysis according to the expression: $r(X, Y) = cov(X, Y) / \sigma_X \sigma_Y$. All analyzes were developed using SAS 9.2 software.

Results. Sixteen ejaculates were collected and evaluated, of which 10 were processed and cryopreserved by freezing and vitrification. A total of 32 straws and 35 cryovials were post-thaw assessed (Table 1), for an average of 3.35 repetitions per processed ejaculate and treatment. For the progressive

motility was found an average of 43.7% with a variation coefficient of 18.6%. The determination coefficient (r^2) of the fitted model was 0.84, and the effects of stallion, HOS and vitality were statistically significant ($P \leq 0.05$).

For the vitality was found an average of 56.47% with a variation coefficient of 12.6%. The determination coefficient (r^2) for the fitted model was 0.80, with statistically significant effects of stallion, treatment and motility. For the normal morphology was found an average of 82.95% with a variation coefficient of 31.57%. The main abnormalities observed were proximal and distal cytoplasmic droplets, micro

heads, reflected mid piece and double head. The determination coefficient (r^2) of the fitted model was 0.29, being the stallion the only statistically significant effect. And for the HOS was found an average of 44.71% of reacted spermatozoa with a variation coefficient of 15%, the determination coefficient (r^2) of the fitted model was 0.84, and were statistically significant the effects of stallion, treatment and progressive motility.

The results of the treatments for the variables of progressive motility, vitality, normal morphology and HOS are described in the Table 1.

Table 1. Results for the different parameters of fertilizing capacity by treatment in equine semen. ($\bar{x} \pm DE$).

Treatment	n	Progressive motility	Vitality	Normal morphology	HOS
Fresh semen	16	78.7 \pm 6.2 a	80 \pm 7.5 a	85.9 \pm 5.7 a	74.4 \pm 10.3 a
Freezing	32	41.6 \pm 11.9 b	54.3 \pm 10.3 b	83.1 \pm 5.4 a	41.7 \pm 9.7 b
Vitrification	35	37 \pm 8.5 b	52.2 \pm 7.9 b	83.6 \pm 5.8 a	38.8 \pm 3.6 b

Averages with different letters indicate statistically significant difference ($P \leq 0.05$) between treatments, according to Tukey test.

Correlation coefficients indicated a high and positive association between the variables progressive motility, vitality and HOS. While between progressive motility and vitality with the normal morphology were found

low levels of association. No significant association was found between normal morphology and HOS. The correlation coefficients between the variables are presented in the Table 2.

Table 2. Matrix of correlation coefficients between the parameters of fertilizing capacity of equine semen.

<i>Pearson correlation coefficients</i>				
	Motility	Vitality	Morphology	HOS
	1	0.78	0.24	0.79
Motility		<.0001	0.03	<.0001
	86	83	83	74
	0.78	1	0.23	0.74
Vitality	<.0001		0.04	<.0001
	83	83	83	71
	0.24	0.23	1	-0.06
Morphology	0.03	0.04		0.62
	83	83	83	71
	0.79	0.74	-0.06	1
HOS	<.0001	<.0001	0.62	
	74	71	71	74

For each association are reported values of correlation (top), P value (middle), and the number of repetitions (bottom). Correlations were considered significant with $P \leq 0.05$.

DISCUSSION

Cryopreservation of stallion semen is known for reducing different parameters of fertilizing capacity of sperm, such as total motility, progressive motility, permeability of the plasma membrane, morphology and even mitochondrial activity (Parks and Graham, 1992; Brum *et al.*, 2008). With the results of this study was observed a detrimental effect of equine semen cryopreservation by the procedures of freezing and vitrification, on the parameters of progressive motility, vitality and membrane integrity, compared to the fresh stallion semen. Salazar *et al.* (2011), observed a reduction in the parameters of total motility, progressive motility, and the curvilinear and linear velocities, in semen cryopreserved with rapid and slow rates of cooling. In this study there was no effect of cryopreservation on the normal morphology of stallion semen, while Blottner *et al.* (2001) reported several alterations in the morphology of cryopreserved equine sperm, and Vlasiu *et al.* (2008) describe changes in the head dimensions of the cryopreserved sperm.

The values for the parameters of fresh semen quality found in this work are comparable and, in some cases, higher than those reported in the literature, mainly in relation to normal morphology and plasma membrane integrity. Results are presented for progressive motility: $41.5\% \pm 5.4\%$ (Kankofer *et al.*, 2005); $68.3\% \pm 3.7\%$ (Pérez *et al.*, 2008) and $73\% \pm 6.3\%$ (Cocchia *et al.*, 2011). Results of vitality of $81\% \pm 5.2\%$ (Cocchia *et al.*, 2011); $55.1\% \pm 14.4\%$ (Henry *et al.*, 2002) and $86.6\% \pm 4.3\%$ (Sieme *et al.*, 2003). And normal morphology values: $43.4\% \pm 19.9\%$ (Neild *et al.*, 2000), and also results are presenten for: $56.1\% \pm 14.2\%$ (Neild *et al.*, 2000) and $56.2\% \pm 8.6\%$ (Perez *et al.*, 2008). Being clear the variability among the different reports, which could be attributed to factors such as race, the environment and the evaluation method.

For the cryopreserved stallion semen were found progressive motility values of $56.1\% \pm 20.9\%$ (Hoffmann *et al.*, 2011); 23% (Squires *et al.*, 2004) and $55.3\% \pm 14.3\%$ (Henry *et al.*, 2002). Results of vitality of $32.6\% \pm 6.52\%$ (Hoffmann *et al.*, 2011); 44.1% (Medeiros *et al.*, 2002) and $12\% \pm 9.9\%$ (Henry *et al.*, 2002); and a HOS value of 52.8% (Mantovani *et al.*, 2002). These results were lower in some cases, and in other cases similar to those obtained in this study, being important to consider the differences in the

cryopreservation protocols, the origin of the animals, as well as, in the quality cryopreserved semen.

Between treatments for equine semen cryopreservation (freezing vs. vitrification), no statistically significant difference was found for any of the parameters evaluated as indicators of the fertilizing capacity: progressive motility, vitality, normal morphology and HOS. Thus can be considered that vitrification is comparable to the rapid freezing regarding equine sperm fertilizing ability after cryopreservation.

As mentioned previously, in different investigations has been evident decline in post-thaw quality of cryopreserved stallion semen compared to fresh semen, being slow freezing procedures (Padilla and Foote, 1991) and rapid freezing in nitrogen vapor (Clulow *et al.*, 2008), the predominantly used. Vitrification of semen has been reported primarily in humans, which has demonstrated great efficiency in maintaining the integrity and fertility of spermatozoa (Isachenko *et al.*, 2004a, 2004b; Peirouvi *et al.*, 2007), for that reason was not available for this analysis reports regarding the vitrification of stallion semen. However, equivalence found between vitrified semen quality compared to the frozen semen quality, may be attributed to a combination of factors such as: the increasing of concentration of the cryoprotective and the use of cryovials for vitrification. Thus could expect a good protection of integrity of the sperm through proper heat transfer during freezing and thawing, as well as, a significant reduction in the appearance of extracellular and intracellular ice crystals.

It is known that the storage system affects the outcome of the semen cryopreserved (Samper and Morris, 1998). Oliveira *et al.* (2006) in a study with donkey semen from the Brazilian breed Pêga, found no statistical difference between the use of French straw of 0.5 mL and the macrotube of 2.5 mL. Kozink *et al.* (2006) found a 39.3% for the motility of equine semen packaged in 0.5 mL straws, and of 31.7% for the motility of semen packaged in 3.6 mL cryovials. While in an investigation from Gomez *et al.* (2011), under conditions of cryopreservation in cryovials similar to those used in this work, found a post-thaw motility rates between 30% and 55.6% . These results show mobility rates fairly close to those reported in this study for both packaging systems.

According to the above, vitrification can be considered as a viable alternative for cryopreservation of stallion

semen, for artificial insemination. Given that addition to providing a fertilizing capacity comparable to that achieved with rapid freezing, could allow the storage of higher doses of semen in the cryovials, facilitating logistics for conducting artificial insemination (Gómez *et al.*, 2011), and reducing the risk of failure when is necessary thaw and join multiple doses of semen (Samper and Morris, 1998). Clulow *et al.* (2008) reported that different fertilizing capacity parameters are not affected by changing the dose of semen packaged.

The high and positive association between the parameters of progressive motility, vitality and HOS, suggests the importance of the interrelationship between them, possibly explained by its nature as indicators of integrity and functionality of the plasma membrane, as well as, fertilizing capacity of sperm (Nie and Wenzel, 2001). The low association found between normal sperm morphology and the other parameters of semen quality would indicate that the morphology could behave independently in semen quality analysis, being to highlight their low impact on the motility of the stallion semen.

CONCLUSION

The fertilizing capacity of stallion semen cryopreserved by vitrification is comparable to that obtained in semen cryopreserved by rapid freezing, so that could be considered the use of vitrification as a viable alternative for the cryopreservation of equine semen intended for the artificial insemination.

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