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Antioxidant effect of Trolox and Crocina on the cryopreservation of Pelibuey ovine semen

Efecto de los antioxidantes Trolox y Crocina sobre la criopreservación del semen de ovino Pelibuey

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ABSTRACT

The objective of the study was to evaluate the effect of the antioxidants Trolox and Crocina in the freezing diluent on the cryopreservation of Pelibuey ovine semen. The ejaculates obtained were mixed, divided and frozen in 3 treatments: T1: 1mM Trolox; T2: 1mM Crocina; TT: Control (without antioxidant). At 0 hours (defrosting) and 6 hours at 37 °C, total (MT) and progressive (MP) motility, sperm viability, mitochondrial activity, integrity of acrosomes and tail membrane were analyzed (Host) The data was analyzed with an ANOVA for a completely randomized design and a Tukey test for the comparison of means. In neither of the two hours were significant differences found in the treatments (P> 0.05). However, from 0 to 6h, MT behaved similarly in T1 and T2, but not in TT (P <0.01), on the contrary, the integrity of acrosomes in all three treatments was similar (P> 0.05). MP, viability, mitochondrial activity and Host decreased over time in a similar way in all three treatments. The antioxidants Trolox and Crocina at 1mM in the freezing diluent, allow to maintain the MT and the integrity of the acrosomes for 6 h of incubation at 37 °C.

Keywords: Free radicals, diluent, sperm and freezing.

RESUMEN

El objetivo fue evaluar el efecto de los antioxidantes Trolox y Crocina en el diluyente de congelación sobre la criopreservación del semen de ovino Pelibuey. Los eyaculados obtenidos se mezclaron, dividieron y congelaron en 3 tratamientos: T1: Trolox 1mM; T2: Crocina 1mM; TT: Testigo (sin antioxidante). Se analizó a las 0 horas (descongelación) y 6 horas a 37 °C, la motilidad total (MT) y progresiva (MP), la viabilidad espermática, la actividad mitocondrial, la integridad de los acrosomas y de la membrana de la cola (Host). Los datos se analizaron con un ANOVA para un diseño completamente al azar y una prueba de Tukey para la comparación de medias. En ninguna de las dos horas se encontraron diferencias significativas en los tratamientos (P>0.05). Sin embargo, de 0 a 6h, la MT se comportó de forma similar en el T1 y T2, pero no así en el TT (P<0.01), por el contrario, la integridad de los acrosomas en los tres tratamientos fue similar (P> 0.05). La MP, viabilidad, actividad mitocondrial y Host disminuyeron con el tiempo de forma similar en los tres tratamientos. Los antioxidantes Trolox y Crocina a 1mM en el diluyente de congelación, permiten mantener la MT y la integridad de los acrosomas durante 6 h de incubación a 37 °C.

Palabras clave: Radicales libres, diluyente, esperma y congelación.

INTRODUCTION

Cryopreservation of sperm is an important tool to conserve genetic material and maintain genetic diversity in wild and domestic species (Lermen *et al*., 2009). However, it is known that the freeze-thaw processes of semen used in most mammalian species cause the death of a large number of cells (Watson, 2000).

In sheep, although a relatively high percentage (40-60%) of sperm retain their motility after thawing, only about 20-30% remain biologically intact (Salamon y Maxwell, 2000). This is because during the refrigeration and cryopreservation process there is an increase in the levels of reactive oxygen species (ROS), which produce biophysical and biochemical changes in the sperm membrane (Chatterjee *et al*., 2001; Kankofer *et al*., 2005) and even damage to its DNA (Aitken, 1999; Agarwal *et al*., 2003), affecting viability and its fertilizing capacity. In recent years, cryopreservation protocols have been substantially improved with the addition of compounds with antioxidant properties, positively impacting the improvement of the quality of thawed semen (Peña *et al.,* 2005; Thuwanut *et al.,* 2008; Domínguez-Rebolledo *et al.,* 2010; Mata-Campuzano *et al.,* 2015).

Antioxidants play an important role in the protection of sperm cells, against the harmful effects produced by ROS (Halliwell, 1997). The function of an antioxidant is based on donating electrons to other molecules that have one or more lost electrons (ROS), and thus preventing alterations in the molecules of lipids, proteins and sperm DNA (Tremellen *et al*., 2008).

The antioxidant Trolox is a water-soluble vitamin E analogue and has been used in a wide variety of cellular systems to avoid the effects of ROS (Halliwel, 1994; Michielse*t al*., 1994). It has been observed that the addition of this antioxidant in the freezing medium improves the quality of the thawed pork sperm (Peña *et al.,* 2005); as well as the sperm viability of thawed semen in sheep of the Churra breed (Mata-Campuzano *et al.,* 2015). In sperm samples from the Iberian deer epididymis (*postmortem*), it was shown that this antioxidant is capable of decreasing the amounts of ROS and lipoperoxidation. In addition to protecting acrosomes and sperm DNA (Domínguez-Rebolledo *et al.,* 2010). Thuwanut *et al.* (2008) observed that the addition of Trolox to the freezing diluent improved motility and viability in cat epididymis sperm in defrosting.

The antioxidant Crocina (glycosyl ester of crocetin), is a water-soluble carotenoid, found in the yellow pigment of saffron (*Crocussativus*). It has been shown that the addition of this antioxidant in the semen incubation medium improves the quality of the thawed sperm of the Iberian deer (Domínguez-Rebolledo *et al.,* 2010) and bull (Sapanidou *et al.,* 2014). In semen of roosters, it improves viability, motility, mitochondrial activity and reduces lipoperoxication to defrosting (Mehdipour *et al*., 2019).

Therefore, the objective of the present study was to evaluate the effect of the addition of the antioxidants Trolox and Crocina to the freezing medium, on the post-cryopreservation sperm characteristics in sheep of the Pelibuey breed.

MATERIAL AND METHODS

Location

The study was carried out at the Pelibuey and Blackbelly Sheep Germplasm Bank, of the National Institute of Forestry, Agricultural and Livestock Research (INIFAP), Mocochá Experimental Field.

Animals

4 adult sheep (2.5 years), of the Pelibuey breed, 40 to 45 kg of live weight, were used with a body condition of 3 to 3.5; to which their health status and sperm quality were previously evaluated.

Obtaining sperm samples

36 ejaculates were obtained from the 4 stallions, using an artificial vagina, and with the help of a sheep that served as a dummy.

Sperm dilution

The ejaculates obtained were mixed (pool) and diluted in Triladyl® + 20% egg yolk, at a final concentration of 400 x 106 sperm/ml. Subsequently, the samples were divided into 3 treatments: T1: 1mM Trolox; T2: 1mM Crocina; TT: Control (without antioxidant), then be packaged in 0.25 ml straws.

Semen freezing

Freezing of the samples was done by placing the straws 4 cm above the surface of the liquid nitrogen (LN2), for 10 minutes. Immediately afterwards the straws were immersed in LN2 and stored until evaluation.

Defrosting semen

The defrosting procedure was performed by immersing the straws in a water bath at 37 °C for 30 seconds; subsequently the samples were evaluated at 0 h (defrosting), at 6 hours of incubation at 37 °C.

Sperm concentration

A small fraction of the semen sample (5 µL) was diluted in 995 µL distilled water; then 9 µL of the sample diluted in water was taken and placed on each of the two sides of the bücker chamber, to estimate its concentration, with the concentration module of the CASA

system (ISAS®v1 (Proiser R + D, Valencia, Spain) 4 fields were captured on each side of the chamber and sperm concentration was obtained.

Sperm motility

Motility was analyzed with the CASA system, placing 5 µL of semen thawed and diluted to~30 x10^6/ml sperm, on a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel), preheated to 37 °C; and at least five fields were captured with a minimum of 300 sperm/sample. The motility parameters evaluated were: Total Motility (MT %) and Progressive Motility (MP %).

Sperm viability

It was evaluated by staining SYBR14-IP (Live/Dead® kit L-7011, InvitrogenTM), adding 1 µL of SYBR14 (10 µM) and IP (12 µM) of the stock solution in 100 µL of sperm sample, diluted in saline solution (PBS); and allowed to incubate for 10 minutes at 37 °C. Subsequently, 5µL of the sample was placed between a slide and a preheated cover slip at 37 °C, and its evaluation was carried out by means of an epifluorescence microscope (LWScientific i40-ADN); counting 200 sperm, which had red (dead) fluorescence, and those that were alive were green.

Acrosome Integrity

It was evaluated by staining FITC-PSA (100 µg/ml, L-0770, Sigma-AldrichTM), adding 5 µL of the stock solution in 100 µL of sperm sample diluted in PBS and allowed to incubate in the dark for 30 minutes at 37 °C . Immediately afterwards 5µL of the sample was placed between a slide and coverslip. Finally, 200 sperm cells were counted with an epifluorescence microscope, which had green fluorescence (damaged acrosomes), and no fluorescence of the intact acrosomes.

Mitochondrial activity

It was analyzed with JC-1 staining (153 µM, Molecular Probes® T-3168, InvitrogenTM), adding 1 µL of the stock solution in 100 µL of sperm sample diluted in PBS and allowed to incubate in the dark for 10 minutes at 37 °C. Then, 5 µL of the sample was placed on a slide and coverslip, and 200 sperm were counted by an epifluorescence microscope, which had an orange fluorescence (active mitochondria) in the middle of the flagellum, and the mitochondria green in color inactive

Integrity of the plasma membrane of the tail (HOST)

It was performed by diluting the 5 µL sperm sample in 50 µL of endosmosis solution (0.735 g of sodium citrate dihydrate and 1,351 g of fructose in 100 ml of distilled water) at 100 mOsm/L and allowed to incubate for 37 °C. Subsequently, 5µL of the sample was placed on a slide and coverslip and 200 sperm were counted with a phase contrast

microscope, which had coiled tails (positive endosmosis), and not coiled (negative endosmosis).

Statistical analysis

The variables expressed as percentages (total motility, progressive motility, viability, mitochondrial activity, intact acrosomes and Host), were transformed to the arc √(variable)/100 before analysis. It was subsequently analyzed with a general linear model (GLM) with PROC GLM procedure; and to find the statistical differences between treatments was Tuckey´s test used through the statistical package of Statistical Analysis System (SAS Inst. Inc., 2003).

RESULTS AND DISCUSSION

At 0 and 6 h of incubation, none of the parameters evaluated showed differences (P> 0.05) between treatments (Table 1). These results differ from those obtained by Thuwanut *et al*. (2008) with cat epididymal sperm supplemented with the antioxidant Trolox at 1mM; since MT, MP and sperm viability improved defrosting. Although they are different species, the difference in results could be attributed to the fact that sperm from cat epididymis by castration, have never been in contact with the secretions of the glands that form the seminal plasma, which is where the greatest antioxidant defense is found (Chen *et al*., 2003). Therefore, there are differences in freezing between sperm from ejaculates and epididymis (Gilmore *et al.,* 1998), which affect sensitivity to cooling, such as resistance to freezing (Schmehl *et al.,* 1986). In this sense, Trolox could have acted directly and without any competition on the sperm of the epididymis, similar to that observed by Domínguez-Rebolledo *et al*. (2007), with seminal sheep plasma on thawed semen.

On the other hand, in synergy with the antioxidants present in the seminal plasma, the seminal samples used in this study could be altered, causing their effect to diminish. Likewise, it has been reported that supplementation of the diluent with Trolox at 1mM, depending on the type of additive used in the diluent, may or may not improve the viability of the frozen semen of sheep of the Churra breed (Mata-Campuzano *et al*., 2015); that is, when the diluent was made from soy lecithin, the antioxidant Trolox had a greater viability in the thawed samples; while when a tris-based diluent was used, such as the one used in this study (Triladyl®), the viability in the sperm did not improve. Soy contains isoflavones, which act as antioxidants; which could have exerted some protection on the sperm during the freeze-thaw process or in combination with Trolox, producing a synergy that helped to enhance the beneficial effects on the sperm. With the addition of the antioxidant Crocina, greater viability, motility, mitochondrial activity and reduction of lipoperoxidation have been reported, in rooster sperm samples to defrost (Mehdipour *et al*., 2019).

Table 1. Percentages (%) of sperm parameters evaluated at 0 h and 6 (mean ± standard error).

Hour	Treatment	Total motility	Progressive motility	Viability	Mitochondrial activity	Intact acrosomes	Host
0	T1	57.5±5.3^{aA}	17.1±1.6^{aA}	39.5 ± 3.9 ^{aA}	50.7 ± 6.2 ^{aA}	49.6 ± 6.4 ^{aA}	23.6 ± 3.1 ^{aA}
	T2	49.9±5.6^{aA}	15.8±2.2 ^{aA}	45.2 ± 5.2 ^{aA}	42.3 ± 5.4 ^{aA}	44.6 ± 6.1 ^{aA}	23.4 ± 3.1 ^{aA}
	TT	58.6±3.9^{aA}	15.3±2.2 ^{aA}	41.4 ± 5.2 ^{aA}	40.5 ± 6.0 ^{aA}	47.7 ± 6.1 ^{aA}	19.3 ± 2.1 ^{aA}
6	T1	36.6±7.0^{aA}	8.6±7.0^{aB}	24.6 ± 4.0 ^{aB}	15.7 ± 1.9 ^{aB}	46. 0 ±2.7 ^{aA}	10.2 ± 1.0 ^{aB}
	T2	39.2±5.8^{aA}	8.9±5.8 ^{aB}	22.8 ± 2.7 ^{aB}	17.1 ± 1.5 ^{aB}	43.7 ± 4.0 ^{aA}	13.1 ± 1.1 ^{aB}
	TT	26.7±4.3$^{aB\ **}$	8.2±4.3 ^{aB}	27.2 ± 4.0 ^{aB}	20.0 ± 2.2 ^{aB}	41.7 ± 3.5 ^{aA}	12.4 ± 1.2 ^{aB}

T1: 1mM Trolox; T2: 1mM Crocina; TT: Control (without antioxidant). (ab) Different literals within the same column indicate significant differences between treatments within each hour (P<0.05). (AB) Different literals within the same column indicate significant differences (P <0.01 **) between the same treatment over time.

Through the incubation time (0 to 6 hours), the MT behaved similarly in T1 and T2, but not in the TT (P <0.01); on the contrary, the integrity of acrosomes in all three treatments was similar (P>0.05). MP, viability, mitochondrial activity and Host decreased over time in a similar way in the three treatments (table 1). The results are similar to those reported by Domínguez-Rebolledo *et al*. (2010) in sperm samples of epididymis thawed from the Iberian deer, where MT and acrosome integrity were maintained during incubation at 37 °C with the antioxidant Trolox and Crocina at 1mM. Also, Sapanidou *et al*. (2014) observed that the motility, viability and integrity of acrosomes in bovine sperm were maintained during incubation at 37 ° C with 1mM Crocina. In view, of the results after 6 h of incubation where the T1 and T2 maintained the MT and the integrity of the acrosomes, it can be said that the antioxidants tested are efficient and recommended for incorporation into the diluent prior to freezing; in such a way, that the seminal dose will be in force in the genital tract, after insemination until the moment close to ovulation.

CONCLUSION

The addition of the antioxidants Trolox and Crocina to 1mM in the freezing diluent, allows to maintain the total motility and integrity of the acrosomes during 6 h of incubation at 37 °C.

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