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Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreservation

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Summary

Background: Sperm cryopreservation is an important tool for breed improvement, nonetheless, spermatozoids of rams are extremely sensitive to cryopreservation. **Aims:** The present research was to compare a liposome-based (OptiXcell: OX) diluent, a commercial TRIS-egg yolk (Optidyl: OP) and a citrate egg yolk-based (CY) diluent on ovine semen quality through the cryopreservation process. **Methods:** Semen was collected from four sexually mature Dorper rams during the natural breeding season. After collection, semen was evaluated and diluted in OX, OP or CY diluent and was cooled from 37°C to 4°C for 2 h (refrigerated semen, RS), after that semen-filled straws were placed in liquid nitrogen (LN) vapour for 10 min, then immersed into LN at -196°C (cryopreserved semen, CS) and stored until evaluation. **Results:** For fresh semen (FS), similar values (P>0.05) were obtained from the 3 diluents [motility (4.2 ± 0.3), viability (75.4 ± 3.2), hypo-osmotic swelling test (HOST) (59.2 ± 2.1), and normality (84.7 ± 3.5)]. The motility values were higher for RS with OX and CY (4.0 ± 0.2 and 3.6 ± 0.3, respectively) compared to OP (3.0 ± 0.21; P<0.05). The viability was reduced after refrigeration and freezing (P<0.05). Refrigerated semen viability was similar for OX (65%), CY (63%) and OP diluents (60%; P>0.05), but for frozen semen, viability was lower in the CY diluent (P<0.05). Membrane integrity (HOST) in OX (53.6 ± 1.7) was similar to that in OP (50.7 ± 1.5; P>0.05) but higher than in CY (48.7 ± 1.5; P<0.05). **Conclusion:** No difference was found between the OX diluents and those made with egg yolk in terms of sperm parameters; however, the OX diluent was more efficient in protecting the integrity of membrane in freezing/thawing semen.

Key words: Cryopreservation, Diluent, Egg yolk, Liposome

Introduction

Sperm cryopreservation is an important tool for breed improvement and conservation programmes for several species, including small ruminants (Jiménez-Rabadán *et al*., 2016); however, there are some issues to consider with its use, among them, the fact that the response of sperm to cryopreservation varies between individuals of the same species as well as between different species, and, in general, spermatozoids of small ruminants are extremely sensitive to cryopreservation compared to those of other species (Aisen *et al*., 2002; Küçük *et al*., 2014).

To date, it has not been possible to standardize the process of freezing and thawing to avoid the biochemical and structural damage in sperm cells (acrosome, nucleus, mitochondria, axoneme, and plasmatic membrane) that is produced by temperature changes, osmotic stress and the formation of ice crystals, which cause a decrease in viability, motility, DNA integrity and oxidative stress (Amirat *et al*., 2004; Gürler *et al*., 2016).

Because of the aforementioned reasons, different diluents have been studied to increase the spermatic quality, prevent the formation of intracellular ice crystals and reduce damage to the membrane during and after cryopreservation (Amirat *et al*., 2004; Alcay *et al*., 2015; Arando *et al*., 2017). Egg yolk is a common ingredient for semen diluents. The components of egg yolk (high molecular weight and the fraction of low-density lipoproteins) provide excellent protection to semen against cold shock (Salamon and Maxwell, 2000), and it has been demonstrated that they work as a non-permeable cryoprotector for cryopreservation. Because 66% of its proteins are of low density, they reduce the loss of acrosomal enzymes and prevent degenerative changes in the acrosome during liquid storage (Salamon and Maxwell, 2000; Amirat *et al*., 2004; Ansari *et al*., 2016; Murphy *et al*., 2018). Nonetheless, the composition of chicken eggs depends on management and nutritional practises, and its use implicates some microbial contamination risks (Ansari *et al*., 2016; Belala *et al*., 2016), which makes it difficult to reproduce trials. Thus, partial or total substitution of semen diluents with proteins of animal origin or other diluents composed of liposomes and containing no proteins of animal origin has been attempted (Valente *et al*., 2010;

Ansari *et al*., 2016).

Other diluents have been developed that do not contain proteins of animal origin and are chemically composed of liposomes (Ansari *et al*., 2016). It has been reported that liposomes have protection properties for semen during the freezing process, which can modify conformation and permeability of the plasmatic membrane to water for the physical and chemical properties of the lipids that compose them (Röpke *et al*., 2011), favouring the cryopreservation process. These lipids, unlike the animal protein in yolk, are not a vector of infectious agents (Kumar *et al*., 2015) and have been widely used in the cryopreservation of bull semen (Ansari *et al*., 2016; Fleisch *et al*., 2017; Yang *et al*., 2018) and buffalo (Kumar *et al*., 2015; Swami *et al*., 2017). However, the effects of these liposome-based (OptiXcell: OX) diluents on the quality of cryopreserved semen (CS) of ram are poorly known. Therefore, the impact of a diluent based on liposomes and those based on egg yolk protein on the quality of ram semen, preserved by refrigeration and freezing, was evaluated *in vitro*.

Materials and Methods

Study site

The present research took place during the natural breeding season (fall-winter) in the Northern Mexico under an intensive ovine production system (25° N, 103° W). The climate of the region is semi-desert, with a mean annual rainfall of 203 mm, and the average daily maximum temperature is 41°C in May and June and the lowest (-3°C) occurring in December and January (CONAGUA, 2015). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Animal management

Four adult Dorper rams, averaging 2 to 4 years of age and proven fertility (used for natural mating), were used for donor semen. Rams were fed twice a day (10:00 and 18:00 h) with a total mixed ration (17% CP and 1.5 ME) and had mineral blocks and water *ad libitum* with an adaptation period of 3 weeks prior to the research.

Collection and processing of semen

Semen was collected from the four rams in the morning (08:00 h) every 7 days with an artificial vagina at 37°C and using an oestrogenised sheep treated with 2 mg of oestradiol benzoate (Sincrodiol, Ourofino, Brazil) by intramuscular (IM) route 12 h before semen collection.

After each extraction, semen was placed in a water bath at 37°C and immediately evaluated for volume, concentration, mass motility and viability. The volume was measured using a conic, graduated glass tube (0.1 ml), and the concentration was measured with a photometric analysis (SDM 1 Minitube, Landshut, Germany), using undiluted semen and expressed in 10^6 cells. Mass motility was calculated using the arbitrary scale of 0 to 5 (0=0% to 5=100% motile spermatozoa), using a pre-warmer (37°C) slide and a microscope with the 10X objective. Sperm viability was calculated using eosin-nigrosin staining, as previously described by Kafi *et al*. (2004). Only ejaculates with a volume of ≥0.5 ml, a concentration of $\geq 2.5 \times 10^9$ ml, mass motility of ≥3.0 and a viability of ≥85% were considered for the experiment. All the previous measurements were performed by only one person.

Diluents preparation and freezing process

For the cryopreservation process, 3 diluents were used, and their composition was as follows: to prepare the citrate egg yolk-based (CY) diluent, fresh chicken eggs were used. The eggs were broken manually, separated and carefully rolled over filter paper to eliminate any traces of albumin that adhered to the vitelline membrane. This membrane was punctured with a syringe needle, and 15 ml of the yolk was collected and placed in a beaker. Then, 2.37 g of sodium citrate, 0.50 g of glucose, 7 ml of glycerol, 100 000 IU of penicillin and 100 mg of streptomycin were added to a glass containing 100 ml of distilled water (Salamon and Maxwell, 2000). The Optidyl (OP) diluent (Optidyl®, CRYO-VET, France) and OptiXcell (OX) (OptiXcell, CRYO-VET, France) were prepared according to the manufacturer's recommendations.

After evaluation, semen samples were extended in one of the three diluents to obtain a final concentration of 800×10^6 cells per ml. Extended semen was cooled from 37°C to 4°C for 2 h (refrigerated semen, RS). Straws (0.25 ml) were filled with semen, properly identified and sealed with polvinilic alcohol. Straws were placed in 4 cm of liquid nitrogen (LN) (vapour) in styrofoam for 10 min and then immersed into LN at -196°C (CS) and stored until analysis (Jerez *et al*., 2016). For each of the conservation states (FS, RS and CS), the semen was evaluated for motility, viability, morphology, and membrane integrity. Frozen straws were thawed by immersion in tempered water at 37°C for 26 s.

Motility was evaluated through a floating drop, consisting of placing a drop of semen of approximately 10 µL on a warmer slide that is subsequently rotated on its vertical axis; the drop remained suspended and was then evaluated with a binocular microscope with a 10X objective, according to the technique of Salamon and Maxwell (2000). Motility was subjectively scored from 0 to 5, where 5 denoted the greatest forward progressive movement and 0 denoted no motility. Sperm viability was evaluated with eosin-nigrosin staining as previously described by Kafi *et al*. (2004). A total of 200 cells were counted, and the live/dead ratio was calculated. Sperm stains were considered dead. The functional integrity of the sperm membrane was determined by a hypo-osmotic swelling test (HOST). First, 10 µL of semen was incubated with 100 µL of a hypo-osmotic solution of 100 mOsm (9 g of fructose, 4.9 g of sodium citrate per litre of distilled water) at 37°C for 60 min (Vásquez *et al*., 2013). After incubation, 20 µL of the sample was placed on a slide tempered at 37°C and spread with a coverslip,

Table 1: Means (±SEM) for motility (scale of 0-5), viability (%), normality (%), hypo-osmotic swelling test (HOST) (%) of Dorper ram semen diluted with egg yolk citrate (CY), Optidyl (OP) or OptiXcell (OX) and subsequently evaluated fresh semen (FS), after being refrigerated semen (RS) for 2 h and when thawed, the cryopreserved semen (CS)

Parameters	Motility (%)	Viability (%)	Normality (%)	HOST (%)
Fresh semen (FS)				
CY	4.2 ± 0.2^{a}	73.2 ± 3.4^{abc}	82.5 ± 2.8^{a}	59.0 ± 1.5^{a}
OP	4.0 ± 0.3^{a}	78.3 ± 3.9^{a}	85.5 ± 2.8^{a}	59.3 ± 1.7^{a}
OX	4.3 ± 0.2^{a}	74.5 ± 3.4^{ab}	85.5 ± 2.8^{a}	59.2 ± 1.5^{a}
Refrigerated semen (RS)				
CY	3.6 ± 0.2^{ab}	63.2 ± 3.4^{cd}	84.5 ± 2.8^{a}	53.7 ± 1.5^{bc}
OP	3.0 ± 0.2^{bc}	60.5 ± 3.4^{de}	87.0 ± 3.2^{a}	54.0 ± 1.5^{bc}
OX	4.0 ± 0.3^{a}	66.7 ± 3.4^{bcd}	84.7 ± 2.8^{a}	56.7 ± 1.5^{ab}
Cryopreserved semen (CS)				
CY	2.2 ± 0.2^{cd}	52.7 ± 3.4^{e}	87.2 ± 2.8^{a}	48.7 ± 1.5^{d}
OP	2.5 ± 0.2^{cd}	57.7 ± 3.4^{de}	80.7 ± 2.8^{a}	50.7 ± 1.5^{cd}
OX	2.0 ± 0.2^{d}	59.0 ± 3.9^{de}	85.0 ± 3.0^{a}	53.6 ± 1.7^{bc}

$^{a, b, c, d, e}$ Within a column, means without a common superscript differ (P<0.05) between the diluents used (CY, OP, OX) and the states of conservation of the semen (FS, RS and CS)

and 200 sperm cells were evaluated under a microscope at ×100. The sperm with the tail rolled were recorded as intact membranes (Fonseca *et al*., 2005; Alcay *et al*., 2015).

Statistical analysis

The data were analysed using a variance analysis (ANOVA) via the General Linear Model (GLM) procedure. The means obtained from the seminal parameters were compared using a t-test. The ANOVA of the repeated measures was performed comparing the results to the different diluents, the states of the cryopreservation process and the interaction of these. All data were analysed using the SAS (2005). The differences were considered significant at a value of P≤0.05.

Results

The results of the different sperm quality parameters of Dorper sheep semen diluted with CY, OP, or OX that were evaluated in fresh semen (FS), refrigerated for 2 h (RS) and thawed (CS) semen are summarized in Table 1.

When analysing the effects of the different diluents, regardless of semen conditions, no significant differences were observed in each of the measured variables (P>0.05). For FS, similar values (P>0.05) were obtained independently of the added diluent in each of the variables evaluated [motility (4.19 ± 0.28), viability (75.44 ± 3.28), HOST (59.19 ± 2.12), and normality (84.77 ± 3.49)] in this category. Sperm motility decreased progressively after the refrigeration and freezing process (P<0.0001). The values of motility were higher in the RS when it was diluted with OX and CY (4.00 ± 0.23 and 3.66 ± 0.31, P>0.05), in comparison with the OP diluent, which presented less cellular movement (3.00 ± 0.21; P<0.05).

The percentage of live sperm was reduced after the refrigeration and freezing process (P<0.05). The viability of the RS and diluted semen with OX was higher than 65%, followed by the CY and OP diluents (63.25 ± 4.07 and 60.50 ± 4.07, P>0.05); however, only significant differences (P<0.05) were shown with the semen frozen and diluted with CY (52.75 ± 2.89), which obtained a lower percentage of living cells.

The integrity of the sperm membrane determined by HOST was negatively affected by the refrigeration and freezing process (P<0.0002). The OX diluent affected the integrity of the plasma membrane to a lesser degree (59.00 ± 1.76), and there were no significant differences when this diluent was compared in the RS and frozen semen (56.75 ± 1.76 vs 53.66 ± 0.82, P>0.05).

Discussion

The results from the present research show that there were no effects of using either egg yolk-based or liposome diluents on the mobility, viability and normality of ram semen after freezing. Nonetheless, it has been reported in other species, such as horses and deer, that after freezing the semen, seminal mobility is higher when it has been diluted with an egg yolk-based diluent rather than with liposomes (Pillet *et al*., 2012; Stewart *et al*., 2018). However, our results are in agreement with Fleisch *et al*. (2017), who found that for bull semen, there were no significant differences when using diluents based on egg yolk or liposomes. The aforementioned is also in agreement with reports from Forouzanfar *et al*. (2010), who found no differences upon sperm motility and viability for ram semen cryopreserved with diluents based on egg yolk or liposomes. Additionally, Stewart *et al*. (2018) showed that diluents based on liposomes used in white-tailed deer semen are effective for cryoconservation of the semen but are superior to those based on egg yolk. A possible explanation for these differences in sperm motility between species across the different studies could be due to the differences in composition of diluents based on egg yolk and liposomes and species differences, especially when considering the lipoid composition of the plasmatic membrane (Gharibi *et al*., 2014; Kumar *et al*., 2015). In contrast, Kumar *et al*. (2015), in a study conducted in buffalo semen, found that diluents based on liposomes are more efficient than diluents based on egg

yolk and could be used as cryoprotectors. These results are in agreement with findings reported by Ansari *et al*. (2016), who showed that liposome diluents based on soy grain lipids are efficient for replacing diluents based on egg yolk for freezing bovine semen. It is known that liposome diluents are free of animal origin proteins, which enhance fertility, especially in small ruminants (Murphy *et al*., 2018). The protective properties of liposomes are attributed to lipids and cholesterol that are transferred between liposomes and cellular membranes (Ansari *et al*., 2016). In such a way, the cryoprotective effect of the liposomes depends on their size, and the relative success of each method will depend on the sensitivity of each cell to the damage by the process (Garret *et al*., 1999; Röpke *et al*., 2011). In this regard, under our experimental conditions, greater sperm survival was maintained in the RS and diluted semen based on liposomes than the diluted semen based on egg yolk. Indeed, it has been reported that bulbourethral enzymes react with egg yolk in goat and sheep semen, causing hydrolysis of lecithin and triglycerides present in the egg yolk, which causes high semen toxicity (Murphy *et al*., 2018).

On the other hand, after the freezing and thawing process, we found no differences in our research results with the different diluents used; it is probable that this was because the cryoprotectors used in this research enhanced the sperm cold shock resistance on both treatments (Osuagwuh and Palomo, 2017). Another possibility is that, in both treatments, glycerol was used, a cryoprotectant that could have prevented cell damage on the different diluents used in our research. This was probably because glycerol augmented the fluidity of the spermatic membrane, which could have improved the resistance to cell damage and prevented the formation of ice crystals in the membrane, which could have improved motility and sperm capacitation at thawing (Taghilou *et al*., 2017). In this regard, it has been shown that sperm survival is compromised by intracellular ice in both freezing and thawing; sperm can suffer loss of integrity of cell membranes, as well as the inactivation of acrosomal enzymes and the loss of phospholipids (Murphy *et al*., 2018).

However, our results, when evaluating the percentage of sperm plasmatic membrane functionality through the HOST, showed that for thawed semen, the percentage was higher for the OX diluent. It is likely that this was because the OX diluents helped decrease the lipid peroxidation of the polyunsaturated fatty acids that are found to protect the spermatic membrane during the cryopreservation process (Cerolini *et al*., 2001; Kaeoket *et al*., 2010), which may have resulted in greater protection of the sperm plasmatic membrane, which, for the rest of the diluents used, could have decreased the motility and viability of the sperm after freezing (Thagilou *et al*., 2017).

Finally, regarding sperm motility, the OP diluent showed less motility when compared to the other diluents. These results are similar to those reported by Thagilou *et al*. (2017), who found that the mean percentage of sperm motility and viability in ram semen diluted with egg yolk, added with polyunsaturated fatty acids and refrigerated for 3 h, was significantly less (35.6 ± 1.8%) than the control group (43.1 ± 1.8%). The aforementioned could be attributed to the fact that the OP, contrary to CY, has no sodium citrate, even though both are based on egg yolk. This helps decrease the presence of high levels of polyunsaturated fatty acids in the sperm membrane, which makes the sperm more susceptible to peroxidation, coupled with a dispersing action of the fat globules of the egg yolk (Cerolini *et al*., 2001; Kaeoket *et al*., 2010).

Overall, the results shown here and data from the literature show that the level of protection of a diluent strongly depends on several factors and on semen processing (Fleisch *et al*., 2017). In particular, diluents with liposomes can protect the plasma membrane through artificial vesicles composed of one or several biological layers of concentric lipids, which have the ability to encapsulate molecules (Asadpour *et al*., 2012; Pillet *et al*., 2012; Belala *et al*., 2016).

In conclusion, the results of this study demonstrate that the use of diluents based on liposomes is more efficient in protecting RS of ram (viability and integrity of the membrane) compared to diluents based on egg yolk; however, when the semen was frozen, the quality decreased independently of the diluent used.

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Conflict of interest

The authors state that there is no conflict of interest that could damage the impartiality of this experiment.

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