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Determination of the quality of semen cryopreserved with soy lecithin or egg yolk, in male goats

Determinación de la calidad del semen criopreservado con lecitina de soya o yema de huevo, en machos cabríos

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ABSTRACT

The objective was to compare the quality of cryopreserved goat semen with soy lecithin or egg yolk. The semen was collected from male goats (n=4), two commercial diluents AndroMed® (1% soy lecithin, SL); Optidyl (20% (v/v) Tris-egg yolk; TY), and a citrate-egg yolk-based diluent (CY) were used in fresh semen (FS) and then cooled from 37 to 4 °C for 2 h (refrigerated semen, RS), afterwards straws were filled with semen and frozen in liquid nitrogen at -196 °C (FS). There were no differences (p>0.05) between diluents in the FS in the mass motility (MM; 4.7±0.26), sperm viability (SV; 74.1±1.66) and individual motility (MI; 62.3±4.0). In the same sense, for the RS there was no difference (p>0.05) between diluents with respect to MM (3.83±0.4) and MI (52.1±6.0), however, the SV varied (p<0.05) according to the diluent, observing the lowest viability in SL vs CY and TY (51.0±13.0 vs 71.3±3.0 and 69.0±3.1). Regarding FS the MM, MI and SV obtained better values (p<0.05) with the diluent TY vs SL and CY (2.4±0.5, 32.5±8.3, 41.3±13.0). The results showed a better cryopreservation of goat semen with the diluent Tris-yolk compared to that of soy lecithin.

Keywords: Soy lecithin, diluent, goat semen

RESUMEN

El objetivo fue comparar la calidad del semen caprino criopreservado con diferentes tratamientos a base de lecitina de soya o yema de huevo. El semen fue colectado de machos cabríos Alpinos (n=4), se utilizaron dos diluyentes comerciales: AndroMed® (1% de lecitina de soya, LS); Optidyl® con 20% (v/v) de Tris-yema de huevo; TY), y un tercer diluyente a base de citrato-yema de huevo (CY), en semen fresco (SF) y después fue enfriado de 37 a 4 °C durante 2 h; semen refrigerado (SR), posteriormente se llenaron pajillas con semen y se congelaron en nitrógeno líquido a -196 °C (SC). No existieron diferencias (p>0.05) entre diluyentes en el SC respecto a motilidad masal (MM; 4.7±0.26), viabilidad espermática (VE; 74.1±1.66) y motilidad individual (MI; 62.3±4.0). En el mismo sentido, para el SR no existió diferencia (p>0.05) entre diluyentes respecto a MM=3.83±0.4, y MI= 52.1±6.0, sin embargo, la VE varió (p<0.05) de acuerdo al diluyente, observando la menor viabilidad en LS vs. CY y TY (51.0±13.0 vs 71.3±3.0 y 69.0±3.1). Respecto al SC, la MM, MI y VE favorecieron (p<0.05) al diluyente TY vs. LS y CY (2.4±0.5, 32.5±8.3, 41.3±13.0). Los resultados mostraron una mejor crio-preservación del semen caprino con el diluente Tris-yema respecto al de lecitina de soya.

Palabras clave: Lecitina de soya, diluyente, semen caprino

INTRODUCTION

Traditional diluents that are added to semen for the preservation of sperm viability and fertility during cryopreservation include egg yolk (Lima-Verde *et al.,* 2017). This is due to the fact that the egg yolk protects the sperm from the damage induced by cryopreservation during cooling, freezing and thawing by interacting directly with the plasma membrane (Andrabi *et al*., 2008; Akçay *et al*., 2012; Sieme *et al.,* 2016). However, in recent years, there has been frequent opinion against the use of egg yolk due to the great variability of its components, which makes the evaluation of its benefits complex (Kulaksız *et al*., 2010); Furthermore, an attempt has been made to avoid the use of diluents of animal origin, since they could be a possible route of disease transmission (Lima-Verde *et al*., 2017; Ansari *et al*., 2017).

Regarding the egg yolk, some undesirable components (steroid hormones and their precursor molecules) are considered detrimental to the integrity of the sperm (Akhter *et al.,* 2012; Lima-Verde *et al*., 2017). The main component of egg yolk that protects the sperm membrane is low-density lipoprotein (LDL). Phospholipids or lecithin in egg yolk have been shown to make sperm less sensitive to cooling (Zeron *et al.,* 2002). In particular, in the male goat, there are negative interactions between the phospholipids of the egg yolk and the bulbourethral gland, this gland secretes with the seminal plasma a coagulating enzyme of the egg yolk, which catalyzes the hydrolysis of the lecithin of the yolk in fatty acids and lysolecithin, which are cytotoxic (Ngoma *et al*., 2016). Therefore, chemically defined substitutes for egg yolk have been used without being of animal origin (El-Sisy *et al*., 2018; Gamal *et al.,* 2016), made from soy lecithin and which can be alternatives potentials for the cryopreservation of semen (Akhter *et al*., 2012).

Liposomes are believed to act similarly to lecithins in egg yolk or milk (Belala *et al*., 2016). In water buffalo, no differences were found in terms of acrosomal integrity when diluents based on egg yolk, lecithin or soy liposomes were used (Kumar *et al.,* 2015; Singh *et al.,* 2013). In this context, diluents based on egg yolk deserve special attention in terms of their components and their effect compared to diluents based on liposomes. Due to the fact that the effect of the aforementioned components is little known on the quality of cryopreserved semen in the male goat, we set ourselves the objective of comparing the effects of diluents based on soy lecithin or egg yolk on the quality of semen, preserved by refrigeration and freezing.

MATERIAL AND METHODS

General

All the methods and management of the experimental units used in this study were in strict accordance with the guidelines for the ethical use, care and welfare of animals in research at the international level (FASS, 2010) and national level (NAM, 2002) with number UAAAN-UL institutional approval reference with code 38111-425501002-2431.

Location and animals

The experiment was carried out in northern Mexico, at the Goat Center of the Antonio Narro Autonomous Agrarian University (26° North Latitude and 104° West Longitude), during the reproductive season (January). The study area is at an altitude of 1120 meters above sea level, with an average annual rainfall of 230 mm and an average temperature of 24 °C, a maximum of 41 °C in May and June, and a minimum of -1° in December and January. (CONAGUA, 2015). Adult male goats of the Alpine-French breed (n = 4, 1.5 to 2 years old), homogeneous in terms of live weight (LW; 75.0 ± 0.32 kg) and body condition (CC; 3.5 ± 0.10 units) were used; with proven fertility prior to the experimental study through frequent evaluations of seminal quality. During the experimental period, males were fed twice a day (800 h and 1800 h), with free access, with a diet based on alfalfa hay (18% PC, 1.95 Mcal of ME) and 100 g of commercial concentrate (21% PC, 1.7 Mcal ME) based on their nutritional requirements (NRC, 2007). The males had free access to clean water and mineral salts and an adaptation period of 2 weeks prior to the investigation.

Collection and processing of semen

The semen was collected in the morning (800 to 1000 h) every 3 days, for three weeks, a female in oestrous activity was used as a stimulus for the extraction of semen. The semen was collected with a standard artificial vagina for sheep and goats, kept at a temperature of 38 °C, so it was preheated to 42 °C prior to collection. A total of 24 ejaculates were collected, after each extraction the semen was immediately immersed in a water bath at 37 °C for subsequent macroscopic and microscopic analysis during the next 10 minutes.

Preparation of diluents and freezing process

Out of a total of 24 ejaculates (6 ejaculates per male) and each ejaculate was divided into three equal parts aliquots to be processed for cryopreservation, using two commercial diluents: AndroMed® (Minitübe, Tiefenbach, Germany; with 1% lecithin from soy; **SL**); Optidyl® (CRYO-VET, France; with 20% (v/v) of Tris-egg yolk; **TY**), and a third diluent based on citrate-fresh egg yolk (**CY**) obtained according to the technique used by Salamon and Maxwell, (2000).

Only samples with a volume> 0.5 mL, a concentration of $2.5x10^9$ mL, mass motility ≥ 3.0, and viability ≥ 70% were considered in the experiment. Subsequently, the already diluted

samples were submitted to 3 processes for evaluation: fresh semen (**FS**); chilled semen (RS, equilibrated at 4 °C for 2 hours); and frozen semen (**FS**). After refrigerating the semen, the 0.25 mL straws were filled and for the freezing process they were placed in 7 cm of liquid nitrogen (NL, -140 °C) for 10 min; then they were immersed directly in the NL (-196 °C) and stored until analysis (Jerez *et al.,* 2016). In each of the conservation states (FS, RS and FS), the semen was analyzed, immediately and every 15 minutes during the conservation process, to evaluate the mass and individual motility and viability. In the case of FS, it was analyzed 24 hours later, for which the straw was thawed, immersing it in tempered water (37º C) for 30 s.

Variables evaluated

Mass motility (MM;%) was evaluated with the use of a preheated platform (37 °C), placing a drop of pure semen (20 µl) on a slide in the optical microscope with a 10x objective, and according to With the observed movement, an arbitrary scale score of 1 to 5 was assigned, where 1=25% and 5=100% motile sperm (Mahsud *et al*., 2013). *Individual motility* (MI ;%) was determined based on the proportion of progressively mobile spermatozoa. For this, a drop (10µL) of semen was placed on a slide and covered with a coverslip slide; later it was observed under a microscope with a 40x objective. Sperm viability (SV;%), was evaluated by using the eosin-nigrosin staining technique (Kafi *et al*., 2004), at least 200 sperm per sample were observed by light microscope, using the 100X objective, and the percentage of live cells (unstained) and dead cells (stained pink) was calculated. All evaluations were always carried out by the same qualified evaluator.

Statistical analysis

Data were analyzed by ANOVA using the General Linear Model (GLM) procedure. The means obtained from the seminal parameters were compared using a t test. The effect of the use of different diluents, the states of the cryopreservation process and their interaction were considered. All data were analyzed using the statistical package SAS V9.1 (SAS, 2005). Differences were considered significant at a value of $P \leq 0.05$.

RESULTS AND DISCUSSION

The results of the different parameters evaluated to determine the quality of semen diluted with SL, TY or CY, in fresh semen (FS), refrigerated for 2 h (RS) and frozen semen (FS) are summarized in Table 1.

Table 1. Means (± SEM) for the quality of cryopreserved semen from French Alpine goats diluted with soy lecithin or egg yolk

Parameters	MM (escala,1-5)	MI (%)	SV (%)
Fresh Semen			
SL	4.6±0.2^{a}	63.0±1.2^{a}	76.0±2.0^{a}
TY	4.8±0.3^{a}	62.5±1.4^{a}	75.0±0.0^{a}
CY	4.8±0.3^{a}	61.3±4.3^{a}	71.3±4.0^{a}
Refrigerated Semen			
SL	3.1±1.0^{a}	44.0±12.0^{ab}	51.0±13.0^{bc}
TY	4.4±0.1^{a}	57.5±2.5^{a}	71.3±2.4^{a}
CY	4.0±0.2^{a}	55.0±3.5^{a}	69.0±3.1^{ab}
Frozen Semen			
SL	1.0±0.4^{c}	11.0±6.4^{c}	11.0±7.1^{d}
TY	2.4±0.5^{b}	32.5±8.3^{b}	41.3±13.0^{c}
CY	0.5±0.3^{c}	6.3±3.8^{c}	2.5±2.5^{d}

Treatments: Andromed® (**SL**; 1% soy lecithin), Optidyl® (**TY**: Tris- 20% egg yolk) or citrate- egg yolk (**CY**). Mass motility (**MM**; scale, 1-5), Individual motility (**MI**;%), Sperm viability (SV;%). ^{abcd} Unequal superscripts between rows indicate significant statistical difference (P≤0.05).

In the FS, similar values were obtained between the groups (P> 0.05) in each of the evaluated variables [MM (4.7 ± 0.26%), SV (74.1 ± 1.66%) and MI (62.3 ± 4.0%)]. The results suggest that the composition of the diluents used in this study affects the quality of the sperm after the thawing process. However, post-thaw semen quality was more affected when SL-based diluent was used compared to TY. These results are similar to those reported in horses and cervids; in which a higher seminal quality is shown when using egg yolk-based diluents (Pillet *et al*., 2012; Stewart *et al*., 2018). It is likely that the results found are due to the fact that the Tris-egg yolk component helps to reduce the generation of reactive oxygen species (ROS), thus maintaining the integrity potential of the membrane by reducing the thermal shock caused by temperature changes in the cryopreservation process (Alcay *et al*., 2016; Seifi-Jamadi *et al*., 2017). This may be related to the effective component of the egg yolk which is the low-density lipoprotein (20%), which contains the TY and functions as a cryoprotective fraction that helps to

maintain the better quality semen (Amirat *et al*., 2004; Forouzanfar *et al*., 2010; Alcay *et al*., 2016).

When comparing the effects of the diluents in the RS conditions, there were no differences (P> 0.05) in the MM and MI (3.83 ± 0.4% and 52.1 ± 6.0%, respectively); however, the SV percentage was lower with the SL-based diluent compared to TY and CY (51.0 ± 13.0 vs 71.3 ± 3.0 and 69.0 ± 3.1 respectively; P <0.05).

The results reported in the present experiment regarding the TY diluent agree with that reported by Celeghini *et al*. (2008), which indicate greater integrity of the bull sperm acrosome, and Konyak *et al*. (2018) who found that after balancing and freezing the semen of goats, sperm motility is significantly higher when using the Tris diluent with 20% egg yolk, compared to that made up of 1% soy lecithin. In this regard, it has been proven that soy lecithin is not capable of preventing lipid peroxidation that occurs during the sperm cooling process (Salmani *et al*., 2013).

Previous research in sheep has shown that semen diluted with soy lecithin presents damage to the sperm membrane, and consequently damage at the mitochondrial level, which results in less mobility and fertility of sperm (Del Valle *et al*., 2012; Lima-Verde *et al*., 2017). Konyak *et al*. (2018). (2018) attributes the low sperm quality of semen exposed to SL to differences in the concentration of soy lecithin used, in relation to this, Forouzanfar *et al*. (2010) observed in rams semen that diluents containing concentrations of 1% lecithin had higher sperm viability, compared to 2% lecithin, and also that the range 1 to 1.5% soy lecithin in the diluent showed better semen characteristics after preservation.

In FS, MM, MI and SV values were higher in semen diluted with TY (2.4 ± 0.5, 32.5 ± 8.3, 41.3 ± 13.0, respectively; P≤0.05) compared to semen diluted with SL and CY that their MM, MI and SV drastically decreased (1.0.0 ± 0.4, 11.0 ± 6.4, 11.0 ± 7.1 and 0.5 ± 0.3 ±, 6.3 ± 3.8 and 2.5 ± 2.5 respectively; P≤0.05). Similarly, the results in our study in post-thaw CY were lower compared to TY. It is likely that these results are associated with the components of the diluent, specifically the percentage of egg yolk, in the CY diluent it was at a concentration of 15%, and in the TY at 20%. These results agree with Fourouzanfar *et al*. (2010), who report that post-thaw sperm motility and viability are higher when using a 20% egg yolk concentration than when using 15%. Similarly, other studies confirm that the increase in the concentration of egg yolk improves the preservation of sperm quality (Amirat *et al*., 2004; Forouzanfar *et al*., 2010; Alcay *et al*., 2016). The reason for this improvement may be due to the fact that the phospholipids contained in the egg yolk, such as phosphatidylcholine, are important for the maintenance of the integrity of the sperm membrane during the freezing and post-thawing process (Mousa *et al*., 2002; Amirat *et al*., 2004; Forouzanfar *et al*., 2010; Alcay *et al*., 2016). Therefore, it is likely that a high percentage of egg yolk improves the sperm viability observed in TY, and this contributes

to maintaining the levels of polinsaturated fatty acids necessary for the sperm membrane, being less susceptible to destructive lipid peroxidation (Cerolini *et al*., 2001; Kaeoket *et al*., 2010).

On the other hand, the poor post-thaw semen quality of CY could be due to the fact that the egg yolk has a high risk of suffering microbial contamination, which could decrease post-thaw semen quality (Aboagla *et al*., 2004; Kulakzis *et al*., 2010) and being the commercial egg yolk (CY), it could not have a good sanitary quality, which impaired post-freezing semen quality. Another factor that could affect the quality of the CY semen is the diet and management of the producing birds (Lima-Verde *et al*., 2017). Indeed, several studies have shown that the egg yolks of different species of birds show a variation in their components, resulting in different effects in the cryopreservation process on sperm (Trimeche *et al*., 1997; Bathgate *et al*., 2006; Singh *et al.,* 2013). Furthermore, egg yolk can contain harmful metabolites and endotoxins that affect sperm viability (Vidal *et al.,* 2013), as well as steroid hormones that reduce sperm motility (El-Sisy *et al*., 2018). Previous laboratory studies reveal that, by eliminating some components in the egg yolk by centrifugation; in addition to certain substances in the yolk that inhibit respiration and sperm motility, suggesting the replacement of the whole egg yolk by the cryoprotective fraction (Amirat *et al.,* 2004).

CONCLUSION

The results of the present study did not show statistically significant differences between the use of the different diluents for the conservation of FS and RS. However, Tris-yolk obtained higher individual and mass motility and post-thaw viability compared to soy lecithin-based diluent during the cryopreservation process of Alpine goat semen.

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