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Short Communication
Survival to Freezing of Sperm Collected by Vas Deferens from *Vicugna pacos* with Tempol Antioxidant Addition

[1]Oscar E. Gómez-Quispe, [2]Manuel Guido Pérez and [3]Yadsen G. Ojeda-Gutierrez

[1]Faculty of Veterinary Medicine and Animal Science, Universidad Nacional Micaela Bastidas de Apurímac, Patibamba baja, Abancay, Apurimac, Peru
[2]Faculty of Veterinary Medicine and Animal Science, Universidad Nacional del Altiplano, Puno, Peru
[3]Representaciones Agropecuarias Nacional SRL, Lima, Peru

Abstract

Background: The viscosity of semen alpaca and oxidative stress are some of the difficulties of cryopreservation. Is continuous, the search of methods to reduce and even avoid this viscosity, as well as decrease the effect of oxidative stress of the alpaca semen. The purpose of the study was to determine the effect of the adding of tempol antioxidant on spermatozoa of epididymal alpaca obtained by desviation of vas deferens. **Methodology:** The vas deferens from three alpacas with adequate sperm parameters were deviated surgically. Sperm samples were collected three times a week and were mixed to annul the individual effect. Collective sample was divided into four aliquots and it was placed in a refrigerator up to 5°C for 2 h. Vitality and membrane integrity sperm were evaluated immediately harvested, cooled and post freezing/thawing. When the temperature reached 10°C in cooling phase was added tempol antioxidant at a final concentration of 0.0, 0.5, 1.0 and 1.5 mM. These were packaged in 0.25 mL straws and placed on liquid nitrogen vapors during 6 min and then submerged. **Results:** The initial sperm vitality was 79.30%, after cooling 66.68, 75.15, 70.03 and 55.03% and post freeze/thawing 42.45, 47.96, 32.06 and 26.13%. The initial sperm membrane integrity was 75.69%, after cooling 63.33, 66.14, 57.56 and 51.83% and 36.26, 44.20, 34.43 and 27.94% post freezing/thawing. In all cases, there was statistical difference ($p \leq 0.05$). **Conclusion:** The sperm collection technique by vas deferens from alpaca and low concentrations of tempol antioxidant (0.5 mM) improves the sperm vitality and the functional sperm membrane integrity.

Key words: Sperm, vas deferens, epididymis, cryopreservation

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Corresponding Author: Oscar E. Gómez-Quispe, Faculty of Veterinary Medicine and Animal Science, Universidad Nacional Micaela Bastidas de Apurímac, Patibamba baja, Abancay, Apurimac, Peru

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

South American camelidae raising constitutes a source of income in the family economy of the population of the high areas of the Andes. However, this activity takes place with some reproductive difficulties. Among other factors, the alpacas have shown limited reproductive performance. In the like manner, alpaca spermatozoa are highly susceptible to cryopreservation[1], which has caused the lack of routine artificial insemination procedures at field level. In this context, currently low sperm parameters were reported in semen thawed using the artificial vagina[1,2] and similarly in epididimal spermatozoa[3-5], as well as in the llama[6] attributed to the high viscosity of semen, which has hampered their handling and processing[7,8]. This had generated the development of alternative techniques, such as the collection of sperm directly from vas deferens of camelids, although devoid of seminal plasma, proposed by Perez *et al.*[9], which has improved sperm parameters after freezing/thawing with the advantage that allows to use available sperm in any season and permanently.

Generally, sperm are vulnerable to washing, dilution, osmotic changes and temperature variations, increasing sperm with damaged membranes or organelles[10]. Another factor of the low sperm survival is attributed to oxidative stress semen, due to an alteration between the generation of Reactive Oxygen Species (ROS) and antioxidant mechanisms, causing lipid peroxidation of sperm membrane, which results low motility and morphology alterations in sperm[11] causing cell damage and affecting sperm viability[12] during the process of semen cryopreservation[13]. During the process of freezing/thawing, a high proportion of spermatozoa suffer irreversible damage to the structure and functional integrity of plasmatic membrane[14]. To counteract this effect, some antioxidants have been used[15], including tempol, which apparently prevents loss of motility and functional integrity of the sperm membrane during cooling sperm[16] and improves semen parameters after freezing/thawing of the semen[2].

The sperm damage can be valued at the level of membrane[10]. The hypoosmotic swelling (HOS) test, that evaluates the functionality and biochemical integrity of sperm membrane[17] and the sperm vitality that measure the amount of living and dead immobilized sperm[18] are some methods. The aim of this study was to determine the effect of the addition of tempol antioxidant on sperm vitality and functional sperm membrane integrity, collected by vas deferens, in fresh, after cooling and freezing/thawing in alpaca sperm.

MATERIALS AND METHODS

Localization: The study was conducted in the animal reproduction Laboratory of Micaela Bastidas University of Apurimac (UNAMBA), located in the district and province of Abancay, department of Apurimac, at 2378 m of altitude, where the rainy season is November to March and the maximum and minimum average temperatures range is 23.8-11.7°C.

Population and sample: Sperm samples of three male alpacas with better seminal characteristics as progressive motility >50%, membrane integrity >60% and percentages of live >80% previously suggested[19] was used, which were mixed to cancel the male effect.

Preparation of animals: The animals were transferred from populated center of San Miguel de Mestizas (Iscahuaca, Cotaruse, Apurimac) to the campus of the UNAMBA, where they were exposed to a adaptation period, which included new climatic conditions, different system feeding, habituated to handling and improved docility for eight months. The animals were fed to grazing on pastures with a predominance of *Pennisetum clandestinum*, *Trifolium repens* and *Bromus unioloides* and the addition daily of 2 kg of *Medicago sativa*. University Committee of Researchs, who also performed functions of Ethic Committee, approved this study. Before, during and after the surgical procedure, the animals have shown no alteration in behavior and welfare.

Surgery technique: According to Perez *et al.*[9,20], the surgical procedure was performed. Previosly, the animals were placed under fasting state for 24 h. Then, the tranquilization was perfomed using 0.1 mg/KPV of acepromazine maleate. In dorsal decubitus position was placed the animal and then prepared the surgical area in the inguinal region with local anesthesia. In the skin side of the penis was performed a small cut of 4 cm. The vas deferens of the left and right sides were located and dissected in a length of about 7 cm. Directed the vas deferens below the subcutaneous tissue and the skin were fixed at the inside of the femoral region being protected by a temporary patch.

Sperm collection: Three times per week was performed semen collection, after habituation of animals. Subsequently followed, the downing of animals, clamping and cleaning of the collection area. The sperm output through vas deferens,

facilitated by gentle massage and sperm aspirated with a tuberculin syringe, which discharged into collection tube containing 0.5 mL of tris based extender[9,20].

Fresh sperm (initial): The spermatozoa samples collected in water bath to 35°C were tempered. Sperm vitality and functional sperm membrane integrity were determined.

Cooling: The collective sample of spermatozoa manteined to 37°C, these were dilute with extenser tris based and 20% v/v of egg yolk in 1:2 proportion. Then placed to temperature of refrigeration (5°C) with the purpose of cooling[9] until 5°C in 2 h. At the time that sperm reached 10°C, tempol antioxidant (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; Sigma-Aldrich Co., St., Louis, MO, USA)[2] was added to each aliquot to a final concentration of 0.0, 0.5, 1.0 and 1.5 mL, then further cooled until reaching at 5°C. At the end of the cooling time were evaluated the sperm vitality and the functional sperm membrane integrity.

Freezing and thawing: Samples kept at this temperature for 30 min period of stabilization. The extended spermatozoa were loaded into 0.25 mL plastic French straws and sealed with polyvinyl alcohol. Straws to liquid nitrogen vapor at a height of 5 cm by 6 min were exposed, then plunged and later stored. After 3 h of freezing, straw frozen was extracted the cryogenic thermo with help of a long gripper and quickly placed in water bath at 37°C for 30 sec. Sperm vitality and membrane integrity assessment was performed.

Sperm evaluation: The sperm vitality was determined for eosin-nigrosine (Sigma-Aldrich Co., St., Louis, MO, USA) staining technique. For this purpose, one eosin drop and other of nigrosine were puts on a preheated slide to 37°C and straight away mixed. A drop of pre-diluted sperm was added, homogenized and dried in heat[21] for 30 sec, then counted 100 spermatozoa. Live sperm were those without coloration and dead who had pink coloration. Sperm count was done with a microscope with exit of image to screen computer with 1000X magnification (BA210, clinical MOTIC, China Group Ltd.). In addition, using the HOS test was evaluated the functional sperm membrane integrity. Therefore, mixed 0.01 mL of sperm with 0.1 mL of hypo-osmotic solution. The HOS solution was prepared mixed fructose (BDH Laboratory supplies, England) 1351 and 735 mg of sodium citrate dihydrated (Merck Germany) and dissolved in 100 mL of bi-distilled water[21]. Immediately it was cultivated at 37°C for 30 min in a water bath[22]. A drop of this, it was placed onto a glass slide with a coverslip sheet protecting it. Sperm endosmosis positive percentage by counting of 100 sperm per sample was estimated[23] using the microscope previously described.

Statistical analysis: Analysis of sperm vitality and membrane integrity in fresh after cooling and post freezing/thawing was realized using the completely randomized design, where the antioxidant tempol (4 levels: 0.0, 0.5, 1.0 and 1.5 mM) was the factor. The differences between means were evaluate with Tukey test. Previosly, data in percentage were transformed (arcsine-square root transformation) to approach a normal distribution. Evaluation of parameters changes were realized using variation rate of sperm vitality or funtional sperm membrane integrity = [(Final value-Initial value)/Final value] p.100 and the differences between rates were evaluated with comparison of proportions test. The processing and analysis of data it realized using the statistical software R-3.1.2 for Windows.

RESULTS

The addition of low antioxidant concentration in cooling phase, improved the sperm vitality with respect to control, however decreased with use of higher concentration (Table 1). In the freezing/thawing phase, sperm vitality it followed the same behavior as in the cooling phase, 47.96% for 0.5 mM with respect to 41.05% for control. Also, a higher sperm mortality in freezing/thawing that in the cooling phase was found, similarly it was observed when higher concentrations

Table 1: Alpaca sperm vitality and funcional membrane integrity to cooling and freezing/thawing with tempol antioxidant addition

			Means±Standard Deviation (%)			
Parameters	Phase	n	Control (0.0 mM)	0.5 (mM)	1.0 (mM)	1.5 (mm)
Vitality	Initial	8	79.30±6.06			
	Cooling*	8	66.68±3.26^{b}	75.15±3.95^{a}	70.03±3.20^{b}	55.03±4.64^{c}
	Freezing/thawing	8	41.05±4.24^{b}	47.96±2.36^{a}	32.06±3.85^{c}	26.13±3.08^{d}
Funcional	Initial	8	75.69±3.53			
membrane	Cooling*	8	63.33±3.74^{a}	66.14±3.48^{a}	57.56±4.57^{b}	51.83±4.43^{c}
integrity	Freezing/thawing	8	36.26±3.51^{b}	44.20±4.69^{a}	34.43±4.60^{b}	22.94± 3.92^{c}

Different superscripts within rows indicate significant differences ($p \leq 0.05$), *Samples with addition of antioxidant, this was done when the temperature dropped to 10°C

Table 2: Parameters variation rate between criopreservation phases with tempol antioxidant addition

Parameters	Phase	0.0 (mM)	0.5 (mM)	1.0 (mM)	1.5 (mM)
Vitality	Initial-cooling	-18.93^{ac}	-5.52^{a}	-13.24^{ac}	-44.10^{bc}
	Cooling-freezing/thawing	-62.44^{a}	-56.69^{a}	-118.43^{bd}	-110.60^{cd}
Funtional membrane integrity	Initial-cooling	-19.52^{a}	-14.44^{a}	-31.50^{a}	-46.04^{a}
	Cooling-freezing/thawing	-74.66^{a}	-49.64^{a}	-67.18^{a}	-125.94^{b}

Different superscripts within rows indicate significant differences ($p \leq 0.05$)

of antioxidant was added (Table 1). Further, variation rate between cooling-freezing/thawing was similar between control and adding of 0.5 mM of antioxidant (Table 2), in spite of having different averages (Table 1).

The functional integrity sperm membrane was unchanged between control and adding of a low antioxidant concentration (0.5 mM) in cooling phase, but with this concentration, after freezing/thawing the percentage achieved was 44.20% (Table 1). Both concentrations, also had a similar initial-cooling rate (Table 2), but were different in the rate of cooling-freezing/thawing.

DISCUSSION

In the conditions of this study, there is scarce information about the sperm vitality in cooling step. Using sperm collected by artificial vagina, which was added 1.0 mM of tempol antioxidant during cooling step, it was found a decrease of live sperm up to 32.71% after freezing/thawing[2], although it was evaluated by different methodology. Similar results was reported in a previous experiment with sperm from the epididymis[4]. In the present study, a higher percentage of live sperm was found with addition of lower concentration (0.5 mM) of antioxidant in cooling step, but was similar to that reported by Santiani *et al.*[1] when was added 1.0 mM. Apparently, could not be significantly affected this parameter in presence of the antioxidant by the absence of seminal plasma in cryopreservation process. In this respect, it showed that the added of 10% of seminal plasma to epididymal alpaca sperm, decreases the detrimental effect during sperm preservation[24]. This is consistent with another study, where removal of a large portion of seminal plasma, improved sperm survival and adding small amounts increased survival to cryopreservation[25]. However, variations in the composition of seminal plasma could affect the freezability of the semen from alpaca[26]. Aditionally, low concentrations of antoixidant could produce a protective effect on the vitality of spermatozoa stripped of seminal plasma.

Information about the behaviour to functional sperm membrane integrity in cooling step for this conditions is little available. With respect to freezing/thawing in similar study but with sperm collected by artificial vagina, this parameter declined to 13.12% with the addition of 1 mM of the antioxidant tempol in cooling[2]. However, using 0.5 mM of the same antioxidant, decreased to 44.22% this parameter. Based on this and another studies, it would be able to explain of the following way: First, it is know that cryopreservation directly affects the sperm plasma membrane[12,27]. This would cause some damage especially in the freezing/thawing phase[28] affecting their integrity[29,30] due to osmotic stress, lipid peroxidation[31] and the formation-disolution of ice in the extracellular[32] and intracellular medium. The addition of the antioxidant could prevent these deleterious effects[33], avoiding the production of ROS and lipid peroxidation by blocking the formation of OH as from H_2O_2 to form non-toxic components such as H_2O and O_2[13,34]. Second, it has been shown that sperm of epididymis (sperm devoid of seminal plasma, as it is in this case) are more resistant to cryogenic stress in comparison with the sperm of the semen[35]. Consistent with this, the partial removal of seminal plasma of the bull semen enhances the freezability[36]. In the case of the alpaca, when it was incubated sperm at 37°C with only 10% of the seminal plasma, the sperm percentage with membrane functionality[24] was 75.3%, showing an improvement of 35%.

In general, if the initial parameters are fixed as reference to low rates of negative variation correspond better parameters, but the establishment of cooling data as reference, besides the above could make that two concentration levels (control and 0.5 mM) with different parameters (sperm vitality after freezing/thawing), may have similar variation rates. In relation to the behavior of the rates, Santiani *et al.*[1] reported similar experience. This would indicate that in the evaluation of sperm parameters after freezing/thawing is important to consider initial or reference values.

CONCLUSION

The sperm collection technique through alpaca vas deferens and the low concentration of tempol antioxidant (0.5 mM), improves the sperm vitality and the sperm membrane functional integrity in the cryopreservation phases. These parameters decrease with freezing/thawing approximately twice the cooling phase and this increases with the high concentration of antioxidants.

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