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SHORT COMMUNICATION

Evaluation of two diluents for the storage of fresh and cryopreserved semen of Harris hawk (*Parabuteo unicinctus*)

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ABSTRACT. Seminal storage, both fresh and cryopreserved, has contributed to the reproduction of wild birds in captivity, however, a method of predicting the fertilization capacity is needed. Indicators of the sperm acrosome reaction (AR) have been associated with its fertilization capacity. Therefore, the aim of this study was to evaluate the *in vitro* AR of fresh and cryopreserved Harris hawk sperm using Beltsville Poultry Semen Extender (BPSE) and Lake as diluents. 64 ejaculates were obtained and examined fresh and after thawing. The sperm basic evaluation for each ejaculate was made using eosin-nigrosin, while the ability of AR was assessed by co-incubation with perivitelline layer (PVL). Sperm motility of fresh semen was higher (P<0.05) in fresh semen in BPSE (37.9±1.7) than in Lake (30.9±1.7) diluent. However, the motility decreased (P<0.05) in both diluents after thawing. For fresh semen, the percentage of sperm that underwent an AR without incubation with PVL was higher (P<0.05) with BPSE (14.1±1.7) than with Lake (6.8±2.5) diluent, however, AR was similar between tow diluents (P>0.05) after thawing. The percentage of sperm that underwent an AR when incubated with PVL post thawing in Lake (45.4±2.7) was lower (P<0.05) than that of fresh semen (55.3±3.1), whereas there were no differences (P>0.05) with BPSE. It is concluded that Lake diluent was more efficient for fresh seminal storage, while BPSE diluent was more efficient for seminal cryopreservation in Harris hawk.

Keywords: acrosome, cryopreservation, sperm.

RESUMEN. El almacenamiento seminal *in vitro* ha contribuido a la reproducción de aves silvestres en cautiverio. Sin embargo es necesario predecir su capacidad fertilizante. Los indicadores de reacción acrosomal (RA) espermática se han relacionado con su capacidad fertilizante. Por lo anterior, el objetivo de este trabajo fue evaluar la capacidad de reacción acrosomal *in vitro* en espermatozoides de halcón Harris, conservados en fresco y criopreservados. Se obtuvieron 64 eyaculados, los cuales fueron evaluados en fresco y posdescongelación, 32 se diluyeron con medio *Beltsville Poultry Semen Extender* (BPSE) y 32 utilizando medio Lake. Se evaluaron 64 eyaculados en fresco y posdescongelación; para la evaluación espermática básica se utilizó la tinción de eosina-nigrosina, y capacidad de RA fue mediante su inducción con membrana perivitelina (MPV). La movilidad espermática en semen fresco fue mayor (P<0,05) cuando se usó el medio BPSE (37,9±1,7) en comparación con el medio Lake (30,9±1,7). Sin embargo, la movilidad en ambos diluyentes disminuyó (P<0,05) posdescongelación. En semen fresco, el porcentaje de RA sin incubar con MPV fue mayor (P<0,05) con medio BPSE (14,1±1,7) al porcentaje con medio Lake (6,8±2,5). En semen descongelado los promedios de RA fueron similares (P>0,05). El porcentaje de espermatozoides con RA en el medio Lake, incubados con MPV posdescongelación (45,4±2,7), fue menor (P<0,05), comparado en fresco (55,3±3,1). Se concluye que el diluyente Lake fue más eficaz para la conservación en fresco y el diluyente BPSE para la criopreservación seminal de halcón Harris.

Palabras clave: acrosoma, criopreservación, espermatozoide.

INTRODUCTION

There are approximately 10,500 species of birds in the world, of which 5% have been placed in a risk category or are in danger of extinction. Moreover, 52 of these species are raptors (Navarro *et al* 2014). Assisted reproductive techniques in birds have contributed to their conservation and sustainable use for purposes such as falconry, which has been declared a Cultural Heritage of Humanity (UNESCO 2010). The Harris hawk (*Parabuteo unicinctus*) is the most common in falconry worldwide. Different methods of seminal conservation that have been used for roosters and turkeys (Sexton 1977, Lake and Ravie 1984) have been used to preserve the sperm of different raptors with varying results, and the outcomes of their fertilization ability have not been reported (Herrera *et al* 2005). Various seminal freezing protocols have also been implemented, regardless of the physiological differences between species, such as the alkaline and acidic urinary pH of roosters (pH 8.0) and raptors (pH 6.5; Umaphaty *et al* 2005). The use of a basic assessment and *in vitro* seminal indicators of the acrosome reaction (AR), allow to predict the *in vivo* fertilization ability of roosters (Lemoine *et al* 2008), mammals (Ricart *et al* 2015), and turkeys (Ochoa *et al* 2014).

The AR is associated with the entry of Ca^{2+} into the sperm, which induces the function of the ATP*ases* in addition to an increase in the intracellular sodium level, which results in the export of hydrogen and consequently, an increase in the intra-acrosomal pH; changes in membrane glycoproteins also occur (Arenas *et al* 2010). The AR in rooster sperm can be induced *in vitro* in a simple saline medium containing a perivitelline layer (PVL) and Ca^{2+} (Lemoine *et al* 2009). Therefore, the aim of this study was to evaluate the *in vitro* ability to undergo an AR of

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Harris hawk sperm, stored both fresh and cryopreserved in two diluents.

MATERIAL AND METHODS

The animal procedures were performed according to the protocols for the care and welfare of animals in the Official Mexican Regulation 062-ZOO-1999. The study was carried out in the native range of the central region of Mexico (21.849044, -102.286742), during March and April, in the natural reproduction station. 64 ejaculates of eight specimens of Harris hawk aged betwen 5 at 8 years old were obtained, using the dorsoventral massage technique (Burrows and Quinn 1937).

MANAGEMENT AND EVALUATION OF SEMEN

The semen was collected from the spermatic groove of the erectile papilla by aspiration with a graduated micropipette (RAININ™) and the ejaculated volume was determined. Thirty two ejaculates were diluted in 50 µl of Beltsville Poultry Semen Extender (BPSE) at 25 °C, described by Sexton and Rivers (1977), containing 0.072 M dibasic potassium phosphate, 0.046 M sodium glutamate, 0.027 M fructose, 0.031 M sodium acetate, 0.016 M N-Tris (hydroxymethyl/aminomethane), 0.0019 M potassium citrate, 0.0047 M monobasic potassium phosphate, and 0.0027 M tetrahydrated magnesium chloride, whereas other 32 ejaculates were diluted in Lake diluent (Lake and Ravie 1984) containing 0.09 M sodium glutamate, 0.04 M fructose, 0.003 M magnesium acetate, and 0.05 M potassium acetate. Fifty percent of the volume of the diluted semen of each sample was used for the evaluation of fresh sperm, and the remaining sample was frozen for later evaluation. The samples were maintained for a maximum of 10 min, at 5 °C prior to freezing in both diluents.

SEMEN FREEZING

Semen aliquots containing 5 X 10^6 sperm were prepared with the BPSE or Lake diluents with 64.1 M dimethyl sulfoxide adjusting to 50 µl in 0.25-ml straws. The samples were cooled at a rate of -5 °C/minute, equilibrated for one minute at 5 °C and then frozen for 10 minutes in liquid nitrogen vapors at 3 cm from the surface (Peláez *et al* 2011). The aliquots were then immersed in liquid nitrogen for at least 30 days before thawing, which was performed at 37 °C for 30 s.

BASIC SEMEN EVALUATION

The percentage of sperm with straight progressive motility in 10 µl semen aliquots was estimated by microscopy (OLYMPUS BX51) with a 40x objective. The sperm concentration was quantified using a Neubauer chamber (Herrera *et al* 2005, Ricart *et al* 2015). Moreover, sperms of a 10-µL-aliquots were stained with eosin-nigrosin (QCA, 996518, USA) and 100 spermatozoa from each sample were analysed using an optical microscope (OLYMPUS BX51) with a 100x objective to assess its viability and morphology (Blanco *et al* 2008, 2012 Ricart *et al* 2015).

ACROSOME REACTION DETERMINATION

The AR pattern of the sperm was determined in fresh and thawed semen (Ochoa *et al* 2014, Ricart *et al* 2015), using aliquots of 5 X 10^6 sperm, which were incubated with 0.9 M chlortetracycline (CTC) in the dark at 38 °C (Lemoine *et al* 2011, Cruz *et al* 2015). For both conditions (fresh and thawed semen), aliquots were adjusted to 50 µL with the BPSE or Lake diluents with or without PVL (20 µg) obtained from fresh egg of hen after removing the vitelo by washing with BPSE medium, and incubated for 30 min. Then, 25 µL of CTC were added, and the mixture was incubated for 10 min (Lemoine *et al* 2008). After the incubation period in both experiments, slides were prepared to observe the samples under an Olympus BX51 fluorescence microscope with a 100x objective (488 nm excitation and > 560 nm emission). Image analysis was performed using the Image-Pro Plus software, version 6.2.1. Two hundred sperm per preparation were counted to determine the proportion of sperm that underwent an AR (Ochoa *et al* 2014).

STATISTICAL ANALYSIS

An analysis of variance (ANOVA) was done to determine differences. The differences in motility, live sperm and AR in presence or not of PVL where analyzed by a factorial design using the sample type (fresh or thawed), the diluent and its interaction as the factors. Later with the Tukey test, determined the difference between variables ($P>0.05$).

RESULTS AND DISCUSSION

The fertilization ability of sperm depends on several parameters, particularly sperm motility and the ability of sperm to undergo an AR (Mocé *et al* 2010, Ahammad *et al* 2013), which makes *in vitro* evaluations necessary. In this study, the spermatic parameters determinated are shown table 1. The percentage of motile fresh sperm was not different ($P>0.05$) in the BPSE diluent (63.7±2.3) to the peprcentage in Lake diluent (68.4±1.9), however, motility was higher ($P<0.05$) post-thawing in the BPSE diluent (37.9±1.7) than in the Lake diluent (30.9±1.7). The motility in both diluents was also significantly lower post-thawing ($P<0.05$) than in the fresh condition. The results obtained in this study during the basic sperm evaluation demonstrated that the cryopreservation process affects sperm motility, resulting in a 50% decrease in both diluents; these data are consistent with reports by other

Table 1. Basic indicators of sperm assessment of Harris hawk (*Parabuteo unicinctus)* fresh and post thawing using sperm diluents Beltsville Poultry Semen Extender (BPSE) and Lake.

Sperm indicator	Semen fresh X±EE		Semen thawed X±EE	
	BPSE n = 32	Lake n = 32	BPSE n = 32	Lake n = 32
% Mobility	63.7±2.3^{a,1}	68.4±1.9$^{b,+}$	37.9±1,7^{a,2}	30.9±1.7^{b,x}
% Live	98.0±0.2^{a,1}	96.0±1.0$^{a,+}$	95.0±0,5^{a,2}	94.0±0.5$^{a,+}$
% Normal morphology	98.6±0.1^{a,1}	94.6±1.4$^{b,+}$	95.3±0,7^{a,2}	93.4±0.4$^{a,+}$
% Abnormalities:				
Dead	0.5±0.1^{a,1}	2.2±0.6$^{b,+}$	2.0±0.4^{a,2}	1.6±0.3$^{a,+}$
Neck	0.6±0.2^{a,1}	1.5±0.4$^{a,+}$	1.3±0.3^{a,2}	1.6±0.2$^{a,+}$
Tail	0.1±0.1^{a,1}	1.5±0.5$^{a,+}$	1.1±0.2^{a,2}	3.2±0.3$^{b,+}$

When comparing the same variable between means, different literal (a,b), indicates statistical difference (P>0.05).
When comparing in the medium BPSE, indicators in fresh semen against thawed, different numbers (1, 2) indicate statistical difference (P>0.05).
When comparing in the medium Lake, indicators in fresh semen against thawed, different symbols (+, X) indicate statistical difference (P>0.05).
Values represent the mean ± standard deviation (X±SD).

authors (Herrera *et al* 2005, Froman 2013), who report that motility is the only parameter in the basic evaluation of sperm that is affected by the process of freezing bird sperm. The percentage of live sperm did not differ (P>0.05) between the BPSE and Lake diluents, and in fresh (98.0±0.2 and 96.0±1.0) and thawed (95.0±0.5 and 94.0±0.5) semen. In this study, after 10 min of storage of semen in fresh, we observed viability percentages above 90% were observed. Viability percentages greater than 90% were also observed in both diluents after thawing, which is consistent with other studies that have evaluated the resistance of raptor sperm to osmotic stress and membrane fluidity (Blanco *et al* 2008). Therefore, it is suggested that the sperm of raptors can withstand the freezing and thawing processes (Blesbois *et al* 2008). The percentage of sperm with normal morphology in fresh samples in BPSE diluent (98.6±0.1) was higher (P<0.05) than in Lake diluent (94.6±1.4). There were no differences (P>0.05) between the diluents post-thawing. Studies conducted by Blanco *et al* (2012) showed that thawed turkey sperm presented low survival rates, with only approximately 50% recovery of viable sperm. Regarding abnormal sperm morphology, in this study damage was only found in the tail. However, up to 41% of abnormalities in the sperm head have been reported in the sperm of golden eagles (*Aquila chrysaetos*) and peregrine falcons (*Falco peregrinus*), suggesting that abnormalities may be a genetically determined feature (Wishart *et al* 2002). Other studies have suggested that bird sperm is more susceptible to morphological injuries and ultrastructural abnormalities after freezing than that of mammals, and damage is commonly reported to occur in the tail. It has been suggested that in most studies damage occurs during the handling and manipulation of the samples (Donoghue *et al* 2000, Agca and Critser 2002). It has also been reported that bird spermatozoa are more susceptible to osmotic stress than mammalian sperm (Watson *et al* 1992). In studies of Griffon vultures, it has been reported that the sperm of this species can tolerate freezing and thawing procedures, remaining viable for four hours *in vitro* after thawing (Madeddu *et al* 2009).

There is evidence that AR in bird sperm is unique because the release of the acrosomal proteolytic enzyme may occur through a single circular opening formed at the base of the acrosomal cap, which is detached in an intact form from the posterior acrosomal region of the sperm (Ahammad *et al* 2013). This result is consistent with the fluorescence patterns identified in this study and those reported by Ochoa *et al* (2014), and is referred to as the sperm AR because when we assessed the intracellular calcium mobilization with CTC staining, we obtained a sperm pattern with a fluorescent band in the equatorial region, indicating loss of the acrosomal membrane without loss of the inner membrane.

The results of this study regarding the ability of sperm to undergo an AR are shown in table 2, which shows that the percentage of sperm undergoing an AR without PVL was higher (P<0.05) in the BPSE diluent (by approximately 50%) than in Lake diluent in fresh semen, but not in thawed semen. Other studies have reported that 30% of sperm in fresh semen undergo an AR (Lemoine *et al* 2011). These findings can be explained by the preservation medium, which causes a change in the phospholipid composition and an increase in the permeability in the acrosomal region, leading to increased membrane fluidity, which allows the release of sperm enzymes, inducing a premature AR (Donoghue and Walker 1999). Interestingly, the sperms in Lake diluent had less premature AR which suggests that this diluent is more efficient that BPSE diluent for storage of fresh semen. In presence of PVL the ability of sperm to perform the AR was not different (P>0.05)

Table 2. Percentages of sperm showing an acrosome reaction in fresh and post-thawed Harris hawk (*Parabuteo unicinctus*) sperm samples preserved in the Beltsville Poultry Semen Extender (BPSE) and Lake diluents.

Semen condition	Semen Without PVL X±SD		Semen incubated with PVL X±SD	
	%			
	BPSE n=32	Lake n=32	BPSE n=32	Lake n=32
Fresh	14.1±1.7[a]	6.8±2.5[b]	52.0±2.6[ab]	55.3±3.1[a]
Thawed	17.3±2.0[a]	13.6±1.9[a]	51.9±2.7[ab]	45.4±2.7[b]

PVL - Perivitelline layer - Membrana perivitelina.
Values represent the mean ± standard deviation (X±SD).
Between different diluents and times of evaluation (fresh vs thawed semen) values with different letters (a,b) indicate significant differences ($P<0.05$).

in fresh semen than thawed semen using BPSE diluent, however with Lake diluent the ability of sperm to perform the AR was lower ($P<0.05$) after thawed. Some studies have reported that cryopreservation impairs the ability of sperm to undergo an AR due to the reduced production of cAMP, lack of mobilization of Ca^{2+}, or oxidative stress and changes in the proportion of cholesterol and phospholipids in the membranes before the AR (Ashizawa *et al* 2006).

The percentage of sperm that underwent an AR was always higher ($P<0.05$) when they were induced with PVL than the percentage that underwent an AR without PVL. Results suggest that the incubation of sperm with heterologous PVL induces an AR in Harris hawk sperm. This phenomenon occurs because the PVL contains progesterone. When progesterone interacts with its receptor on the sperm membrane, it causes Ca^{2+} to enter the cell, leading to an AR (Sasanami *et al* 2007, Lemoine *et al* 2009, Ickowicz *et al* 2012).

The increased capacity of the Lake diluent to store fresh semen may be explained because the pH of the diluent is 7.0, which is similar to the pH value of semen from several raptor species, including Harris hawk (pH 7.0), *Gyps africanus* (pH 7.1), *Aquila fasciata* (pH 6.86), *Aquila chrysaetos* (pH 7.25), and *Aquila adalberti* (pH 6.8) (Umapathy *et al* 2005). However, the BPSE diluent has a pH of 8.6, which is similar to the pH value of *G. gallus* semen. The semen of *G. gallus* has shown better results after cryopreservation, which can be attributed to the greater variety of salts in its composition that might contribute to the stabilization of the osmolarity conditions, in addition to producing a cryoprotective effect.

Based on the results of this study, we conclude that BPSE and Lake diluents have different efficacies for the storage of Harris hawk semen in fresh and cryopreserved. Based on the *in vitro* ability to produce an AR in the presence of PVL and the basic sperm evaluation, the Lake diluent is more efficient for the storage of fresh semen, whereas the BPSE diluent is more effective for cryopreserved semen.

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