rccp
Revista Colombiana de Ciencias Pecuarias

Original Research Article

Relationship between cholesterol and sperm quality in Chino-Santandereano (*Bos taurus*) bulls

Relación entre colesterol y calidad espermática en toros Chino Santandereano (Bos taurus)

Relação entre o colesterol e a qualidade espermática em touros Chino Santandereano (Bos taurus)

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To cite this article:
Villa-Duque N, Terraza-Martinez R, Franco-Rodriguez JE, Romero-Cárdenas E, Valencia JA, Rueda FL. Relationship between cholesterol and sperm quality in Chino-Santandereano (*Bos taurus*) bulls. Rev Colomb Cienc Pecu; 38(4):e358073. DOI: https://doi.org/10.17533/udea.rccp.e358073

Received: August 12, 2024.
Accepted: February 10, 2025.
Published: October 20, 2025.

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Abstract

Background: The assessment of seminal quality parameters is often the initial step in bull selection for siring or conservation purposes. While these parameters provide insights into fertility, delving into the molecular dynamics occurring within sperm cells can complement this primary reproductive evaluation. Cholesterol, one of the most active molecules on the sperm membrane surface, has been linked to critical processes that confer functional features required for reaching the oocyte and achieving fertilization. The complexity of cholesterol dynamics in the sperm surface, including membrane lipid remodeling, is relevant to numerous processes that precede fertilization. **Objective:** To elucidate the correlation between sperm quality parameters and cholesterol levels in sperm cells from Chino-Santandereano bulls, a Colombian creole breed renowned for its high sperm quality, yet unfortunately facing imminent extinction. **Methods:** Semen samples were collected from ten Chino-Santandereano bulls to evaluate sperm motility, viability, and sperm functional competence (SFC) immediately after collection. Simultaneously, cholesterol was extracted from sperm membranes using a methanol-chloroform solution, and cholesterol levels were measured by spectrophotometry using a specific commercial kit. A Pearson's correlation test was used to determine the relationship between cholesterol concentration and seminal quality parameters. **Results:** Our results revealed a strong negative correlation between cholesterol quantity and the average SFC. Additionally, cholesterol levels were also negatively associated with sperm motility. This suggests that cholesterol dynamics, particularly cholesterol output, play a more significant role in sperm functionality than cholesterol content. **Conclusions:** Monitoring cholesterol output from the sperm membrane appears to be an interesting alternative to complement the initial reproductive evaluation in bulls. The intricate nature of

these relationships deserves further experimental work to fully comprehend the implications of cholesterol for the reproductive potential of Chino-Santandereano bulls' semen and offer insights for enhancing sperm quality in other breeds.

Keywords: *bovine reproduction; bull; bull selection; cattle; membrane lipids; seminal quality; sperm; sperm functional competence; sperm membrane; sperm motility; sperm quality.*

Resumen

Antecedentes: La evaluación de los parámetros de calidad seminal es a menudo el paso inicial en la selección de reproductores. Si bien dichos parámetros brindan información sobre la fertilidad, profundizar en la dinámica molecular que ocurre dentro de los espermatozoides puede complementar esta evaluación reproductiva primaria. El colesterol, una de las moléculas más activas en la superficie de la membrana espermática, ha sido relacionado con procesos críticos que anteceden a la fecundación. La complejidad de la dinámica del colesterol en la superficie del espermatozoide, así como la remodelación de los lípidos que se presenta en la membrana espermática, tiene una relevancia especial en los procesos que anteceden a la fecundación. **Objetivo:** Elucidar la correlación entre los parámetros de calidad espermática y los niveles de colesterol en espermatozoides de toros Chino Santandereano, una raza colombiana conocida por su alta calidad espermática, pero que desafortunadamente enfrenta un riesgo inminente de extinción. **Métodos:** Se recolectaron muestras de semen de diez toros Chino Santandereano para evaluar la motilidad, viabilidad y competencia funcional espermática (CFE) inmediatamente después de la colecta. Simultáneamente, el colesterol fue extraído de las membranas espermáticas mediante una solución metanol-cloroformo, y sus niveles fueron medidos por espectrofotometría utilizando un kit comercial específico. Se aplicó una prueba de correlación de Pearson para determinar la relación entre la concentración de colesterol y los parámetros de calidad seminal. **Resultados:** Se evidenció una fuerte correlación negativa entre cantidad de colesterol y porcentaje de CFE. Adicionalmente, los niveles de colesterol se asociaron negativamente con la motilidad espermática. Esto sugiere que la salida del colesterol de la membrana espermática es más relevante para la competencia funcional del espermatozoide que el contenido de colesterol. **Conclusión:** Monitorear la salida de colesterol de la célula espermática podría complementar la evaluación reproductiva en toros destinados a la reproducción. La naturaleza intrincada de estas relaciones merece mayor exploración para comprender las implicaciones del colesterol en el potencial reproductivo de los toros Chino Santandereano, y mejorar la calidad del semen de otras razas bovinas.

Palabras clave: *calidad espermática; calidad seminal; competencia funcional espermática; espermatozoides; ganadería; lípidos de membrana; membrana espermática; motilidad espermática; selección de toros; reproducción bovina; toros.*

Resumo

Antecedentes: A avaliação dos parâmetros de qualidade seminais é muitas vezes o passo inicial na seleção touros para fins de reprodução ou conservação. Embora esses parâmetros forneçam perspectivas sobre competência em fertilidade, pesquisando na dinâmica molecular que ocorre dentro dos espermatozoides pode-se complementar esta avaliação reprodutiva primária. O colesterol, como uma das moléculas mais ativas na superfície da membrana do esperma, tem sido associado a processos críticos que conferem características funcionais necessárias para atingir o ovócito e realizar a fertilização. A complexidade da dinâmica do colesterol na superfície do esperma, incluindo remodelação lipídica da membrana, é relevante para numerosos processos, como maturação espermática, capacitação e reação acrossômica. **Objetivo:** Estabelecer a correlação entre parâmetros de qualidade espermática e níveis de colesterol em espermatozoides de touros Chino Santandereano, uma raça crioula colombiana conhecida pela sua alta qualidade de esperma, mas infelizmente enfrentando extinção iminente. **Métodos:** Foram coletadas amostras de sêmen de dez Chino Santandereano Touros para avaliar motilidade, viabilidade e competência de funcionalidade espermática (SFC) imediatamente após a coleta. Simultaneamente, o colesterol foi extraído das membranas dos espermatozoides usando uma solução de metanol-clorofórmio, e os níveis de colesterol foram medidos por espectrofotometria, utilizando um pacote comercial específico. Foi empregado um Teste de correlação Pearson para determinar a relação entre concentrações de colesterol e parâmetros de qualidade seminal. **Resultados:** Os resultados revelaram uma forte correlação negativa entre a quantidade de colesterol e a media

de SFC. Adicionalmente, as quantidades de colesterol também foram associadas negativamente à motilidade dos espermatozoides. Isso sugere que a dinâmica do colesterol, especialmente a saída do colesterol, desempenha um papel mais significativo na funcionalidade do esperma do que o conteúdo de colesterol. **Conclusões:** Monitorar o colesterol que sai da membrana do esperma parece ser uma alternativa interessante para complementar a avaliação reprodutiva inicial em touros. A natureza complexa dessas relações merece mais trabalho experimental para compreender completamente as implicações do colesterol no potencial reprodutivo de touros Chino Santandereano e oferecem perspectivas sobre como melhorar a qualidade do esperma em outras raças também.

Palavras-chave: *competência de funcionalidade espermática; esperma; lipídios de membrana; membrana do esperma; motilidade espermática; pecuária; qualidade do esperma; qualidade seminal; reprodução bovina; seleção de touro; touros.*

Introduction

Bovine seminal quality remains a paramount consideration in male selection within breeding programs geared towards production or conservation endeavors. Traditionally, the evaluation of reproductive capacity has focused on the assessment of sperm viability and motility, as these characteristics reflect the functional and structural attributes of sperm cells. Nonetheless, from a molecular standpoint, the landscape is revealed as a significantly intricate map of interactions. The interplay between sperm cells and seminal plasma or oviductal fluid promotes a multitude of transformations within the sperm membrane, encompassing an array of biochemical and physiological phenomena such as the efflux of intracellular Ca++ and the reorganization of membrane lipid distribution (Hung and Suarez, 2012). These alterations in the cholesterol/phospholipid ratio bear relevance to numerous processes, including sperm maturation (Keber et al., 2013), capacitation, acrosome reaction, and even cryopreservation survival capacity, commonly referred to as freezability (Travis and Kopf, 2002; Purdy and Graham, 2004). In addition, other studies have suggested that the cholesterol content in the sperm membrane directly affects the acrosomal response and *in vitro* fertility (Shan et al., 2021). The above may be due to cholesterol molecules being well embedded between the phospholipid's tails to enhance membrane fluidity and decrease the deleterious effects of cold and heat shocks (White, 1993). In this sense, cholesterol acts as a temperature buffer avoiding fluidity reduction at low temperatures and, simultaneously, excessive fluidity at high temperatures, making the sperm cells functional and healthy within a temperature range (Lee et al., 2013; Parsons and Rock, 2013; Ernst et al., 2016; Yang et al., 2016). In most species, cholesterol is tightly related to sperm maturation (Keber et al., 2013), sperm capacitation, and male fertility (Leahy and Gadella, 2015; Saez and Drevet, 2019). In bulls, cholesterol levels and other lipids have shown an association with sperm quality (Beer-Ljubić et al., 2009). Moreover, some studies have indicated that sperm motility and viability improve upon incubation with cholesterol (Díaz et al., 2019). Consequently, this work aimed to elucidate the correlation between sperm quality parameters and cholesterol levels in sperm from Chino-Santandereano bulls, a creole breed historically raised under Colombian lowland tropical conditions and recognized by local farmers for its superior sperm quality. As the Chino-Santandereano breed faces the imminent threat of extinction, all breeding investigations are oriented towards conservation objectives as well.

Materials and methods

Animals and semen samples

Semen was collected from 10 Chino-Santandereano bulls aged between 3-6 years. Sampling was performed by electroejaculation

(ElectroJac 5®, Ideal Instruments. Lansing MI, USA). All animals were under the same feeding conditions at the Santa Lucia research center of UNIPAZ (Barrancabermeja, Santander, Colombia) and in optimal health conditions. Three semen samples per bull were collected over three months (one per month).

As electroejaculation is considered a non-invasive procedure, bioethical authorization was not necessary. Semen samples were maintained at 37 °C, and semen quality parameters such as sperm motility, vitality, and sperm functional competence (SFC) were measured immediately after ejaculation. Afterward, sperm cells were separated by centrifugation at 4000 x g for 15 min at 4 °C and then adjusted to 50 x 106 spermatozoa (SPZ) with a 1:5 dilution with PBS buffer for further cholesterol determination.

Sperm quality parameters

Sperm motility was determined through a computer-assisted sperm analysis (CASA; MICROPTIC S.L., Barcelona, Spain). Semen samples were diluted with OptiXcell® medium and incubated at 37 °C for 5 min. Then, diluted samples were carefully placed into disposable 20 µm LEJA chambers with four counting areas (Minitube GmbH, Tiefenbach, Germany). The CASA system was connected to a phase contrast Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan) with a high-density eco-illumination system.

Sperm vitality was assessed by staining with Hoechst 33342-based solutions: trihydrochloride trihydrate (330/380) and propidium iodide (536/617), allowing differentiation between dead and live sperm cells. Sperm counting was performed using a microscope with a UV standard DAPI filter (EX 330-380, DM 400, BA 420).

Finally, sperm functional competence (SFC) was established as a combined test of membrane functionality, acrosome integrity, morphology, and vitality in accordance with Valencia et al. (2019) modified by Pérez-Llano et al. (2009). Briefly, after induced hypoosmotic stress at 75 mOsm/Kg, 10 µl of semen were stained with Hoechst-trihydrochloride trihydrate (FluoVit® MICROPTIC S.L., Barcelona, Spain) previously warmed to 37 °C, and the mixture was incubated for 5 min. Then, 1.0 µL of a propidium iodide solution (FluoVit® MICROPTIC S.L.) was added. Stained samples were fixed with 2 % glutaraldehyde, and cell counting (200 cells per duplicate) was performed using a phase contrast microscope (membrane functionality, and morphology) and a fluorescence microscope with a DAPI standard filter (sperm vitality), at 100 X magnification. To establish the SFC percentage, spermatozoa that showed no morphological defects, had a functional acrosome, an intact plasma membrane, and were alive were considered.

Cholesterol determination

Lipid extraction from sperm was performed using a methanol-chloroform solution (2:1) and centrifugation at 500 g, 4 °C, for 10 min. The upper layer was carefully removed with a micropipette, and the mixture was centrifuged again. The cholesterol levels were measured by semi-automated spectrophotometry using a commercial Kit (IHR Diagnostics, Ref 01639, lot 021A36), according to the procedure proposed by the manufacturer.

Statistical analysis

Data analysis was performed through a Pearson´s correlation test to determine the relationship between cholesterol concentration and seminal quality parameters. Before the correlation test, a Shapiro-Wilk test was used to corroborate the normal distribution of data. The statistical analysis was performed with SPSS software (IBM Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp).

Results

The semen from Chino-Santandereano bulls was promptly assessed for sperm quality following sample collection, and the results are shown in Table 1.

Table 1. Sperm quality parameters, including sperm functional competence (SFC), in fresh semen from Chino-Santandereano bulls.

Sperm vitality (%)	Individual motility (%)	Progressive motility (%)	SFC (%)
81.98 ± 12.68	94.46 ± 11.8	84.27 ± 7.23	71.06 ±12.37

Average cholesterol in sperm cells from Chino-Santandereano was 29.45 ± 5.48 nmol/50 x 106 SPZ. Pearson's correlation test revealed positive correlations between vitality and individual motility (r = 0.66; P < 0.001), as well as between individual motility and SFC (r = 0.76; P < 0.001). Conversely, negative correlations were observed between cholesterol levels and individual motility (r = -0.47; P < 0.001), as well as between cholesterol levels and SFC (r = -0.38; P < 0.05) (Figure 1).

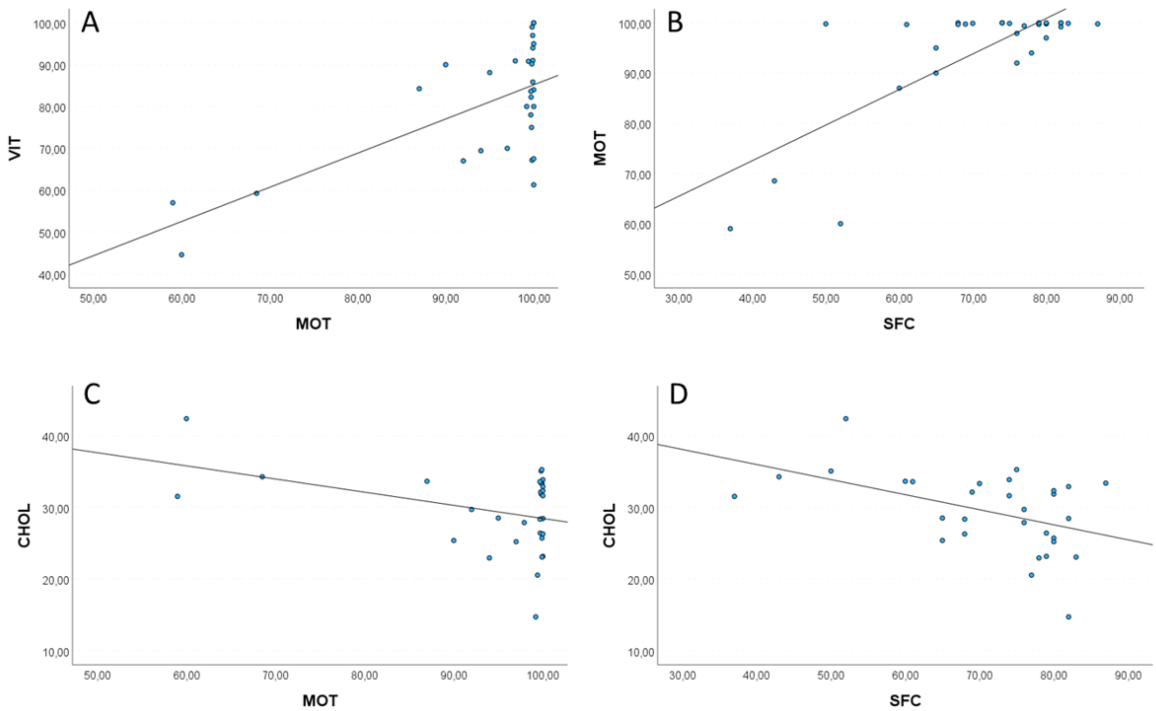

Figure 1. Linear correlation test between: A) sperm vitality percentage (VIT), and individual motility percentage (MOT); B) individual motility percentage (MOT) and sperm functional competence (SFC %); C) cholesterol quantity (CHOL; nmol/50 x 106) and individual motility percentage (MOT); and D) cholesterol quantity (CHOL; nmol/50 x 106) and sperm functional competence (SFC; %).

Discussion

Sperm quality is often the first evaluation and serves as a fundamental assessment of the reproductive status in bulls. Notably, Chino-Santandereano's semen displays greater sperm motility and viability compared to other creole breeds through several locations in the low Colombian tropics (Rueda et al., 2013; Fernández et al., 2022). These values are higher than those reported in *Bos indicus* semen (Brito et al., 2002; Leite et al., 2021). In addition, the Chino-Santandereano semen exhibited

significantly elevated levels of SFC. This test, rooted in hypotonic resistance and functional competence, discerns a sperm subpopulation possessing optimal functional traits, indicative of a heightened potential to fertilize the oocyte (Valencia et al., 2019).

Regarding correlation, the Pearson´s test evidenced a strong association between sperm vitality, motility, and SFC parameters. This is expected, given that functional competence heavily relies on sperm movement and membrane integrity. In fact, the correlation reveals that approximately 76% of SFC behavior can be explained by individual motility values. However, it is widely acknowledged that these quality parameters do not solely determine sperm fertilizing ability. As numerous molecules and interactions are involved, the sperm quality assessment could be followed by additional measurements in sperm cells and seminal plasma. In this sense, cholesterol levels in sperm cells have been proposed as an indirect marker of sperm functionality, freezability, and fertility (Shan et al., 2021). Moreover, experiments involving incubation of sperm cells with oviductal fluid revealed that cholesterol losses of approximately 25% occur in the sperm membrane (Díaz et al., 2019). The cholesterol levels detected in the present study were approximately ten-fold higher compared to Parks et al. (1987) (~ 2.4 nmol/50 x 10^6 spz), ranging between 20.55 and 42.37 nmol/50 x 10^6 spz. Nevertheless, this discrepancy might stem from variations in the cholesterol measurement technique. The values in our study closely resemble those by Girouard et al. (2008) - approximately 30 nmol/50 x 10^6 spz- using the same method.

Cholesterol in the sperm membrane has been closely linked to capacitation, a critical process for oocyte fertilization (Beer-Ljubić et al., 2009). During sperm capacitation, lipid reorganization occurs, and cholesterol leaves the membrane to promote the binding of proteins from seminal plasma and oviductal fluid (Witte and Sch, 2008). Additionally, cholesterol supplementation has shown positive effects in protecting sperm cells from damage during the freezing and thawing process (Maldjian et al., 2005).

Considering these findings, the correlation found in the present study evidences a negative relationship between cholesterol amounts in sperm cells and quality parameters ($P<0.001$). This suggests that high cholesterol in sperm membranes may result in low motility and SFC values, despite these values not being particularly low in fresh semen from Chino-Santandereano bulls (ranging between 87 -100 % and 65 -83 %, respectively). It is known that high SFC values increase hypotonic resistance in cells, indicating a greater ability to regulate cell volume and membrane fluidity, particularly in adapting to osmotic changes and progressive sperm motility during epididymal maturation (Yeung et al., 2004, 2006; Keber et al., 2013). The concurrent release of cholesterol from the membrane aligns with the surge in these sperm characteristics (Keber et al., 2013), potentially explaining the negative correlations observed in our study when assessing sperm quality immediately post-ejaculation. It is known that transit through the epididymis induces biochemical and functional transformations in spermatozoa, involving a decrease in cholesterol content and heightened membrane fluidity (Caballero et al., 2011; Keber et al., 2013). Cholesterol efflux in sperm plays a pivotal role in regulating fertilization potential through various membrane proteins (Travis and Kopf, 2002).

Hypotonic resistance, crucial for countering osmotic changes, is linked to sperm fertilization potential and relies on different channels and membrane transporters within regulatory volume decrease and regulatory volume increase systems (Petrunkina et al., 2007). Immediately after ejaculation, a shift from a hyperosmotic to an iso-osmotic environment occurs as sperm and seminal plasma mix during ejaculation, leading to an osmotic shock (Yeung et al., 2004; 2006). The low cholesterol content at this stage may influence hypotonic resistance percentages due to increased membrane fluidity, flexibility, and activation of membrane channels.

Our results align with the notion that cholesterol output (rather than cholesterol content) is crucial for sperm capacitation and, consequently, for key sperm competence parameters like movement and functional membrane integrity. Indeed, cholesterol output may be related to sperm membrane capability to interplay with the components of other fluids during the natural journey of sperm cells after ejaculation towards finding the oocyte. This dynamic process involves intricate interactions with seminal plasma and oviductal fluid as spermatozoa navigate towards its ultimate destination for fertilization. In this sense, these cholesterol losses are also associated with the composition of the oviductal fluid, which might facilitate the transfer of sperm cholesterol to high-density lipoproteins. The rebuilding of lipids in the sperm membrane also depends on exogenous factors, including the contents of seminal plasma and the microenvironment of the female tract (Sheriff and Ali, 2010). These complex interactions emphasize the significance of cholesterol dynamics in sperm functionality and warrant further research to understand its impact on fertility and sperm performance.

Conclusion

Despite the high relevance of an initial seminal quality evaluation, complementing it with its molecular determinants could prove valuable in refining male selection in siring or conservation programs. Cholesterol content in the sperm membrane may not be as useful as monitoring cholesterol loss from the sperm membrane. This can be valuable to complement the initial reproductive evaluation in bulls. Additional research is needed to assess the relationship between both subjects. Such studies hold the potential for advancing reproductive outcomes across diverse breeds. As we delve deeper into this subject, we can gain valuable insights into optimizing male selection strategies and bolstering overall breeding programs.

Declarations

Acknowledgments

The study was conducted with the valuable help of the team at the Animal Reproduction Laboratory of the Veterinary Medicine Faculty of UNIPAZ, and AGROSAVIA C.I Tibaitatá (agreement memorandum S275-2021).

Funding

This study was funded by Instituto Universitario de la Paz- UNIPAZ, Colombia.

Conflicts of interest

The authors declare that they have no known financial interest or personal relationships that could have influenced the work presented in this article.

Author contributions

NVD: conception and design, experimental development, writing and editing, critical revision of the manuscript. RTM: experimental development, data analysis. JEFR: experimental development, writing and editing. LCA: experimental development. JV: conception and design, data analysis, writing and editing, drafting, critical revision of the manuscript. FLRA: data analysis, writing and editing, drafting, critical revision of the manuscript.

Use of artificial intelligence (AI)

No AI or AI-assisted technologies were used during the preparation of this work.

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