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Leukocyte profiles in bull semen and their relationship with sperm quality parameters

Perfis de leucócitos no sêmen de touros e sua relação com os parâmetros de qualidade espermática

Perfiles leucocitarios en semen de toro y su relación con parámetros de calidad espermática

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Abstract
The role of leukocytes in semen remains controversial, with both beneficial effects, such as the removal of microorganisms and abnormal sperm, and harmful effects, including reactive oxygen species (ROS) production and reduced fertility potential. Research on this topic in farm animals is limited. This study investigated correlations between differential leukocyte counts in bull semen and sperm parameters, both fresh and post-thaw. A total of 44 ejaculates from 36 adult bulls were collected using the artificial vagina method. Semen cytology was performed with Diff-Quick staining, and leukocytes were quantified under an optical microscope. Sperm parameters analyzed included ejaculate volume, motility (fresh and post-thaw), concentration, and morphology (major, minor, and total defects). Lymphocytes predominated (82%), followed by macrophages (13%), neutrophils (4%), and eosinophils (1%). Neutrophils showed weak negative correlations with major defects (r = -0.36, P = 0.027) and total defects (r = -0.328, P = 0.047), as well as a moderate negative correlation with post-thaw motility (r = -0.515, P = 0.0012) in Spearman's correlation analysis. In contrast, lymphocytes, macrophages, and eosinophils showed no significant correlations with sperm parameters. These findings suggest that among leukocyte populations, only neutrophils negatively impact sperm morphology in fresh semen and motility in thawed semen.
Keywords: Bovine; Post-thawing; Leukocytospermia; Neutrophils; Sperm-defect.

Resumo
O papel dos leucócitos no sêmen permanece controverso, com efeitos benéficos, como a remoção de microrganismos e espermatozoides anormais, e efeitos prejudiciais, incluindo produção de espécies reativas de oxigênio (ROS) e potencial de fertilidade reduzido. Estudos investigando o perfil leucocitário e sua correlação com os parâmetros seminais em bovinos são limitados. Este estudo objetivou avaliar o perfil leucocitário em sêmen de touros e sua correlação com os parâmetros espermáticos do sêmen fresco e descongelado. Um total de 44 ejaculados de 36 touros adultos foram coletados usando o método de vagina artificial. A citologia seminal foi realizada com coloração Diff-Quick, e os leucócitos foram quantificados em um microscópio óptico. Os parâmetros seminais analisados incluíram volume do ejaculado, motilidade (fresco e pós-descongelamento), concentração e morfologia (defeitos maiores, menores e totais). Os linfócitos predominaram (82%), seguidos por macrófagos (13%), neutrófilos (4%) e eosinófilos (1%). Os neutrófilos apresentaram correlações negativas fracas com defeitos maiores (r = -0,36, P = 0,027) e defeitos totais (r = -0,328, P = 0,047), bem como uma correlação negativa moderada com a motilidade pós-descongelamento (r = -0,515, P = 0,0012) na análise de correlação de Spearman. Em contraste, linfócitos, macrófagos e eosinófilos não

apresentaram correlações significativas com parâmetros espermáticos. Essas descobertas sugerem que, entre as populações de leucócitos, apenas os neutrófilos impactam negativamente a morfologia espermática no sêmen fresco e a motilidade no sêmen descongelado.
Palavras-chave: Bovino; Pós-descongelamento; Leucocitospermia; Neutrófilos; Defeito espermático.

Resumen
El papel de los leucocitos en el semen sigue siendo controvertido, con efectos beneficiosos, como la eliminación de microorganismos y espermatozoides anormales, y efectos perjudiciales, incluida la producción de especies reactivas de oxígeno (ROS) y la reducción del potencial de fertilidad. La investigación sobre este tema en animales de granja es limitada. Este estudio investigó las correlaciones entre los recuentos diferenciales de leucocitos en el semen de toro y los parámetros del esperma, tanto fresco como post-descongelado. Se recogieron un total de 44 eyaculados de 36 toros adultos utilizando el método de vagina artificial. La citología del semen se realizó con tinción Diff-Quick y los leucocitos se cuantificaron bajo un microscopio óptico. Los parámetros del esperma analizados incluyeron el volumen del eyaculado, la motilidad (fresco y post-descongelado), la concentración y la morfología (defectos mayores, menores y totales). Predominaron los linfocitos (82%), seguidos de los macrófagos (13%), los neutrófilos (4%) y los eosinófilos (1%). Los neutrófilos mostraron correlaciones negativas débiles con los defectos mayores (r = -0,36, P = 0,027) y los defectos totales (r = -0,328, P = 0,047), así como una correlación negativa moderada con la motilidad posterior a la descongelación (r = -0,515, P = 0,0012) en el análisis de correlación de Spearman. Por el contrario, los linfocitos, macrófagos y eosinófilos no mostraron correlaciones significativas con los parámetros espermáticos. Estos hallazgos sugieren que, entre las poblaciones de leucocitos, solo los neutrófilos afectan negativamente la morfología espermática en el semen fresco y la motilidad en el semen descongelado.
Palabras clave: Bovino; Post-descongelación; Leucocitospermia; Neutrófilos; Defecto en los espermatozoides.

1. Introduction

Evaluating bull reproductive efficiency is essential for the success of breeding and genetic improvement programs, as bulls contribute to over 90% of the herd's genetic makeup (Menegassi et al., 2011). Consequently, semen quality is a critical determinant of reproductive success in both natural breeding (Chenoweth & McPherson, 2016) and artificial insemination programs (Al Makhzoomi et al., 2008).

The role of leukocytes in semen and their association with bull fertility remains a topic of debate. Leukocytes can have both beneficial effects, such as contributing to microorganism clearance, removing defective sperm, and enhancing fertilization capacity (Kiessling et al., 1995; Omu et al., 1999; Saleh et al., 2002; Menkveld, 2004), and detrimental impacts, including reduced sperm concentration and motility, increased morphological defects, and the induction of oxidative stress (Tomlinson et al., 1992; Omu et al., 1999; Arata de Bellabarba et al., 2000; Li et al., 2020).

The predominant leukocyte type in semen also plays a significant role in fertility outcomes. In humans, polymorphonuclear leukocytes, particularly neutrophils, are the most abundant, followed by macrophages and lymphocytes (WHO, 1999; Stanislavov, 1999; Saleh et al., 2002). Similarly, in bulls, neutrophils are the predominant leukocyte type, followed by lymphocytes and macrophages (Zart et al., 2014; Ferrer et al., 2024). However, studies investigating the phenotypic profile of bull seminal leukocytes and their correlation with sperm parameters in fresh (Sprecher et al., 1999; Zart et al., 2014; Ferrer et al., 2024) and especially thawed semen remain limited in veterinary science.

To address this gap, the present study aimed to evaluate differential leukocyte counts in bull semen and their correlation with fresh and thawed seminal parameters.

2. Methodology

2.1 Animals and semen collection

For this study, experimental, laboratory research was carried out, of a quantitative nature (Pereira et al., 2018). A total of 44 ejaculates were obtained from 36 healthy adult bulls (3–6 years old) of different breeds at a Semen Processing Center (Central Bela Vista, CRV Group®, Botucatu, SP, Brazil). Semen collection was performed routinely at the Semen Processing

Center using an artificial vagina heated to 40–42°C. Before collection, the foreskin was washed and dried, and semen was collected in sterile 15 mL tubes preheated to 37°C.

2.2 Semen analysis

Fresh semen was evaluated for total motility (subjective analysis) by diluting samples in OptiXcell® extender (IMV Technologies) at 37°C, followed by observation under an optical microscope at 10x magnification. Sperm morphology was assessed in formalin-fixed samples using differential interference contrast microscopy, counting 200 cells per sample at 100x magnification (Olympus Life Science®, Tokyo, Japan), and categorizing defects as major or minor (Bloom, 1973). Sperm concentration was determined by diluting 40 µL aliquots of each ejaculate in microcuvettes containing 960 µL of ultrapure water. The diluted samples were analyzed using a portable photometer (AccuRead®, IMV Technologies, L'Aigle, France), following the methodology described by Silva et al. (2022).

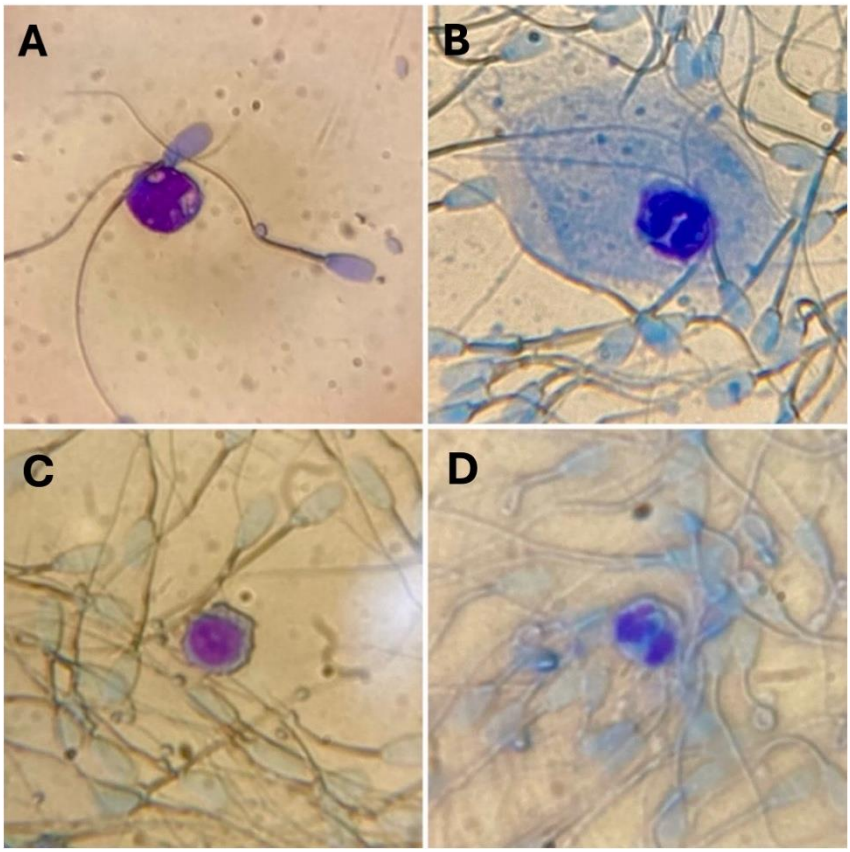
2.3 Post-thawing evaluation

Semen cryopreservation was performed according to the protocol described by Gürler et al. (2016). Straws were thawed at 37°C for 30 seconds, and motility was assessed using a computer-assisted sperm analysis system (IVOS® II, Hamilton Thorne).

2.4 Determination of leukocyte morphology

Smears were prepared from aliquots of semen on glass slides, which were air-dried and stained using Diff-Quick staining (NewProv®, Paraná, Brazil). Neutrophil, lymphocyte, macrophage, and eosinophil counts for leukocyte profile were conducted by identifying ten leukocytes per slide under a light microscope at 400x magnification, with additional confirmation performed at 1,000x magnification (Figure 1).

Figure 1 - Differential count of leukocytes observed under an optical microscope in bovine semen (1,000X magnification) show as (A) macrophage; (B) neutrophil; (C) lymphocyte; (D) eosinophil.



Source: Personal archive.

2.5 Statistical analysis

The results obtained were supported by descriptive statistics (Shitsuka et al., 2018) and statistical analysis (Vieira, 2021), were analyzed using Sigma Plot for Windows software, version 11.0. Since the data did not pass the Kolmogorov-Smirnov normality test, Spearman's correlation test was applied. The strength of the correlation was classified as very weak (0–0.19), weak (0.2–0.39), moderate (0.40–0.59), strong (0.6–0.79), and very strong (0.8–1), according to Doria-Filho (1999). Correlations with $P < 0.05$ were considered statistically significant.

3. Results and Discussion

Semen smears revealed a predominance of lymphocytes (82%), followed by macrophages (13%), neutrophils (4%), and scarce eosinophils (1%) (Figure 1). These findings differ from previous reports in bulls, where neutrophils were identified as the most common leukocyte type, followed by lymphocytes and macrophages (Zart et al., 2014; Ferrer et al., 2024). In humans, polymorphonuclear neutrophils (PMNs) account for 50–60% of seminal leukocytes, followed by macrophages (20–30%) and T lymphocytes (2–5%) (Aitken et al., 1995; Tremellen, 2008).

Sperm morphology plays a critical role in fertilization success and early embryonic development (Enciso et al., 2011). Morphological abnormalities in sperm are negatively correlated with fertility rates (Söderquist et al., 1991). The presence of leukocytes in semen has been associated with both beneficial and detrimental effects on sperm parameters. While leukocyte phenotypes can enhance fertilization potential by eliminating defective sperm through phagocytosis (Tomlinson et al., 1992;

Kiessling et al., 1995), they are also known to negatively affect sperm concentration, motility (Kortebani et al., 1992; Arata de Bellabarba et al., 2000; Tortolero et al., 2004; Arjadi et al., 2022), and morphology (Tomlinson et al., 1992; Thomas et al., 1997; Aziz et al., 2004), primarily due to the excessive production of reactive oxygen species (ROS) (Omu et al., 1999; Saleh, 2002; Henkel et al., 2005; Li et al., 2020).

In this study, neutrophils exhibited a weak but significant negative correlation with major defects (r = -0.36, P = 0.027) and total defects (r = -0.328, P = 0.047), suggesting their potential role in increasing sperm abnormalities (Table 1). Interestingly, lymphocytes, macrophages and eosinophils showed no significant correlation with sperm parameters in this study (Table 1), suggesting their minimal impact on semen quality.

Table 1 - The Spearman rank-order correlation coefficients of leukocyte phenotypes and seminal parameters.

Leukocyte phenotype	Major defects	Minor defects	Total abnormalities	Volume	Sperm concentration	Fresh motility	Thawed motility
EO	0.19	0.05	0.18	0.01	0.02	0.12	0.11
LYMPH	0.13	-0.08	0.13	-0.22	-0.05	-0.21	0.18
Mϕ	-0.15	0.11	- 0.14	0.13	0.07	0.24	- 0.05
NEUTR	-0.36*	0.17	- 0.33*	0.23	-0.26	0.02	-0.52*

EO: eosinophils; LYMPH: lymphocytes; Mϕ: macrophages; NEUTR: neutrophiles. * Correlation is significant, P < 0.05.
Source: Personal archive.

Neutrophils are recognized as the predominant producers of ROS among leukocytes (Saleh et al., 2002) and have been linked to reduced sperm motility across various species, including beef bulls (Ferrer et al., 2024), stallions (Baumber et al., 2002; Ferrer et al., 2023), and humans (Kovalski et al., 1992; Plante et al., 1994; Castellini et al., 2020). These studies have demonstrated that incubating sperm with activated neutrophils significantly reduces motility, with the effect becoming more pronounced at higher PMN concentrations. Consistent with these findings, this study identified a moderate negative correlation (r = -0.515, P = 0.0012) between neutrophils and post-thaw motility (Table 1), supporting the hypothesis that neutrophils contribute to oxidative stress during cryopreservation through excessive ROS production.

Oxidative stress is a key factor compromising semen quality during and after cryopreservation (Tremellen, 2008; Kumar et al., 2020), largely due to excessive ROS production. Neutrophils generate significantly higher ROS levels than spermatozoa themselves (Plante et al., 1994; Henkel et al., 2005; Li et al., 2020). During cryopreservation, oxidative damage intensifies during cooling and thawing phases, particularly in samples with higher leukocyte concentrations (Wang et al., 1997). ROS produced by neutrophils can overwhelm the antioxidant defenses of seminal plasma, inducing lipid peroxidation in sperm membranes, impairing motility, and reducing fertilization potential (Henkel et al., 2005; Aitken et al., 2022). Additionally, oxidative imbalance may activate caspases in germ cells, exacerbating cellular damage and further compromising sperm quality (Mupfiga et al., 2013). These findings highlight the importance of mitigating oxidative stress through strategies such as leukocyte removal or antioxidant supplementation, which have been shown to improve sperm viability and functionality after cryopreservation.

4. Conclusion

In conclusion, this study indicates a predominance of lymphocytes, macrophages, neutrophils and scarce eosinophils. Lymphocytes, macrophages and eosinophils did not demonstrate a significant impact on seminal parameters. Neutrophils are suggested to be the main type of leukocyte responsible for the adverse effects observed on sperm morphology in fresh semen

and on motility in thawed semen and may impact the success of reproductive biotechnology techniques involving cryopreservation.

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