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Kinetic and Morpho-Functional Analyses of Refrigerated and Cryopreserved Semen of 5/8 Girolando Bulls

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MSC, MALO and CCB developed the experimental protocol, conducted the analyses, and drafted the initial version of the manuscript. Authors SK, JCFS, IMLA, NABN, AFBS, LMM and VJFF conducted writing, review and editing. Author CCB supervised the study analysis, reviewed, and revised the final version of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

This study aimed to investigate the effects of refrigeration and cryopreservation on the sperm kinetic and morpho-functional parameters of 5/8 Girolando bulls. Eleven ejaculates from each bull were collected, diluted, and divided into two portions: one portion was subjected to refrigeration at 5ºC for 24 and 48 hours, while the second portion underwent cryopreservation(-196ºC). Post-thawing, various kinetic parameters were analyzed using the Computer Assisted Sperm Analysis (CASA) system. These parameters included total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness coefficient (STR), wobble coefficient (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Additionally, plasma membrane integrity (PMI), acrosomal membrane integrity (AMI), mitochondrial membrane potential (MMP), and chromatin condensation (CC) were examined for morpho-functional analyses. The results indicated that kinetic parameters (TM, PM, VCL, VSL, VAP) and morpho-functional analyses (PMI, MMP) differed significantly ($P < 0.05$) between cryopreserved and refrigerated semen, as well as other parameters such as LIN, ALH, and BCF across all groups. The acrosomal membrane integrity showed no differences, but CC varied significantly among all groups. In conclusion, semen from 5/8 Girolando bulls can be preserved for up to 48 hours at 5ºC, providing an alternative for the short-term use of genetic material from this breed.

Keywords: Bovine; CASA; crossbred; frozen spermatozoa; zebu semen.

1. INTRODUCTION

Artificial insemination (AI) has become a standard practice in livestock breeding, enabling rapid genetic improvement (Baruselli et al., 2018). Semen quality plays a significant role in the success of reproductive biotechnologies, such as AI and *in vitro* embryo production (Almeida et al., 2021: Vale et al., 2025). As the bull contributes half of the genetic material to its offspring, the quality of its semen directly impacts reproductive success and productivity. Preserving semen through refrigeration and cryopreservation is essential for ensuring its viability and enabling long-distance transport or storage (Bryła et al., 2024). However, both methods have distinct challenges. Cryopreservation, while allowing long-term storage, often causes cryoinjury, which impairs sperm motility, viability, and overall fertilizing potential (Sharafi et al., 2022). Refrigerated semen, stored at 4°C, causes less damage and has shown improved pregnancy rates compared to frozen semen, particularly in short-term applications. Despite these advantages, refrigerated semen has a limited storage life, making its timely use essential (Almeida et al., 2021).

The Girolando breed, a cross between Holstein and Gyr cattle (Indian Zebu), is increasingly recognized for its high milk production and adaptability to tropical environments (Vieira et al., 2022). Representing a significant portion of Brazil’s milk production, the 5/8 Girolando breed is a key player in the dairy industry (Silva et al., 2015). The demand for this crossbred Girolando is increasing worldwide due to its better performance (Alkoiret et al., 2011; Daltro et al., 2021). However, limited research exists on the reproductive aspects of this breed, especially regarding the impact of semen preservation techniques on breeding outcomes.

This study aims to investigate the effects of refrigeration and cryopreservation on the sperm kinetic and morpho-functional parameters of 5/8 Girolando bulls. By comparing these preservation methods, we can improve the reproductive efficiency of this crossbred breed by improving fertility outcomes, enabling better genetic selection, and contributing to the sustainable development of the dairy and beef industries.

2. MATERIALS AND METHODS

A schematic experimental design is presented in Fig. 1.

2.1 Location and Bulls

The study took place at the Institute of Agronomy in Pernambuco, Brazil, latitude 08º25'08" south and longitude 37º03'14" west. Two fertility-proven bulls aged 3 to 4 years were used. The bulls were maintained in a rotational grazing system, providing additional cane silage with ad libitum access to water and mineral salt.

2.2 Semen Collection, Refrigeration or Cryopreservation and Grouping

Despite proven fertility in the natural covering system, the bulls had previously undergone andrological examinations. They were trained to collect semen via the artificial vagina once a week for two months before the start of the experiment. During the experiment, semen was collected weekly in the morning (5:30 AM), following two false mounts. Eleven ejaculates from each bull (more than 70% progressive motility and 3 of vigour) were collected and diluted in Botu-bov® (SKU:30503, Botufarma Ltda, Botucatu, São Paulo, Brazil) to obtain a final concentration of 25 x 10^6. Afterwards, the semen was packaged in 0.25 mL French straws and further divided into three groups: refrigerated (RS-24 and RS-48) and cryopreserved (CS). The straws were stored at 5ºC (cooling rate of -1.4ºC/min) for subsequent analysis at 24 and 48 hours. Another portion was cryopreserved using an automated system (Cryogen®, Neovet, Brazil; curve of -5ºC/min).

2.3 Kinetics Analysis

Semen straws were thawed in a 37°C water bath, and a 5µl sample was analyzed using Sperm Class Analyzer-SCA-TM software v. 5.1, Microptics, SL Barcelona, Spain (equipped with phase contrast microscope). A minimum of 400 spermatozoa from five random non-consecutive fields were analyzed. Parameters assessed included total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness coefficient (STR), wobble coefficient (WOB), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), plasma membrane integrity (PMI), acrosomal membrane integrity (AMI), mitochondrial membrane potential (MMP), chromatin condensation (CC).

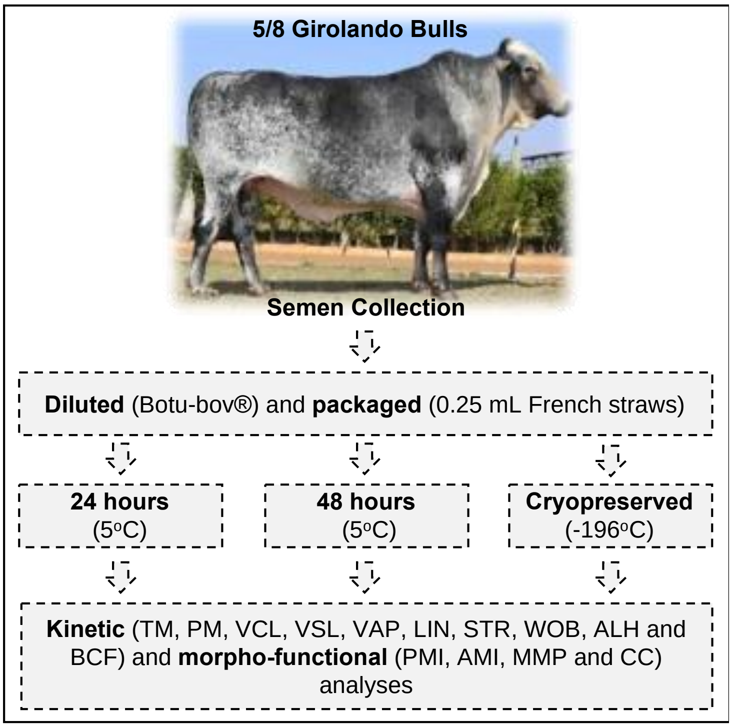

Fig. 1. Schematic presentation of experimental design

Total motility (TM); progressive motility (PM); curvilinear velocity (VCL); straight line velocity (VSL); average path velocity (VAP); linearity (LIN); straightness coefficient (STR); wobble coefficient (WOB); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); plasma membrane integrity (PMI); acrosomal membrane integrity (AMI); mitochondrial membrane potential (MMP) and chromatin condensation (CC)

2.4 Morpho-Functional Analyses

2.4.1 Plasma membrane integrity (PMI)

The PMI of sperm cells was assessed by staining with carboxyfluorescein diacetate (0.46 mg / mL in DMSO) and propidium iodide (0.5 mg / mL in PBS) using an epifluorescence microscope (Carl Zeiss) as per Silva et al. (2013). 200 spermatozoa were assessed at 400× magnification with specific emission (580-630 nm DBP) and (excitation 485-520 nm DBP) filters. Spermatozoa were classified as intact (green) or with damaged membranes (red).

2.4.2 Acrosomal membrane integrity (AMI)

Spermatozoa were stained using fluorescein isothiocyanate-peanut agglutinin (FITC-PNA) following Câmara et al. (2011). Briefly, semen samples (10 µL from each group) were smeared and air-dried. A 30 µL FITC-PNA (1 mg/mL FITC-PNA diluted in 900 µL PBS) was added to the smears and incubated for 20 minutes in a humid chamber at 4 °C, protected from light. After immersion in PBS and drying in the dark, 5 µL of mounting medium (4.5 mL glycerol, 0.5 mL PBS, 5 mg p-phenylenediamine) was placed and covered with a coverslip. Two hundred spermatozoa were examined at 1000× magnification using an epifluorescence microscope, classifying sperms as having an intact acrosome if stained fluorescent green, and reacted if the equatorial region showed a green band or no green at all.

2.4.3 Mitochondrial membrane potential (MMP)

MMP was assessed using the lipophilic cationic fluorochrome JC-1 (Silva et al., 2012). Briefly, 30 µl of semen was incubated with 5 µl of JC-1 (0.15 mM in DMSO) at 37ºC for 10 min. Two hundred spermatozoa were analyzed under a 1000x epifluorescence microscope at 400x magnification. An emission filter of 515 nm (long-pass) and an excitation filter of 450-490 nm (band-pass) were employed. Sperm with orange-stained midpieces indicated a high MMP, while green-stained midpieces indicated a low MMP.

2.4.4 Chromatin condensation (CC)

The CC assessment followed Hiraiwa et al. (2021). Briefly, Semen samples (10 µl per group) were prepared and air-dried. After, slides were immersed for 1 minute in a 3:1 ethanol and acetic acid solution, then for 3 minutes in 70% ethanol, and 25 minutes in 4M HCl. The slides were rinsed with distilled water and dried again. To observe CC, a drop of dye solution (0.025 g toluidine blue, 0.1 M citric acid, and 0.2 M Na_2HPO_4 in 1000 mL distilled water) was placed between the slide and coverslip. One hundred sperms were counted in duplicates using a brightfield microscope (1000X, Nikon Eclipse E200, Melville, USA). Sperm with dark blue to violet heads were classified as flawed, while those with light blue heads were considered normal.

2.5 Statistical Analysis

The percentage data was transformed into arcsine $\sqrt{P}$ / 100. The results were expressed as mean ± standard error of the mean. Further, the assumptions of normality (Shapiro-Wilk) and homoscedasticity (Lilliefors) were verified. ANOVA was performed, followed by Tukey's test, using SPSS version 16.0. A P-value of less than 0.05 was considered significant.

3. RESULTS

The variables of semen are presented in Tables 1 and 2. The kinetic parameters, including TM, PM, VCL, VSL, and VAP, showed significant differences ($P < 0.05$) between cryopreserved and refrigerated semen. Additionally, the LIN, ALH, and BCF parameters differed significantly ($P < 0.05$) across all groups (Table 1). In terms of morpho-functional analyses, PMI and MMP demonstrated significant differences ($P < 0.05$) between the cryopreserved and refrigerated semen groups, except for the integrity of the acrosomal membrane (Table 1). Furthermore, the CC also showed significant differences ($P < 0.05$) across all groups (Table 2).

4. DISCUSSION

The present study on "Kinetic and morpho-functional analyses of Refrigerated and Cryopreserved Semen of 5/8 Girolando Bulls" is significant as it evaluates the semen quality of bulls under refrigerated and cryopreserved conditions, crucial for improving artificial insemination success. Further, it helps identify optimal preservation methods for enhanced fertility outcomes. Kinetic and morpho-functional parameters of refrigerated (RS-24 and RS-48) and cryopreserved semen (CS) reveal significant

Table 1. Kinetic analyses of refrigerated (24 and 48 hours at 5ºC) and cryopreserved (-196ºC) semen of 5/8 Girolando bulls

Variables	Forms of conservation		
	RS-24	**RS-48**	**CS**
TM (%)	64.3±0.6[a]	64.3±0.8[a]	39.4±1.2[b]
PM (%)	49.6±0.7[a]	49.5±0.9[a]	29.0±0.9[b]
VCL (µm/s)	87.6±2.6[a]	88.6±1.6[a]	59.2±1.5[b]
VSL(µm/s)	52.0±1.8[a]	52.4±0.7[a]	40.4±1.6[b]
VAP (µm/s)	63.6±2.7[a]	65.6±1.6[a]	48.0±1.8[b]
LIN (%)	53.2±0.3[a]	49.6±0.7[b]	55.5±0.6[c]
STR (%)	65.5±0.4[ab]	64.5±0.7[a]	66.8±0.3[b]
WOB (%)	62.1±0.8[ab]	59.5±0.7[a]	63.8±0.9[b]
ALH (µm)	2.3±0.1[a]	2.9±0.1[b]	1.9±0.04[c]
BCF(Hz)	11.5±0.2[a]	12.6±0.3[b]	9.6±0.3[c]

** Different lower-case letters in the same line indicate statistical difference (P <0.05); semen refrigerated for 24 hours (RS-24); semen refrigerated for 48 hours (RS-48); cryopreserved semen (CS); total motility (TM); progressive motility (PM); curvilinear velocity (VCL); straight line velocity (VSL); average path velocity (VAP); linearity (LIN); straightness coefficient (STR); wobble coefficient (WOB); amplitude of lateral head displacement (ALH) and beat cross frequency (BCF)*

Table 2. Morpho-functional analyses of refrigerated (24 and 48 hours at 5ºC) and cryopreserved (-196ºC) semen of 5/8 Girolando bulls

Variables (%)	Forms of conservation		
	RS-24	**RS-48**	**CS**
PMI	53.6±1.1[a]	52.8±1.1[a]	35.7±0.9[b]
AMI	63.3±2.0[a]	64.9±1.8[a]	60.9±1.2[a]
MMP	58.7±1.4[a]	55.9±2.8[a]	39.0±3.7[b]
CC	80.7±0.2[a]	79.6±0.2[b]	78.8±0.2[c]

** Different lower-case letters in the same line indicate statistical difference (P <0.05); semen refrigerated for 24 hours (RS-24); semen refrigerated for 48 hours (RS-48); cryopreserved semen (CS); plasma membrane integrity (PMI); acrosomal membrane integrity (AMI); mitochondrial membrane potential (MMP) and chromatin condensation (CC)*

differences among the conservation methods. Overall, refrigerated semen demonstrated superior motility and functional parameters compared to frozen semen. However, further studies involving molecular markers related to cryoinjuries could yield more conclusive results and identify additional areas for research.

Motility is a prerequisite for the sperm to reach and fertilize the oocyte, and it is used primarily to qualify the semen (Cojkic et al., 2017). Our results showed that cryopreservation alters the pattern of sperm motility compared to refrigeration. According to Ullah et al. (2019), this processing increases the generation of reactive oxygen species, which causes lipid peroxidation and subsequently diminishes the quality of sperm cells after thawing.

Previous studies have shown that kinetic parameters are indicative of the fertilization capability of sperm (Koray et al., 2015; Tanga et al., 2021). In this study, we observed significantly better values for VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF for refrigerated semen than cryopreserved semen. Earlier research reported that VCL and VAP had a higher correlation with *in vitro* embryo production. LIN and BCF provide better migration and penetration into the cervical mucus during the reproductive process *in vivo* (Cezar et al., 2020). Moreover, the values of VCL, VAP, and VSL indicate the penetrating ability of the spermatozoa into the ovum (Inanc et al., 2018), and higher values of other kinetic parameters indicate good fertile ability of bull (Belala et al., 2019). STR and WOB demonstrated similar values for both RS-24 and CS groups, suggesting these parameters are not affected by the conservation method.

A significant decline of PMI and MMP in the frozen semen group to refrigerated groups suggests that short-term storage can preserve sperm viability better than freezing. This observation aligns with the understanding that cryopreservation can cause severe osmotic and cryo-injurious effects. The spermatozoa with higher PMI are more capable of penetrating the zona pellucida and fusing with the ooplasm to fertilize the oocyte (Celeghini et al., 2010; Segabinazzi et al., 2024). The MMP value affects the energy production capabilities of spermatozoa and is closely related to sperm motility and overall fertility potential. The significant difference between the different groups for CC indicates where CC decreases as the duration of refrigeration increases or when semen is frozen. Hallam et al. (2024) observed that fragmented cromatins indicate dead sperm or low fertility. Corroborating with Anzar et al. (2002), that cryopreservation causes chromatin condensation failures. According to Fleming and Thomson, (2025) and Hassan et al. (2025), the products generated by sperm metabolism, among which reactive oxygen species, represent one of the main factors that lead to the inevitable reduction in sperm viability when subjected to low temperatures and that cause protein oxidation and damage to sperm DNA, impairing fertilization. Likewise, Yánez-Ortiz et al. (2022) found that physical injury caused by the formation of intracellular ice crystals leads to structural changes in chromatin.

5. CONCLUSION

The semen from 5/8 Girolando bulls can be preserved for up to 48 hours at 5ºC, providing an alternative for the short-term use of genetic material from this breed. Therefore, after collection and dilution, the semen can be transported at 5ºC from one farm to another or remote areas for artificial insemination or *in vitro* fertilization, thereby eliminating the need for cryopreservation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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