ASPRA Asociación Peruana de Reproducción Animal

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SPERMOVA

Spermova 2022; 12(2): 203-209

Artículo original – original article

DOI. 10.18548/aspe/0010.27

EFFECT OF BREED AND DILUENT ON KINETIC PARAMETERS OF CHILLED AND CRYOPRESERVED RAM SEMEN UNDER CONDITIONS OF PERUVIAN HIGHLANDS

Efecto de la raza y del diluyente sobre los parámetros cinéticos del semen de refrigerado y crioconservado en condiciones de altamontaña peruana

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Recibido: 27/11/2022

Aceptado: 15/02/2023

Publicado: 31/03/2023

ABSTRACT

The cryo-sensitivity of the ram allows us to further investigate kinetic parameters to improve cryopreservation protocols using commercially available diluents. An investigation was carried out with the objective of evaluating the kinetic parameters of chilled and cryopreserved semen from Criollo and Corriedale rams using three diluents under conditions of the Peruvian highlands. Four rams suitable for reproduction of each breed were used. Semen collection was performed four times from each ram with an artificial vagina and during the reproductive season. Semen evaluation was performed after collection and three diluents were used: Andromed, Triladyl, and Tris-egg yolk. The diluted samples were aspirated into 0.25 mL straws, slowly cooled to a temperature of 5°C and, with an equilibration time of 2 hours, cryopreservation in liquid nitrogen vapors was performed. Kinetic parameters (total motility, progressive motility, curvilinear velocity, rectilinear velocity, mean velocity, straightness, linearity, ALH and BCF) were evaluated in chilled and post-thaw cryopreserved semen using computer-assisted sperm analysis (CASA). Higher values of total motility and progressive motility of chilled semen were observed for Triladyl diluent with 92.31% and 89.31%. Although the highest values for rectilinear velocity and mean velocity were for Tris-egg yolk with 69.99 µm/s and 83.90 µm/s. For post-thaw cryopreserved semen, total motility was higher for Triladyl and Tris-egg yolk diluents with 40.61% and 33.70%, respectively. In addition, progressive motility was higher for Triladyl diluent with 33,47%. Total motility and progressive motility values were higher for chilled semen of the Criolla breed with 89.48% and 85.10%, respectively. For the post-thaw cryopreserved semen, total motility and progressive motility were also higher for the Criolla breed with 42.89% and 35.32%, respectively. The Triladyl diluent provides greater protection during refrigeration and cryopreservation of ram semen. The Criolla breed presented higher values of total motility and progressive motility in both chilled and cryopreserved semen.

Keywords: CASA, progressive motility, cryopreservation, sheep, semen

RESUMEN

La criosensibilidad del carnero nos permite profundizar la investigación de los parámetros cinéticos para mejorar los protocolos de criopreservación mediante el uso de diluyentes comerciales disponibles. Se llevó a cabo una investigación con el objetivo de evaluar los parámetros cinéticos del semen refrigerado y criopreservado de carneros de raza Criolla y Corriedale mediante el uso de tres diluyentes bajo condiciones del altiplano de Perú. Se utilizaron cuatro carneros aptos para la reproducción de cada raza. La colecta de semen se realizó cuatro veces de cada carnero con una vagina artificial y durante la estación reproductiva. Se realizó la evaluación del semen después de la colecta y se utilizaron tres diluyentes: Andromed, Triladyl y Tris-yema de huevo. Las muestras diluidas se aspiraron en pajillas de 0.25 mL, se enfriaron lentamente hasta llegar a una temperatura de 5°C y, con un tiempo de equilibrio de 2 horas, se realizó la criopreservación en vapores de nitrógeno líquido. Los parámetros cinéticos (motilidad total, motilidad progresiva, velocidad curvilínea, velocidad rectilínea, velocidad media, rectitud, linearidad, ALH y BCF) se evaluaron en el semen refrigerado y criopreservado post descongelación, utilizando el análisis espermático asistido por ordenador (CASA). Se observaron valores superiores de motilidad total y motilidad progresiva del semen refrigerado para el diluyente Triladyl con 92,31% y 89,31%. Aunque los valores más altos de velocidad rectilínea

y velocidad media fueron para Tris-yema de huevo con 69,99 µm/s y 83,90 µm/s. Para el semen criopresevado, la motilidad total post descongelación fue mayor para los diluyentes Triladyl y Tris-yema de huevo con 40,61% y 33,70%, respectivamente. Además, la motilidad progresiva fue superior para el diluyente Triladyl con 33,47%. Los valores de motilidad total y motilidad progresiva fueron superiores para el semen refrigerado de la raza Criolla, con 89,48% y 85,10%, respectivamente. Para el semen criopreservado post descongelación la motilidad total y motilidad progresiva, también fueron superiores para la raza Criolla con 42.89 y 35.32%, respectivamente. El diluyente Triladyl, brinda una mayor protección durante la refrigeración y criopreservación del semen de carnero. La raza Criolla presentó valores superiores de motilidad total y motilidad progresiva, tanto en el semen refrigerado como el semen criopreservado.

Palabras clave: CASA; motilidad progresiva; criopreservación; ovino; semen

INTRODUCTION

Semen cryopreservation facilitates storage, transport, and long-term storage. Therefore, its use is essential in the artificial insemination of domestic animals, including sheep (Anel et al., 2006). This technique requires increasing the seminal volume using diluents to improve the number of possible gestations. Such diluents or buffering agents help to control pH, and the most commonly used are Tris and sugars (e.g., glucose, raffinose, or trehalose) (Evans and Maxwell, 1987; Purdy, 2006). Penetrating cryoprotective agents such as glycerol are also used (Holt, 2000). In sheep, Tris, Andromed, and Triladyl are used (Salamon and Maxwell, 2000), commonly adding 20% (v:v) egg yolk to Tris and Triladyl diluents (Tonieto et al., 2010), while Andromed contains soy lecithin (Salmani et al., 2014).

However, it is necessary to evaluate the effect of these diluents on sperm quality, which can be done by computer-assisted analysis (CASA). This system allows an assessment of the different characteristics, such as movement, motility, and morphology, with high levels of accuracy and reliability (Tsakmakidis, 2010). A parameter for sperm evaluation is motility because it is strongly related to sperm capacity migration through the female genital tract to interact with and fertilize the oocyte (Holt et al., 1994; Verstegen et al., 2002; Suarez and Pacey, 2006). In addition, progressive motility is correlated with in vitro fertilization (IVF) (Herrara et al., 2005), and low sperm motility has been linked to low fertility rates (Kjæstad et al., 1993; Puglisi et al., 2012). On the other hand, average path velocity (VAP) and straight-line velocity (VSL) are also evaluated, which were significantly related to the number of sperm penetrating the oocyte (Farrell et al., 1998), although curvilinear velocity (VCL) and average path velocity (VAP) are the only sperm kinematic parameters that correlate positively with the capacity of sperm to migrate in the cervical mucus of the ewe (Robayo et al., 2008).

In Peru, cryopreservation results tend to be unsatisfactory in sheep due to the low fertility caused by the reduced number of viable sperm cells and their short lifetime in the female reproductive tract (Sandoval, 2005). Thus, sperm motility values after thawing are lower compared to bovines and pigs (around 40% and 30%, respectively) (Garcia et al., 2017); no more than 50% of sperm survive cryopreservation, providing low fertility rates (Watson, 2000). This is due to the increased sensitivity of ram sperm to extreme temperature changes during the freeze-thaw process (Salamon and Maxwell, 1995; Ashrafi et al., 2012; Ptáček et al., 2019). Consequently, irreversible partial damage to sperm occurs (Purdy et al., 2016).

Currently, post-thaw sperm kinematics results indicate that the use of diluents containing fresh egg yolk presented higher values of individual motility, forward movement, and straight-line velocity (Jiménez et al., 2004) without the need for programmable freezers (Ptáček et al., 2019) and with lower oxidative stress (Souza et al., 2019). On the other hand, soy lecithin used at higher dilution rates serves for ram semen freezing and intrauterine insemination without reducing fertility (Fukui et al., 2008), constituting a viable alternative replacing animal-derived components in freezing extenders for goat semen (Lv et al., 2019).

Therefore, sheep are ideal experimental models to study various aspects of semen freezing. In addition, there is no information on the kinetic parameters of refrigerated and cryopreserved semen from sheep of different breeds evaluated in the CASA system in the Peruvian highlands. Thus, the objective of this research work was to use three diluents to evaluate the kinetic parameters of chilled and cryopreserved semen from Criollo and Corriedale sheep under conditions of the Peruvian highlands.

MATERIAL AND METHODS

Place of Study

The animals were sampled in the field at the Chuquibambilla Experimental Center, belonging to the Universidad Nacional del Altiplano, Umachiri district, Melgar province, Puno region. It is located on the coordinates 13°47'37" south latitude and 70°47'50" west longitude at 3974 m.a.s.l. It has a cold temperate climate. The area has a maximum temperature of 20.4°C in December and a minimum temperature of -18.4°C in June, with an annual average of 8°C; the average annual relative humidity is 53% (maximum 81%, minimum 18%), with an average annual rainfall of 659 mm.

Laboratory analysis was carried out at the IVITA-Maranganí experimental station of the Universidad Nacional Mayor de San Marcos.

Animals

Eight sexually mature rams (4 to 5 years old) were used, four of Corriedale breed and four of Criolla breed, which were selected based on the fertility tested in the artificial insemination campaigns of the Chuquibambilla Experimental

Center. The rams were fed on natural pastures and provided good-quality hay; they consumed water ad libitum.

Semen collection and evaluation

Semen collection was performed during the reproductive season (May, June, and July) once a week. Four replicates per ram were reached. The artificial vagina (Carrera-Chávez et al., 2020) was used at a temperature of 42°C. As a succubus for semen extraction, an ewe in oestrus was used. The collection was performed after five minutes of pre-excitation and false mating. The samples were transferred to a water bath at 37°C for evaluation. In seminal evaluation, samples with a volume > 0.5 mL, mass motility ≥ 4 (range 1 to 5), concentrations greater than 2.5x109 spermatozoa/mL, and abnormal morphology ≤ 15% were considered (Salmani et al., 2014).

For the dilution of the semen samples, two commercial diluents were used, namely, Andromed® and Triladyl®, as well as a diluent composed of Tris (3.639 g), citric acid (1.990 g), glucose (0.500 g), penicillin 100 000 IU, streptomycin 100 mg. Likewise, fresh egg yolk was added to each media at a final concentration of 15 % (v/v). For fractions A and B, glycerol was added at a concentration of 5 % (v/v). For preparing the commercial diluents, the steps indicated by the manufacturer were followed. The samples under consideration were diluted in a 1:1 ratio, and dilution was completed until a dose of $400x10^6$ spermatozoa/mL was obtained (Murawski et al., 2015).

Semen refrigeration

Diluted samples were aspirated through 0.25 mL straws, sealed with polyvinyl alcohol, and slowly cooled to a temperature of 5°C with an equilibrium time of 2 hours (Murawski et al., 2015). After this time, the evaluation of the kinetic parameters of the refrigerated semen was performed.

Semen cryopreservation

Straws were placed horizontally on a grid in a Styrofoam box, frozen in liquid nitrogen vapors (4 cm above the liquid) for 10 min, and immersed in liquid nitrogen at -196°C (Murawski et al., 2015). For storage, they were placed in goblets in a liquid nitrogen container.

Semen thawing

For the evaluation of the kinetic parameters of cryopreserved semen, the straws were thawed individually one week later in a water bath at 37°C for 30 sec.

Evaluation of kinetic parameters

The evaluation of the quantity and quality of sperm movement in the different sperm suspensions after refrigeration and post-thawing was performed using the CASA system (AndroVision®; Minitube, Germany). For this purpose, the different semen samples were diluted in TGC solution (0.3 M Tris, 27.8 mM Glucose, 94.7 mM Citric Acid) to a final concentration of 50 x 10^6 spermatozoa/mL. A 10 µL drop of the sperm suspension was then deposited on a slide, covered with a coverslip (22 x 22 mm), and 4 to 6 fields were collected for each sample using a phase contrast microscope (BH-2 Olympus); a minimum of 200 cells/sample were analyzed. The kinetic parameters evaluated were: total motility (MT, %), progressive motility (MP, %), curvilinear velocity (VCL, µm/s), rectilinear velocity (VSL, µm/s), average velocity (VAP, µm/s), linearity (%LIN=[VSL/VCL]x100), straightness (%STR= [VSL/VAP]x100), amplitude of lateral head displacement (ALH, µm) and beat frequency (BCF, Hz).

Statistical analysis

Data from the eight rams with four observations each were analyzed using the GLM procedure (ANOVA) of SPSS 20.0 (SPSS Inc., Chicago, IL, USA), in a completely randomized design (CRD) with a 2 x 3 factorial arrangement (2 sheep breeds: Criolla and Corriedale) and (3 semen diluents: Andromed, Tris-Yolk and Triladyl).

RESULTS

Kinetic parameters of chilled and cryopreserved sheep semen with three diluents.

Table 1, shows the results of the kinetic parameters of chilled ram semen. Thus, MT and MP showed higher values for Triladyl diluent, followed by Tris-egg yolk and last Andromed diluent. For VCL, STR, and ALH; Triladyl and Tris-egg yolk diluents showed higher values versus Andromed. In addition, it is observed that VSL, VAP, and LIN showed higher values for Tris-egg yolk diluent. Also, the highest BCF value was observed for Triladyl, followed by Tris-egg yolk and Andromed. For cryopreserved semen the highest values of MT, MP, VSL, ALH, and BCF were for Triladyl compared to Tris-egg yolk and Andromed. However, LIN and STR parameters were higher for Tris-egg yolk and Andromed. On the other hand, VCL and VAP parameters showed no differences between diluents.

Motility parameters, MT: % of total motile spermatozoa, MP: % of spermatozoa with progressive movement; VCL: curvilinear velocity (µm/s); VSL: straight-line velocity (µm//s); VAP: average path velocity (µm//s); LIN: linearity (%); STR: straightness (%); ALH: amplitude of lateral spermatozoa head displacement (µm); BCF: head crossing frequency (Hz).

In Table 2, The chilled semen results showed the highest values of MT, MP, and BCF for Criollo rams. However, the kinetic parameters VCL and VSL were higher for the Corriedale breed. In addition, there were no differences between breeds for VAP, LIN, STR, and ALH. Furthermore, the highest values for MT, MP, VCL. VSL, ALH, and BCF of cryopreserved semen were for rams of the Criolla breed in comparison. Although, LIN and STR values were higher for the Corriedale breed. Finally, no differences between breeds were observed for VAP.

Motility parameters, MT: % of total motile spermatozoa, MP: % of spermatozoa with progressive movement; VCL: curvilinear velocity (µm/s); VSL: straight-line velocity (µm//s); VAP: average path velocity (µm//s); LIN: linearity (%); STR: straightness (%); ALH: amplitude of lateral spermatozoa head displacement (µm); BCF: head crossing frequency (Hz).

Table 1. Kinetic parameters of chilled and cryopreserved ram semen using three diluents.

Chilled semen									
Type of diluent	MT	MP	VCL	VSL	VAP	LIN	STR	ALH	BCF
Andromed	82.20^{a}	73.88^{a}	111.42^{a}	41.51^{a}	50.57^{a}	36.63^{a}	73.53^{a}	1.16^{a}	9.62^{a}
TRIS-Yolk	87.69^{b}	83.26^{b}	160.60^{b}	69.99^{c}	83.90^{c}	42.44^{b}	81.81^{b}	1.38^{b}	9.89^{a}
Triladyl	92.31^{c}	89.84^{c}	143.88^{b}	56.00^{b}	69.84^{b}	39.00^{a}	80.06^{b}	1.49^{b}	13.17^{b}
Probability	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Cryopreserved semen									
Andromed	27.70^{a}	21.50^{a}	51.32	25.26^{a}	29.40	55.22^{b}	84.91^{b}	0.50^{a}	3.68^{a}
TRIS-Yolk	33.70^{ab}	24.82^{a}	54.88	26.66^{a}	31.05	55.28^{b}	83.75^{b}	0.57^{a}	4.42^{a}
Triladyl	40.61^{b}	33.47^{b}	62.98	34.62^{b}	29.76	43.31^{a}	78.84^{a}	0.83^{b}	6.34^{b}
Probability	0.001	0.003	0.210	0.003	0.342	0.001	0.001	0.001	0.001

a,b,c Different letters in the rows denote significant differences (P≤0.05).

Table 2. Kinetic parameters of chilled and cryopreserved semen in Corriedale and Criolla breeds.

Chilled Semen									
Breed	MT	MP	VCL	VSL	VAP	%LIN	%STR	ALH	BCF
Corriedale	85.32^{a}	79.56^{a}	146.43^{b}	60.46^{b}	71.28	40.04	79.38	1.33	10.13^{a}
Criolla	89.48^{b}	85.10^{b}	130.84^{a}	51.20^{a}	64.52	38.67	77.56	1.35	11.65^{b}
Probability	0.002	0.001	0.037	0.016	0.117	0.242	0.145	0.742	0.016
Cryopreserved Semen									
Corriedale	25.11^{a}	17.88^{a}	42.74^{a}	24.41^{a}	27.72	57.23^{b}	84.75^{b}	0.54^{a}	3.12^{a}
Criolla	42.89^{b}	35.32^{b}	70.04^{b}	33.28^{b}	30.75	42.65^{a}	80.25^{a}	0.73^{b}	6.51^{b}
Probability	0.001	0.001	0.001	0.001	0.287	0.001	0.001	0.001	0.001

a,b Different letters in the rows denote significant differences (p≤0.05).

DISCUSSION

In the evaluation of kinetic parameters of chilled and cryopreserved ram semen, Triladyl and Tris-egg yolk diluents had a beneficial effect; the egg yolk component is a non-permeable cryoprotective agent, able to bind to the plasma membrane to form a protective film stabilizing the lipid bilayer (Tonieto et al., 2010) and, due to the low density proteins (LDL): phosphatidyl choline and phosphatidyl serine of egg yolk, protects the integrity of the phospholipids of the sperm membrane (Graham and Foote, 1987; Amirat et al., 2004; Bergeron and Manjunath, 2006; Forouzanfar et al., 2010), decreasing the formation of extracellular ice by reducing the freezing temperature of the medium (Holt, 2000) during the freeze-thaw process.

Our results showed that Triladyl diluent, had higher values for MT, MP, VSL, ALH, and BCF, in chilled semen compared with Tris -egg yolk and Andromed. Similarly, Hegedűšová et al. (2012), indicate consistent long-term results with Triladyl diluent, and it appeared to be the best stabilizing agent for ram semen. In addition, Rekha et al. (2016), noted that sperm quality parameters after thawing were better in Triladyl than in the locally prepared extender (Tris, fructose, egg yolk, 7% glycerol). However, Jha et al. (2019), did not find differences between diluents Triladyl and Tris, citrate, fructose, egg yolk with 5% glycerol, on post-thaw sperm motility of ram semen.

On the other hand, Fernandes et al. (2021), found that motility parameters of thawed semen from Merino rams previously diluted with Tris-egg yolk and evaluated in the CASA equipment had a higher quality compared to the Andromed diluent. In addition, according to Moreno-Avalos et al. (2021), during the cryopreservation process of Alpine goat semen, the Tris-yolk diluent obtained higher individual and mass motility after thawing compared to the soy lecithin-based diluent. However, Jha et al. (2019), when using Tris Yolk -Glycerol 5% and Triladyl-based diluents in rams, with no difference between diluents before and after cryopreservation.

The total and progressive motility values obtained post-thawing for the Tris-egg yolk diluent are similar to those reported .by other authors using the same diluent (Leahy et al., 2010; Del Olmo et al., 2013; Mehdipour et al., 2016), although the values of the kinetic parameters VAP, VCL, VSL, ALH, and BCF found for cryopreserved semen using Tris-egg yolk are lower than those found by several authors who worked on other breeds with programmable freezers and semen collection by electroejaculation (Bag et al., 2004; da Silva Maia et al., 2009; Del Olmo et al., 2013; Mehdipour et al., 2016; Gungor et al., 2018). It should be noted that kinetic

parameters are considered an indicator of forward progression at VSL, and at VAP, as an indicator of sperm capacitation (Farrell e.t al., 1998), and that the lower values of VAP, VCL, VSL parameters in this study show that short- or long-term exposure of rams to altitude induces oxidative stress in the blood and deteriorates some sperm characteristics. These changes may be partly responsible for the low fertility of sheep flocks kept at high altitudes (Cofré et al., 2018), as well as ultrastructural, biochemical, and functional damage (Maxwell et al., 1993) caused by factors such as heat shock, ice formation, dehydration, increased salt concentration and osmotic shock (Mazur, 1984; Woelders and Chaveiro, 2004), which induces osmotic stress, high production of reactive oxygen species (ROS), sperm DNA damage, sperm membrane destabilization, and sperm mitochondria dysfunction (Peña et al., 2004) during the sheep semen cryopreservation process.

The lowest values of the kinetic parameters of chilled and cryopreserved semen were for the Andromed diluent. Therefore, under conditions of the peruvian highlands, there were not the same results as other authors who worked with 1% soy lecithin (Masoudi et al., 2016) or Andromed with higher values in progressive motility after thawing of sheep semen (Ebensperger, 2013) even though Andromed is indicated as an alternative to conventional (Tris-based) diluents (Akçay et al., 2012).

Regarding the ram breed, total and progressive motility values were higher for Criollo breed sheep, both for chilled and cryopreserved semen, i.e., the breed would affect seminal characteristics. This was also reported by Carvajal-Serna et al. (2018), when evaluating the total and progressive motility of fresh semen in the Criollo breed. In addition, Kumar et al. (2010), evaluated CASA derived sperm motion characteristics and, revealed that semen quality of native Malpura rams is better compared to crossbred Bharat Merino rams during breeding season. In the same way, the kinetic parameters VSL, VCL, and VAP of fresh semen were higher in the Criollo breed compared to the Romney Marsh breed in a study conducted under high altitude conditions (Carvajal-Serna et al., 2018), which could indicate that this breed would have better cryo-resistance.

However, not only breed would be an influential factor but also geographic location and differences between rams. Furthermore, differences in sperm cryo-sensitivity can also be attributed to individuals within breeds, variations between ejaculates (Medrano et al., 2010; Ramon et al., 2013), i.e., besides the breed, there would be an individual effect of the ram, as demonstrated in Pelibuey sheep, using the commercial diluent Triladyl for semen cryopreservation under tropical conditions, where total motility for rams was obtained: good freezers of 60.6% and poor freezers of 39.9% (Gomez, 2019). Contrary to our results, Castro Bedriñana et al. (2017) indicate that the ram breed factor was not influential or determinant concerning seminal characteristics during refrigeration. Therefore, further breed comparison studies are suggested.

CONCLUSIONS

There is a detrimental effect on sperm kinetic parameters during the cryopreservation process of ram semen. However, the Triladyl and Tris-egg yolk diluents provide better protection during sperm refrigeration and cryopreservation than the Andromed diluent. On the other hand, there is an influence of the breed factor, with higher values of total and progressive motility for the Criollo breed compared to the Corriedale breed, both in refrigerated semen and cryopreserved semen.

ACKNOWLEDGMENTS
Our gratitude to Dr. Daniel Ramos Dueñas (director of the Chuquibambilla Experimental Center) for the completion of this work.

AUTHORS' CONTRIBUTION
Study conception and design (NC), data acquisition (WG; WC), data analysis and interpretation (FR; EC), funding acquisition (NL), article writing and approval (NC; WG).

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