https://doi.org/10.15446/rfmvz.v71n3.113280

Addition of cyclodextrins saturated with cholesterol and its effect on the cooling of goat semen

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Recibido: 01/03/2024. Aprobado: 15/08/2024

ABSTRACT

Caprine spermatozoa exhibit a low cholesterol-to-phospholipid ratio in their plasma membrane, which affects its structure and functionality, as well as sperm survival after the cryopreservation process. The presence and incorporation of cholesterol into the plasma membrane enhances membrane stability, thereby increasing sperm survival. The objective of this study was to evaluate the effect of adding cholesterol-loaded methyl-β-cyclodextrin (CLC) on the viability and membrane integrity of chilled semen. Four Anglo Nubian x Boer crossbred males were used, with 5 ejaculates collected per male, resulting in a total of 20 ejaculates. Each ejaculate was divided into four groups: a control without CLC and three treatment groups (1, 2, and 3 mg of CLC per 120 million spermatozoa). The samples were refrigerated at 5 °C for 24 and 48 hours for evaluation. No statistical differences were observed between the treatment means for semen viability after 24 and 48 hours. However, in treatment 3, a statistically significant decrease in membrane integrity was observed after 24 and 48 hours. It is concluded that, although a biological improvement was observed, it was not statistically significant.

Keywords: reproduction, caprine, spermatozoa, cryopreservation.

Adición de ciclodextrinas saturadas con colesterol y su efecto en la refrigeración de semen caprino

RESUMEN

Los espermatozoides caprinos presentan una baja relación colesterol/fosfolípidos en la membrana plasmática, lo que afecta su estructura y funcionalidad, como también la supervivencia del espermatozoide tras el proceso de criopreservación. La presencia de colesterol y su incorporación a la membrana plasmática aumenta su estabilidad, lo cual incrementa la supervivencia de los espermatozoides. El objetivo de este trabajo fue evaluar el efecto de la adición de metil-β-ciclodextrina cargada de colesterol (CLC) sobre la viabilidad e integridad de membrana del semen refrigerado. Se emplearon 4 machos cruza Anglo Nubian x Boer y se obtuvieron 5 eyaculados por macho, con un total de 20 eyaculados. Cada eyaculado fue dividido en cuatro: control sin CLC y tres tratamientos (1, 2 y 3 mg de CLC/120 millones de espermatozoides). Las muestras fueron refrigeradas a 5 °C durante 24 y 48 horas para su evaluación. No hubo diferencias

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estadísticas entre las medias de los tratamientos para la viabilidad del semen refrigerado luego de 24 y 48 horas. Sin embargo, en el tratamiento 3 se observó una diferencia estadística en la integridad de membrana que disminuyó luego de las 24 y 48 horas. Se concluye que, a pesar de encontrar una mejora desde el punto de vista biológico, no tiene significancia estadística.

Palabras clave: reproducción, caprinos, espermatozoide, crioconservación.

INTRODUCTION

Artificial insemination (AI) is one of the most widely used assisted reproduction techniques worldwide. Due to its numerous benefits, AI has been implemented in breeding and conservation programs for both livestock species of zootechnical interest and wild species. The success of AI outcomes depends on semen quality, as well as processing and preservation methods (Viñán Díaz *et al*. 2019). Semen can be used either fresh or cryopreserved. When inseminating with fresh semen, it must be used shortly after collection, as sperm motility and viability rapidly decline under these conditions due to the accumulation of lactic acid in the ejaculate. Refrigerated semen, however, can be preserved for approximately 48 hours, providing greater flexibility for its use. Regarding storage temperature, numerous studies have been conducted, indicating that semen can be stored at temperatures ranging from 2 to 15 °C. This is based on the rationale that reducing sperm motility and metabolism in a reversible manner extends its fertilizing capacity (Castro Bedriñana *et al.* 2017; Puente *et al.* 2022).

The results described for the viability and fertility of ovine and caprine spermatozoa refrigerated for 5-8 hours are satisfactory, but longer periods (beyond 12-24 hours) show a reduction in fertility (Leboeuf *et al.* 2000; Paulenz *et al.* 2002). Protection of cells against low temperatures is enhanced by the addition of compounds such as egg yolk or skim milk, as they increase resistance to permeability changes and prevent sperm from accumulating calcium due to alterations in membrane exchange systems (Mocé *et al.* 2020). The presence of cholesterol is essential, and its incorporation into plasma membranes prior to refrigeration increases the cholesterol-to-phospholipid ratio, enhancing membrane stability and consequently sperm survival (Ccalta Hancco *et al.* 2022). Improvements in motility, viability, and membrane integrity have been observed when equine, bovine, ovine, and porcine spermatozoa are treated with cholesterol-saturated cyclodextrins prior to cryopreservation (Combes *et al.* 2000; Ferré *et al.* 2018; Moore *et al*. 2005). However, Zahn *et al.* (2002) and Purdy *et al.* (2004) did not observe improvements in motility.

Studies conducted in various animal species have reported improvements in semen quality parameters when spermatozoa were treated with cholesterol-loaded cyclodextrins (CLC), including both methyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin. These improvements have been observed in donkeys (Álvarez *et al.* 2006), sheep (Castillo Cevallos *et al.* 2019; Mocé *et al.* 2010), and swine (Zeng and Terada 2001; Galantino–Homer *et al.* 2006; Souza *et al.* 2021).

The objective of this study was to evaluate the effect of adding cholesterol-loaded methyl-β-cyclodextrin (CLC) to refrigerated semen and its impact on sperm viability and membrane integrity variables.

MATERIALS AND METHODS

Animals

Four sexually mature Anglo Nubian x Boer (Nubor) crossbred male goats were used for this study. The animals were fed a diet consisting of alfalfa pellets, corn, pasture hay, and had *ad-libitum* access to water. The study was conducted at the experimental facility of the Faculty of Agricultural Sciences at the National University of Lomas de Zamora, located in the Santa Catalina field station in Llavallol, Buenos Aires, Argentina.

Semen collection

Semen was collected using an artificial vagina and a female in estrus. The artificial vagina was maintained at a temperature of 42-45 °C. A total of 5 ejaculates were obtained from each male, amounting to 20 ejaculates in total. Each ejaculate was evaluated macroscopically for volume and color. A 10 µl drop was then placed on a glass slide over a heated stage to assess mass motility using an optical microscope (10X). The semen was subsequently diluted at a 1:10 ratio.

Preparation of cyclodextrin

The preparation of cholesterol-loaded cyclodextrin (CLC) followed the technique described by Purdy and Graham (2004). A total of 200 mg of cholesterol (Sigma) was dissolved in 1 ml of chloroform. Separately, 1 g of methyl-β-cyclodextrin (Sigma) was dissolved in 2 ml of methanol in a glass tube.

A 0.45 ml aliquot of the cholesterol solution was added to the cyclodextrin solution, and the mixture was stirred until homogenized into a clear solution, which was then poured into a glass Petri dish. The dish was placed in a fume hood at room temperature for 24 hours, allowing the solution to crystallize. The resulting crystals were removed from the dish, stored, and kept in a glass container with an airtight lid at room temperature.

Treatments

The ejaculates were centrifuged at 1,500 rpm for 5 minutes at room temperature. The supernatant was then removed, and the sperm pellet was resuspended in Tris-citric acid-fructose (TCF) diluent, restoring it to the initial volume. Afterward, 20% egg yolk was added. From each sample, one control (Semen + TCF) and three treatments were prepared: 1- (Semen + TCF + 1 mg of CLC per 120 million spermatozoa), 2- (Semen + TCF + 2 mg of CLC per 120 million spermatozoa), and 3- (Semen + TCF + 3 mg of CLC per 120 million spermatozoa).

The semen was evaluated immediately after collection and after storage at 5 °C for 24 and 48 hours. Viability was assessed using the 5% Eosin-Nigrosin staining technique. A 10 µl aliquot of diluted semen was mixed with 10 µl of the stain solution, homogenized, and a smear was prepared. The smear was air-dried at room temperature before being examined under an optical microscope (100x). Membrane integrity was assessed using the hypoosmotic swelling (HOS) test (Van der Ven *et al.* 1986), which involved mixing 10 µl of diluted semen with 490 µl of a hypoosmotic solution containing fructose and sodium citrate. The mixture was incubated at 36 °C in a water bath for 30 minutes. A 10 µl aliquot of the solution was then placed on a glass slide and observed under an optical microscope (40x).

Ethics for animal experimentation

All ethical requirements of the institution where the study was conducted were followed, as outlined by the Comité Institucional para el Cuidado y Uso de Animales de Experimentación (Cicuae, for its acronym in Spanish) under Resolution CAA/123 Expte. A/22839/2017.

Statistical analysis

Statistical analysis was performed using Infostat software (Di Rienzo *et al.* 2020). An analysis of variance (Anova) for a completely randomized design (CRD) was conducted for a fixed effects model. The assumption of normality was tested using the Shapiro–Wilk test, and the assumption of homogeneity was tested using Levene's test. Subsequently, mean comparisons were made using the Duncan's Multiple Range Test (DGC), with a significance level set at 5%.

RESULTS

After refrigeration for 24 hours at 5 °C, there was no significant variation among the three treatments in terms of the percentage of live spermatozoa compared to the control, which exhibited the highest percentage (76.69%). After 48 hours of refrigeration, no significant differences were observed among the three treatments compared to the control group. However, the treatment with 1 mg of CLC showed a higher percentage of live spermatozoa (73.69%) (table 1).

Regarding membrane integrity data, at the 24-hour assessment, a significant difference was observed between the control and treatment 3, which had the lowest percentage (35.23%). Treatments 1 and 2 with CLC did not show significant differences from the control group, which had the highest percentage (63.69%) (table 1).

TABLE 1. Percentage of live sperm and integrity of the plasma membrane, after refrigeration at 5 °C for 24 and 48 h with 0 (control), 1, 2, and 3 mg of CLC

Treatment	Time (hours)	Percentage of live sperm	Percentage of sperm with plasma membrane integrity
Extender with 0 mg CLC	24	76.69 ±12.09 [a]	63.69 ± 7.43 [a]
	48	70.31 ±10.42 [a]	58.54 ± 6.46 [a]
Extender with 1 mg CLC	24	73.23 ± 9.58 [a]	61.69 ± 5.96 [a]
	48	73.69 ±10.98 [a]	58.62 ± 6.98 [a]
Extender with 2 mg CLC	24	72.69 ±11.56 [a]	60.08 ± 7.02 [a]
	48	70.00 ±11.78 [a]	56.31 ± 7.42 [a]
Extender with 3 mg CLC	24	67.85 ±11.64 [a]	35.23 ± 7.62 [b]
	48	64.38 ± 12.06 [a]	51.85 ± 7.55 [b]

Values represent the mean ± SD (n = 20). Treatments with different letters are significantly different from each other ($p > 0.05$).

Source: own elaboration.

Data observed after 48 hours of refrigeration indicate that treatment 3 with CLC had a lower percentage (51.85%), showing significant differences compared to the other treatments. There were no significant differences between treatments 1 and 2 with CLC and the control group; however, treatment with 1 mg of CLC (58.62%) exhibited the highest percentage of membrane integrity (table 1).

DISCUSSION

During cryopreservation, spermatozoa undergo intracellular and extracellular changes that lead to a reorganization of membrane lipids and proteins, as well as osmotic changes, which can damage sperm membranes and result in cell death (Purdy & Graham 2004).

The understanding of the relationship between phospholipids and cholesterol in sperm membranes and their resistance to thermal shock is not recent. Darin–Bennet and White (1977) and Parks and Lynch (1992) established a correlation between the ratio of polyunsaturated to saturated fatty acids in sperm phospholipids and the role of cholesterol in cold tolerance. These observations have spurred research into the amount of cholesterol in spermatozoa and its correlation with sensitivity to thermal decline. The ratio of phospholipids to cholesterol in membranes varies among species. Darin–Bennet and White (1977) and Purdy and Graham (2004) observed differences in the cholesterol-to-phospholipid ratio between species, noting that species with a lower ratio, such as boars (0.26), stallions (0.36), rams (0.38), bulls (0.45), or bucks (0.59), were more susceptible to cryopreservation compared to species with a higher ratio, close to 1, such as rabbits (0.88) or humans (0.99).

The addition of cholesterol improves the survival rate during cryopreservation. Its incorporation into the sperm membrane is facilitated by cyclodextrins, with β-cyclodextrin showing the highest affinity for cholesterol, as demonstrated by Ccalta Hancco *et al.* (2022) in alpacas, using concentrations of 0, 1.5, and 3 mg of cholesterol.

Castillo Cevallos *et al.* (2019) refrigerated and froze ram semen with 1 and 2 mg of cholesterol-loaded cyclodextrin (CLC), finding no differences between treatments after refrigeration. However, differences were observed after freezing, with better results obtained using 2 mg of CLC. These results are consistent with those of Moraes *et al.* (2010) using bull semen, in contrast to the findings of Purdy *et al.* (2010), who did not observe significant differences.

Ferré *et al.* (2018) demonstrated that the process of sexing semen in cattle could exacerbate the detrimental effects of thermal decline on membranes. These authors conducted an experiment involving the stabilization of plasma membranes in sexed spermatozoa by adding cholesterol-loaded cyclodextrin (CLC) at concentrations of 1.5, 3, and 6 mg, followed by freezing. They observed that spermatozoa treated with 3 mg of CLC exhibited higher motility and vigor.

In goats, according to the results of Salmón *et al.* (2016), to test whether treatment with cholesterol-saturated cyclodextrins protects motility and membrane integrity, a study was conducted where fresh semen was treated with 1, 3, and 6 mg of CLC prior to freezing. After thermal decline, the proportions of motile and viable

spermatozoa with intact acrosomes were twice as high as in the control group. This beneficial effect of CLC on cold resistance was evident at all three concentrations of CLC evaluated, with no differences observed between these treatments.

According to our results, with the concentration of 1 mg of CLC, an increase in the evaluated semen quality parameters was observed after refrigeration. Conversely, at a concentration of 3 mg, the evaluated parameters decreased compared to the control group.

The differing results observed in various studies may be attributed to the species of animal or to different individuals within a species who may respond differently to cyclodextrins. Factors contributing to this variability in results include the use of different diluents and cryopreservation protocols. For example, Ccalta Hancco *et al.* (2022) used concentrations of 0, 1.5, and 3 mg of CLC in alpacas, with a tris-based diluent, egg yolk, and dimethylformamide, and performed a freezing curve over 2 hours and 45 minutes. In contrast, Castillo Cevallos *et al.* (2019) used concentrations of 0, 1, and 2 mg of CLC in ram semen, with a freezing procedure lasting 20 hours.

CONCLUSION

It is concluded that refrigeration at 5 °C for 24 hours of goat semen with the addition of CLC at concentrations of 1, 2, and 3 mg does not show a significant difference in viability compared to the control group, with the control group exhibiting the highest results. Regarding plasma membrane integrity, the 3 mg concentration significantly yielded lower results.

After 48 hours, no significant differences in viability were observed among the concentrations of 1, 2, and 3 mg compared to the control group. However, for plasma membrane integrity, the 3 mg concentration again showed significantly lower results.

Although no statistical differences were found among the treatments, refrigeration of semen at 5 °C for 24 or 48 hours provides greater flexibility for use in artificial insemination programs.

CONFLICT OF INTEREST

The authors declare no commercial or personal conflicts of interest related to this research.

FUNDING

The project was funded by a grant provided by the Universidad Nacional de Lomas de Zamora, Secretaría de Ciencia y Técnica (Lomas CYT).

ACKNOWLEDGMENTS

We would like to thank the Faculty of Agricultural Sciences at the Universidad Nacional de Lomas de Zamora, Argentina, for providing access to their laboratory equipment.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

The authors declare that no artificial intelligence tools were used during the development of the research or the preparation of the article resulting from this research.

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Forma de citación del artículo:

Covelo, I., Puente, M., & Tartaglione, M. (2024). Addition of cyclodextrins saturated with cholesterol and its effect on the cooling of goat semen. Rev. Med. Vet. Zoot. 71(3): e113280. https://doi.org/10.15446/rfmvz.v71n3.113280