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Viability of *in vitro* preserved sperm of Harris's hawk (*Parabuteo unicinctus)*

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Abstract

Harris hawks (*Parabuteo unicinctus)* are the raptors with more anthropogenic use, which makes it necessary to improve their reproduction either natural or assisted for sustainable use even though they are not endangered. The aim of this study was to determine fresh and thawed *P. unicinctus* sperm capacitation and acrosome reaction parameters as indicators of sperm viability in ejaculates of birds with injuries. A total of 30 ejaculates were analyzed as fresh and thawed samples. Basic sperm evaluations as well as *in vitro* acrosomal reaction capability through the presence and distribution of Ca^{2+} and N-acetylglucosamine and sialic acid using chlortetracycline and agglutinin-fluorescein isothiocyanate *Triticum vulgaris* were performed, respectively. Tagged samples were observed using epifluorescence microscopy. The results indicated 30.00% decrease in sperm viability following freeze-thaw, while rate of capacitated sperms (10.00%) and the percentage of sperm with acrosomal reaction (40.00%) were increased. The results of the experiment demonstrated that the post-thaw viability of the obtained sperm may be an alternative for use in artificial insemination.

Introduction

Conservation and sustainable use of wild species are of great interest worldwide[1] and most of the raptors in wildlife are included in some endangered categories. [2] It is common to find these birds kept in captivity, taking part in private collections and used either for falconry or biological control of birds in airports.[3]

Harris's hawk (*Parabuteo unicinctus)* is one of the species with most presence in captivity and it is also frequent to find some specimens of this species with injuries, which even though these do not compromise its life, they do avoid the natural reproduction of the species being possible to be done in captivity through assisted reproduction techniques;[4] nevertheless, specific protocols are needed for each species such as artificial insemination.

Sperm fresh preservation[5] and cryopreservation[6] studies in wild birds use similar techniques as those used in poultry; nevertheless, obtained results are diverse. Different irregularities in plasmatic membrane of the sperm have been reported in *Aquila chysaetus*,[5] *Gallus gallus, Meleagris gallopavo, Hieraaetus fasciatus* and *Falco peregrinus* as an effect of osmolarity,[7] on the other hand, *Buteo jamaicensis* sperm membrane showed irregularities in the carbohydrates distribution.[8]

Sperm cryopreservation is the main method of preserving gametes for a long time; nevertheless, it has been proved that this method modifies the lipid organization in the sperm membrane[9,10] along with its fluency[11] and carbohydrate content reductions.[12] Also, it produces damages in mitochondria, nucleus and acrosome affecting the sperm capability of acrosome reaction (AR) modifying its fertilization capability.[9,10]

In avian sperm, AR can be induced naturally using extracts of perivitelline layer (PVL). The capability of AR can be assessed *in vitro* using chlortetracycline to determine the presence and distribution of Ca^{2+}, being associated to the states of capacitation and AR of sperm.[13,14]

Another important aspect to take into consideration is fluency of plasma membrane having a relevant function in physiological restoration of the sperm after thawing.[11]

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In vitro indicators of capacitation and AR provide information about the physiological state of the sperm to predict its fertilization capability *in vivo*.[15] The main objective of this study was to determine fresh and thawed *P. unicinctus* sperm capacitation and acrosome reaction parameters as indicators of sperm viability in ejaculates of birds with injuries.

Materials and Methods

During the natural reproduction season in the months of March and April, 10 clinically healthy males of 4 to 7 years old were selected, which were previously attended because of physical injuries (fractures and amputations). Ten specimens were included in the study because they were donors of semen after one year of recovery. Birds that did not ejaculate were not included in the study. The hawks were kept in Bird of Prey Rehabilitation Center, Aguascalientes, Mexico (21°50´55.5" N 102°17´12.8" W). All the animals were treated worthily and respectfully, according to NOM-062-ZOO-1999[16] and to the management plan authorized by Secretary of Environment and Natural Resources (Mexico), registered as INE/CITES/ DGVS-CR-IN-AV-0035-AGS./00.

Seminal obtaining. The ejaculates were collected through dorso-ventral massage after three training sessions. Three ejaculates were obtained from each specimen, achieving 30 ejaculates in total and ejaculates contaminated with feces or urates were discarded. Each ejaculate was aspirated with a graduated micropipette (Pipetman® 0.20 - 10.00 µL; Gilson, Madrid, Spain) in which the volume of ejaculate was determined and preserved in 50.00 µL of Lake extender (LE) containing 0.1135 M sodium glutamate, 0.04 M fructose, 0.05 M potassium acetate and 0.0056 M magnesium acetate (all reagents used were from Sigma-Aldrich, St. Louis, USA).[17]

Seminal evaluation. The percentage of sperm with progressive motility was estimated with microscopy at 40× (BX51; Olympus, Miami, USA) in aliquots of 15.00 µL of semen at 37.50 ˚C. Sperm concentration was quantified using a Neubauer chamber.[8,18] Sperm from a 10.00 µL aliquot was stained with Eosin-Nigrosin and through an optic microscope, viability and morphology were determined in 100 sperms of each sample.[8,18]

Seminal cryopreservation. Aliquots of 50.00 µL with 5.00×10^6 sperms were prepared with LE and 64.10 M dimethyl sulfoxide (DMSO) was added, preserving them in straws of 0.25 mL. Straws were taken from 37.00 ˚C to 5.00 ˚C in 10 min at a rate of –3.20 ˚C per min, at 5.00 ˚C, they were equilibrated during 5 min, the cooling speed was –5.00 ˚C per min and later freeze was carried out during 10 min in liquid nitrogen vapors 3.00 cm far from surface.[19] The aliquots were sub-merged in liquid nitrogen during 30 days before thawing, which was performed at 37.00 ˚C during 30 sec.

Evaluation conditions. In both, fresh and cryo-preserved samples, aliquots of 5.00×10^6 were adjusted to 50.00 µL with LE for capacitation condition (CC)[20] or with LE and 20.00 µL of PVL to induce AR condition (ARC), both conditions were incubated at 38.00 ˚C for 30 min.[21]

Capacitation and acrosomal reaction indicators. Presence and distribution of Ca^{2+} in the spermatozoa membrane and mitochondria were determined in aliquots with 5.00×10^6 sperms in LE, which were incubated during 10 min with 0.90 M chlortetracycline in darkness. [14,22] To determine membrane carbohydrates, *Triticum vulgaris* (WGA) agglutinin was applied for specific determination of sialic acid and N-acetylglucosamine. The used lectin was conjugated with fluorescein isothiocyanate (Sigma-Aldrich). Aliquots with 5.00×10^6 sperms in LE having 5.00 µL of WGA-FITC (20.00 µg mL^{-1}) were incubated during 30 min at 38.00 ˚C. The aliquots were immediately washed in LE at 600 *g* for 5 min. The formed pellet was fixed in 1.00% paraformaldehyde in LE.[8] In both experiments, after the incubation period, the slides were prepared and observed using the fluorescence microscope (Olympus) at 100× (488 nm excitation and > 560 nm emission). Image analysis was performed using the Image-Pro Plus Software (version 6.2.1; Media Cybernetics, Silver Spring, USA). Two hundred sperms per preparation were counted to determine the proportion of capacitated sperms as well as those with AR capability determined with chlortetracycline.[23] The fluorescent pattern also describes the presence and distribution of sialic acid and N-acetylglucosamine[8] in the sperm surface. In each of the evaluations, the presence and distribution of Ca^{2+} and membrane carbohydrates were determined with the respective fluorescence patterns, which were similar in every evaluation and associated to intact, capacitated and AR sperm (Figs. 1 and 2).

Statistical analysis. The PAST Software (version 3.17; Hammer and Harper, Oslo, Norway) was used to perform the Mann-Whitney test to determine differences among motility, viability, morphology, CC and ARC. A value of $p < 0.05$ was considered significant.[24]

Results

Basic evaluation parameters. Average volume of each obtained ejaculate was 17.00 µL with a range of 10.00 to 20.00 µL; the concentration of each ejaculate was $3,525 \pm 175 \times 10^6$ sperms. In fresh semen, sperm showed motility of 72.20% and viability of 95.30%, both higher ($p < 0.05$) than percentages determined in thawed semen: 16.80% and 58.10%, respectively. According to sperm morphology, there were not significant differences ($p > 0.05$) between fresh and thawed samples (Table 1).

Table 1. Basic evaluation in recently ejaculated and thawed samples of Harris's hawk (*Parabuteo unicinctus)*. Data are presented as mean ± standard deviation.

Parameter	Fresh semen	Thawed semen
Motility	77.20 ± 1.60 [a]	16.80 ± 0.70 [b]
Viability	95.30 ± 0.60 [a]	58.10 ± 8.70 [b]
Normal morphology	97.70 ± 2.00 [a]	97.00 ± 0.50 [a]
Head abnormalities	2.20 ± 0.20 [a]	1.40 ± 0.40 [a]
Neck abnormalities	2.40 ± 0.20 [a]	0.90 ± 0.20 [a]
Tail abnormalities	1.80 ± 0.20 [a]	0.70 ± 0.30 [a]

[ab] different letters indicate statistical significant difference ($p < 0.05$). Same variables are compared in different conditions.

Sperm physiological status. It was showed that in CC assessment of fresh and thawed semen, the percentages of intact sperms were higher ($p < 0.05$) than percentages of sperms with AR. On the contrary, in ARC, the percentages of intact sperms in fresh and thawed semen were lower ($p > 0.05$) than percentages of sperms with AR (Table 2). The percentages of intact sperms in the CC were always higher ($p < 0.05$) than percentages determined in those incubated in ARC. On the other hand, percentages of sperms with AR in CC were lower ($p > 0.05$) than those determined in ARC. The findings showed the AR capability following co-incubation with PVL as a natural inductor of AR, proving different sperm subpopulations in intact sperm, capacitated ones and those with AR. In regard with capacitated sperms, the percentages determined showed always intermediates values, suggesting the presence of intact sperm sub-populations in transition to become sperms with AR.

Discussion

The results demonstrated that cryopreservation affected basic sperm parameters. Viability decreased approximately 40.00%; similar studies have reported that cryopreservation decreases sperm viability significantly up to 85.00% in *G. gallus,* 64.00% in *Numidia meleagris* and 67.00% in *M. gallopavo*. The motility of 16.80% in *P. unicinctus* sperms cryopreserved with DMSO was obtained; compared with other study,[8] in which DMSO was used as a cryoprotectant, a motility of 36.00% in rooster sperms, 42.00% in pheasant sperms and 34.00% in *B. jamaicensis* sperms was showed.

Fluorescence patterns identified with the applied techniques showed a physiological indicator different form the one identified in the basic evaluation. The results of this study regarding fresh and thawed semen demonstrated a similar behavior among sperm parameters of capacitation and AR, associated with assessments performed. The findings showed that the percentages were lower when the parameters were determined in CC and higher when determined in ARC, due to the use of PVL as a natural inductor of AR as it contains ZP1 protein which is an AR inductor in birds sperm.[25]

These results met proportions showed in the study done by Ochoa *et al.*, in recently ejaculated semen of *M. gallopavo* analyzed under the same method, where it was determined that it has the same tendency, in which it is shown an intact sperm percentages of 72.40%, capacitated sperms (9.30%) and sperms with AR (2.70%).[23]

In fresh semen, similar parameters of intact and capacitated sperms could be a result of the eventual collection of the ejaculates in specimens without frequent reproductive activity, due to the fact that mature produced sperms stay in the deferens duct; nevertheless, they do not have the adequate conditions for storage or decapacitation which was mentioned in this study as intact sperms, following their maturation, capacitation and AR which has been demonstrated to be induced by Ca^{2+} ions, naturally present in secretions of the deferens duct[11,12] without these processes being stopped in the male reproductive tract.

The results referred to sperm parameters in CC, can be confirmed as it has been demonstrated *in vivo* that these are associated with the oviduct secretions[26] stabilizing sperm membrane[27] being described *in vivo* in the female reproductive tract.[20]

Table 2. Capacitation and acrosomal reaction percentages in sperms of fresh and thawed Harris's hawk semen (*Parabuteo unicinctus)*. Data are presented as mean ± standard deviation.

Sperm	Fresh semen		Thawed semen	
	CC	ARC	CC	ARC
CTC determination				
Intact	61.50 ± 1.60 [a +]	22.60 ± 1.40 [α -]	44.10 ± 3.00 [b +]	10.60 ± 1.60 [β -]
Capacitated	22.10 ± 1.10 [a +]	25.30 ± 1.60 [α +]	32.70 ± 1.70 [b +]	25.90 ± 2.10 [α -]
Acrosomal reaction	16.80 ± 1.30 [a +]	51.40 ± 1.30 [α -]	23.00 ± 2.40 [b +]	63.40 ± 3.10 [α -]
WGA-FITC determination				
Intact	41.60 ± 2.50 [a +]	21.10 ± 2.40 [α -]	50.80 ± 1.50 [b +]	10.40 ± 1.20 [β -]
Capacitated	42.00 ± 3.60 [a +]	39.00 ± 2.70 [α +]	32.30 ± 2.70 [b +]	39.00 ± 2.00 [α -]
Acrosomal reaction	16.40 ± 1.70 [a +]	39.80 ± 3.10 [α -]	16.70 ± 1.50 [a +]	50.40 ± 2.30 [β -]

CC: Incubation in sperm capacitation condition without perivitelline layer; ARC: Incubation in acrosomal reaction condition co-incubated with perivitelline layer; WGA-FITC: Wheat germ agglutinin-fluorescein isothiocyanate. CTC: Chlortetracycline. In CTC or WGA determinations: When comparing fresh vs thawed CC sperm parameters, different letter (a, b) indicate statistical differences ($p < 0.05$). When comparing fresh vs thawed ARC sperm parameters, different symbol (α, β) indicate statistical differences ($p < 0.05$). In fresh or thawed semen when comparing sperm percentages in CC or ARC, different symbol (+, -) indicate statistical difference ($p < 0.05$).

On the other hand, it has been proved that cryopreservation affects membrane fluidity as well, which could compromise the fertilization capability of sperm[11] and probably induce membrane alterations, which could be related with the capacitation and AR indicators determined in this study. When comparing the tolerance ranges to cryopreservation with different cryoprotectants in different avian species, it has been observed that sperms are physiologically different;[28] the results in this study demonstrated that sperm capacitation and AR capability can be modified by the cryopreservation process. Diverse studies have indicated that avian sperms do not require the capacitation or hyper-activation processes to achieve the AR and fertilization capability as well.[22,29]

The results in this study demonstrated evidence of parameters making possible the measurement, showing the necessity to recognize the physiological status of sperm, in order to perform specific studies in each species allowing to develop protocols and more efficient and specialized sperm preservation extenders, additional to the evaluation technique validation contributing to determine *in vitro* viability to predict *in vivo* fertilization capability.

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Conflict of interest

There are no conflicts of interest of any kind.

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