Short communication: Evaluation of alternative diluting media for cryopreservation of goat semen

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Abstract. *Goat farming is an important source of food production in developing countries, where artificial insemination is difficult due to the low quality of semen cryopreservation. In the present investigation, four diluents, namely cow's milk (T1), goat's milk (T2), egg yolk (T3) and commercial (Minutube, USA) Triladyl diluent (T4), were evaluated for the cryopreservation of semen from a Saanen goat buck. Each medium was assessed during 4 repetitions and each repetition performed 10 aliquots for a total of 160 aliquots in study. The color and sperm concentration were revised and counted in the Neubauer chamber from fresh semen; the motility and the thermoresistance were evaluated every 24 hours for a total of 96 hours post-freezing at −174°C. Post-thaw motility was similar in the 4 mediums in the first 24 hours ($p \geqslant 0.05$). After 96 hours, post-thaw motility in T1 (2.25 ± 0.50) and T2 (2.00 ± 0.51) was lower than in T3 (3.00 ± 0.81) and T4 (3.75 ± 0.50), ($p < 0.05$). The thermoresistance was similar for all treatments up to 48 hours ($p \geqslant 0.05$), but after 72 hours T4 (1.75 ± 0.50) and T3 (1.25 ± 0.50) showed better results ($p < 0.05$) than T1 (0.50 ± 0.50) and T2 (0.25 ± 0.5). Egg yolk and Triladyl diluents showed a better effect (Kruskal Wallis, $p < 0.05$) of cryopreserved Saanen goat semen viability in vitro compared with cow's milk and goat's milk.*

Introduction

Goat farming is important as a food production source for economically deprived populations, especially in developing countries, where the economic resources of small producers are limited and artificial insemination is still insipient, mainly due to the low quality of semen cryopreservation (Ferreira et al., 2014).

In 2019, the Nicaraguan Institute of Agricultural Technology (INTA) created research and innovation farms (INTA, 2019) to study milk and meat production, and reproduction among different breeds in sheep and goat herds, making the last effort in the development to genetic improvement.

Artificial insemination is an important tool for genetic improvement, using stallions with superior productive characteristics, although the improvement success depends largely on the development of satisfactory diluents for semen, which should protect the sperm during freezing and increase its life span with a minimal effect on fertility (Palomino et al., 2014).

Cryopreservation of sperm is an important tool for breeding programs and conservation of breeds of various species, including small ruminants. However, its effectiveness depends on the type of semen extenders and additives used to stabilize sperm cells during the freezing and thawing processes (Ismail et al., 2020) vitality, and fertility of spermatozoa after freezing. Different diluents have been studied to increase sperm quality, prevent intracellular ice crystal formation and reduce damage to the membrane during and after cryopreservation (Jiménez-Rabadán et al., 2016) electroejaculation. The thermoresistance test (TRT) simulates the time of the sperm persisting in the female genital tract by exposure to 38°C for a long time and concomitantly the motility of the sperm after measuring heat stress (Talini et al., 2019).

There is an expansive range of diluents and methods used in the preservation of goat semen (Cuevas & Kevin, 2018; Sun et al., 2020). Mediums used for this purpose extend the viability of the sperm cell for a limited period (refrigeration) or indefinitely (freezing), making the most of the number of doses obtained per ejaculate. Among the natural diluents, egg yolk and skimmed or ultra-pasteurized milk are usually used to preserve goat semen (Amiridis & Fthenakis, 2012). The objective of this study was to evaluate the effect of extenders based on cow's milk, goat's milk, egg yolk and Triladyl diluents for *in vitro* viability of cryopreserved Saanen goat semen.

Materials and methods

The study was carried out in the research facility of the Universidad Católica del Trópico Seco, located at 13°14'39" N, 86°22'29" W, and 865 m above sea level, in the department of Estelí, Nicaragua. Seminal fluid was obtained with an artificial vagina from a Saanen buck. The animal, 4 years old and weighing 70 kg, was in a good general health condition

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according to clinical examination. Its physiological parameters were normal with respiratory rate of 13/min, heart rate of 75/min, and rectal temperature of 39°C. A complete blood count (CBC) was also performed and the normal values were obtained: hematocrit 35%, hemoglobin 11 g/dL, eosinophils 2%, neutrophils 35%, lymphocytes 60%, and monocytes 3%. Additionally, semen color, sperm concentration, motility and heat stress resistance were evaluated, according to previously described methods (Alomar, 2019; Malejane et al., 2014)the formation of hydrogen peroxide (H2O2. Four semen samples were collected at 7-day intervals using an artificial vagina at 37°C, obtaining an average of 4 mL per extraction. From the last extraction, 1:20 dilutions were made in each case, obtaining 160 straws of 0.5 mL, which were prepared with four mediums (T1, UTH cow's milk; T2, goat's milk; T3, egg yolk; and T4, Triladyl) realizing four repetitions (24 h, 48 h, 72 h, and 96 h) post-freezing at −174°C, with 10 straws each. Every 24 h post-freezing, the motility was evaluated, thawing the cryopreserved straws in a water bath at 37°C for 26 s, placing 10 µL on a pre-warmed slide (at 37°C); subsequently, rotating the slide on its vertical axis and observing in a microscope with a 10× objective. Sperm movements were registered on a scale of 0 to 5 (Luna-Orozco et al., 2019). In each evaluation, 10 microscopic fields were analyzed to include at least a total of 300 sperms, according to previously described recommendations (Cardoso et al., 2003). Moreover, a thermoresistance test was performed by incubating an aliquot of 0.5 mL at 38°C in an aerated water bath, as previously described (Schulze et al., 2017). After the incubation period (300 min), motility was determined on a scale of 0 to 5. Table 1 shows the proportion of each ingredient in cryopreserved semen in 100 mL of distilled water.

To compare motility and thermoresistance between the mediums, a non-parametric test (Kruskal-Wallis) was applied to evaluate the statistical significance $p < 0.05$.

Table 1. Proportion of each ingredient in semen cryopreservatives in 100 mL of distilled water

Ingredients	Egg yolk	UHT cow's milk	Goat's milk	Triladyl
Natural diluting	20%	10%	10%	20%
Glycerol	6%	6%	6%	Include
Glucose	0.9%	0.9%	0.9%	Include
Penicillin	100,000 UI	100,000 UI	100,000 UI	Include
Streptomycin	100 mg	100 mg	100 mg	Include
Sodium citrate	0.9%	0.9%	0.9%	Include

Results and discussion

The extracted semen was yellowish in color with a creamy appearance, a similar result obtained by Memon et al. (2011), describing fresh goat semen with yellowish and milky color. This is a satisfactory result since a red color indicates the presence of blood, a gray color indicates the presence of an infection or abnormalities and yellow indicates the presence of urine (Bravo et al., 2002).

The sperm count performed resulted in 6.5 billion sperms per mL of semen, similar data to the parameters proposed by other authors who recommend that the counted sperm range should be between 2 and 6 billion per mL (Cueto, 2016).

The comparison of the post-thaw motility showed significant differences between the four mediums after 48 hours ($p < 0.05$), with the lowest motility in the samples preserved with goat's milk (2.50 ± 0.52) and cow's milk (2.75 ± 0.57) and the highest in the semen preserved with Tryladil (4.00 ± 0.20) and egg yolk (3.50 ± 0.57) (Fig. 1). The results after 96 hours showed that the most effective treatments were egg yolk (3.0 ± 0.81) and Tryladil (3.75 ± 0.50), with similar results for both ($p \geqslant 0.05$), but they were different from those obtained with cow's milk (2.25 ± 0.50) and goat's milk (2.00 ± 0.51), ($p < 0.05$). Similar results were reported by researchers who describe that citrated egg yolk has been shown to function as a non-permeable cryoprotectant for semen, finding sperm motility of 3.6 ± 0.2 in semen after 2 hours at −196°C (Luna-Orozco et al., 2019). Most cryopreservatives contain egg yolk, as it acts as a reservoir for phospholipids and cholesterol that helps to protect the plasma membrane and acrosome against temperature-related injuries (Swelum et al., 2018) pigeon (P. After 96 hours, it was observed that the egg yolk had a better result than goat's milk and cow's milk. Similar results were found in a study carried out in Peru, where the viability of the sperm prepared with skimmed milk was only visible 6 hours after refrigeration vs 48 hours when egg yolk was used (Palomino et al., 2014).

Our data differ from those described by authors who indicate that, although egg yolk or skimmed milk are the most common semen diluents for goats, their interaction between goat seminal plasma is harmful to spermatozoa, a condition which is not seen with other mammalian seminal plasmas (Daramola et al., 2016; Purdy, 2006). Also, bulbourethral enzymes have been reported to react with egg yolk in goat and sheep semen, causing hydrolysis of lecithin and triglycerides present in egg yolk, resulting in high semen toxicity (Luna-Orozco et al., 2019). To address this problem, natural additives such as coconut water (Bottini-Luzardo et al., 2013; Luna-Orozco et al., 2019), or liposome diluents free of animal origin proteins, which enhance fertility, especially in small ruminants (Elodie Pillet et al., 2012) addition of egg yolk in extenders is not without disadvantages and the demand to find cryoprotective alternatives is strong. The objec-

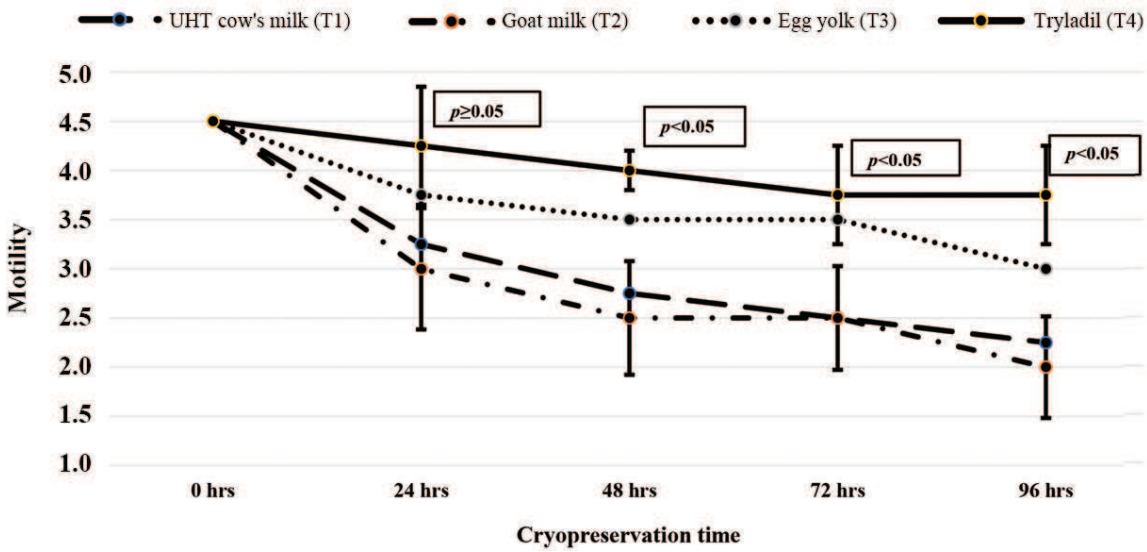

Fig. 1. Sperm motility for the different cryopreservation times.
Only the error bars are shown for the treatments with higher (T4) and lower (T2) motility.
The p values correspond to the comparison between the 4 groups according to the Kruskal Wallis test.

tive of this study was to test the cryoprotective capacities of liposomes composed of egg yolk phospholipids. Two experiments were conducted: 1, were used, despite of their higher cost. Another recommendation is to dilute the goat semen sample in a buffered diluent and then separate the seminal plasma from the sperm, by washing the cells either once or twice, each for 10–15 min at 550–950 g (Purdy, 2006).

The thermoresistance test, by analysis of variance according to Kruskal Wallis, and sperm motility showed significant differences ($p < 0.05$) between the different diluents after 76 hours. Better results were observed in egg yolk (1.25 ± 0.50) and commercial Triladyl diluent (1.75 ± 0.50) compared with cow's milk (0.5 ± 0.57) and goat's milk (0.25 ± 0.50) (Fig. 2). These findings coincide with those described by researchers who observed better motility after the thermal resistance test compared with the skimmed milk diluent (Ferrari & Barnabe, 1999). In addition, a study carried out in Brazil reported better results as the concentration of egg yolk increased; they attributed this result to the fact that egg yolk can provide more protection for cryopreservation of sperm increase osmotic pressure, which results in cell dehydration with consequent reduction of intracellular ice formation (Ferreira et al., 2014). A study testing treolase in goat semen cryopreservatives showed similar results when they managed to maintain high sperm motility in the thermoresistance test during the first hours of cryopreservation (Aboagla & Terada, 2003). Meanwhile, other researchers found that the presence of disaccharide in cryopreservatives did not increase

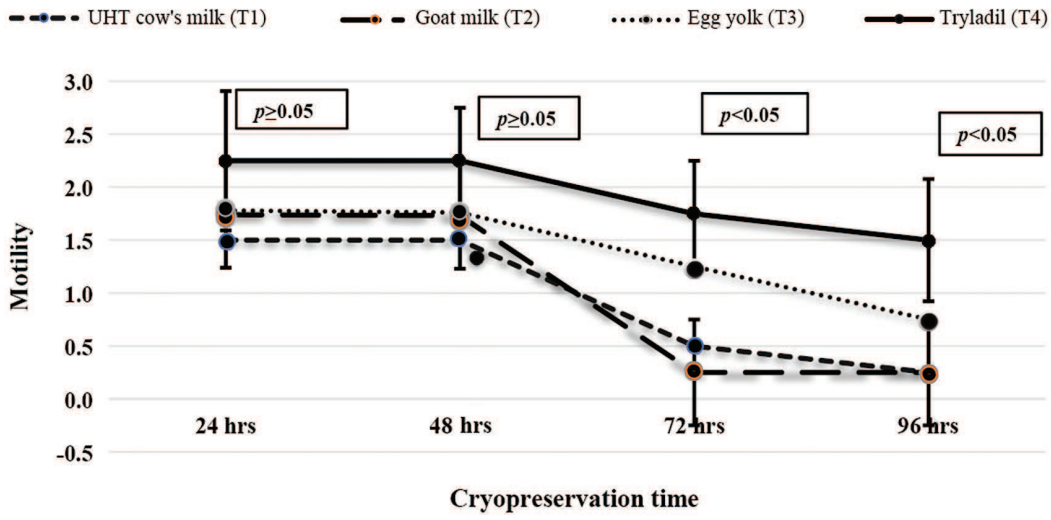

Fig. 2. Sperm motility after different cryopreservation times in the thermoresistance test
Only the error bars are shown for the treatments with higher (T4) and lower (T2) motility.
The p values correspond to the comparison between the 4 groups according to the Kruskal Wallis test.

heat resistance or protect the membrane integrity of frozen goat sperm (Quan et al., 2012).

With the post-thaw semen thermoresistance test, the integrity of the sperm is evaluated during artificial insemination; after the test, the thawed semen must have at least 15% motility to be considered of good quality (Talini et al., 2019). The results of this study showed that both Tryladil and egg yolk prepared semen-maintained motility above 1, even after 96 hours of cryopreservation, thus meeting this requirement.

Conclusions

Sperm motility in cryopreserved Saanen buck semen was higher with Tryladil and egg yolk diluents compared with cow's milk and goat's milk (Kruskal Walli, $p < 0.05$). This difference was marked after 48 hours post-freezing at –170°C, while the sperm motility comparison between Tryladil and egg yolk was similar even after 96 hrs (Kruskal Wallis, $p \geqslant 0.05$). The thermoresistance was also higher in semen cryopreserved with Tryladil and egg yolk diluents compared with cow's milk and goat's milk (Kruskal Wallis, $p < 0.05$). However, this difference was observed until after 72 hours post-freezing. The results of this study revealed that Tryladil diluents and egg yolk were effective in the cryopreservation of Saanen buck semen.

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