Sperm Quality in Cryopreserved Holstein Friesian Bull Semen and Bacterial Load as Affected by Different Extenders

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Abstract. This study compared the effects of two different types of extenders, tris aminomethane egg yolk and modified trico egg yolk, on sperm quality after thawing and bacterial load in cryopreserved semen from a Holstein Friesian bull. Semen samples were divided into two treatment groups, each consisting of five semen tubes (0.25 ml), which were frozen using either Tris aminomethane egg yolk extender or modified trico egg yolk extender. Post-thaw evaluation included sperm motility, viability, abnormalities, membrane and acrosome integrity, capacitation status, DNA fragmentation, sperm kinematics, and bacterial load. Data were analyzed using the Wilcoxon test, while bacterial content was analyzed descriptively. The results showed that Tris aminomethane extender had higher sperm quality parameters after thawing compared to Trico egg yolk extender. Specifically, Tris aminomethane is higher than trico egg yolk in motility (48.20%), viability (68.10%), intact acrosome integrity (70.36%), and membrane integrity (67.92%). Additionally, significantly higher values were observed in parameters related to sperm kinematics, including Curvilinear Distance (DCL, 62.22%), Curvilinear Velocity (VCL, 155.06%), and Amplitude of Lateral Head (ALH, 8.97%). Meanwhile, the trico egg yolk extender caused significantly higher values in sperm straightness (STR, 84.02%), linearity (LIN, 56.22%), and wobble (WOB, 65.40%). The content of E. coli and Mycoplasma bacteria was higher in the trico extender, while Staphylococcus was more dominant in the tris aminomethane extender. These findings indicate that both extenders can maintain the quality of frozen sperm. The results of this study indicate that the Trico egg yolk extender is a reasonable and cost-effective alternative, with comparable performance in several sperm quality parameters; however, the effectiveness of bacterial inhibition needs to be reevaluated in relation to the antibiotic dosage used.

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Keywords: Bacterial contamination, Cryopreservation, Sperm quality, Tris aminomethane extender, Trico extender.

1 Introduction

Artificial insemination (AI) remains the mainstay of genetic improvement and reproductive efficiency in dairy cattle breeding, especially in high-yielding breeds such as Holstein Friesian (HF). The effectiveness of AI programs depends on the quality of the frozen-thawed semen used, as the cryopreservation process can significantly affect sperm viability and fertilization potential. Cryogenic injury during freezing and thawing often reduces sperm motility, viability, membrane integrity, and increased DNA fragmentation, thereby reducing conception rates [1]. To minimize such damage, selecting sperm extenders is critical in maintaining sperm function during cryopreservation.

Among the extenders used in bovine semen processing, Tris-based extenders have proven effective due to their buffering properties, compatibility with cryoprotectants, and ability to maintain sperm quality after thawing [2]. Recently, a modified extender called Trico (Tris-Egg Yolk modified extender) was developed as a more economical alternative to commercial extenders, aiming to enhance the cryogenic protection of sperm. The development of Trico egg yolk was driven by the need to reduce the production cost of AI doses while maintaining or improving sperm quality after thawing, particularly in dairy farming operations with limited resources. However, comparative studies evaluating the performance of this new extender compared to conventional Tris aminomethane egg yolk formulations remain limited.

In addition to maintaining sperm quality, another key consideration in semen cryopreservation is the bacterial load in the final product. Bacterial contamination of semen can occur during collection, handling, and processing [3]. High bacterial counts in semen impair sperm motility, reduce membrane integrity, and contribute to oxidative stress by producing reactive oxygen species (ROS) and endotoxins [4]. Ultimately, this can negatively impact fertilization success in artificial insemination (AI) programs. While many commercial extenders contain antibiotics to control microbial growth, the effectiveness of bacterial suppression can vary depending on the extender composition and processing protocol [5]. Since bacterial contamination is typically evaluated in post-thaw semen, the stage directly used in AI, understanding the interaction between extender type and bacterial load remains an important research priority.

Therefore, the objective of this study was to evaluate the effects of two types of extenders conventional Tris-based extenders and modified Trico extenders on sperm quality in fresh and post-thawed semen from Holstein Friesian bulls and assess bacterial load in semen. This research aims to provide new insights into the potential of modified and cost-effective extenders in maintaining sperm quality and reducing bacterial contamination, thereby supporting more efficient and affordable AI practices in the dairy industry

2 Materials and methods

2.1 Study bulls and semen

This study utilized fresh and frozen semen samples collected from a Holstein Friesian bull (DM Burns; ID: 319120), maintained at Singosari National Artificial Insemination Center (SNAIC). The bull had passed the Breeding Bull Soundness Evaluation (BBSE) and was managed under standardized bull management protocols. A total of 10 frozen semen straws

(0.25 ml each) were used in this study. These straws were divided equally into two treatment groups based on the type of extender used during the cryopreservation process

2.2 Study design and methodology

This study applied a laboratory-based experimental design using a paired comparison approach to evaluate the influence of two types of extenders on the post-thaw sperm quality and bacterial load in cryopreserved semen from a Holstein Friesian bull. The treatments included T1: semen was cryopreserved using a Tris aminomethane egg yolk extender, and T2: semen was cryopreserved using a Trico egg yolk extender. Both extenders were formulated and provided by the SNAIC. Following cryopreservation, all semen straws were stored in liquid nitrogen and thawed for post-thaw evaluation. The paired design minimized biological variability and allowed for a direct comparison of the effects of each extender on semen quality and bacterial contamination.

2.3 Parameters observed

Sperm Quality. *Sperm Motility and Viability*: Post-thaw sperm motility was examined under a microscope. *Viability* and *Abnormality* sperm evaluated using smear technique were differentiated using eosin-nigrosin staining [6] *Membrane Integrity*: Assessed using the hypo-osmotic swelling test. *Intact Acrosome*: The proportion of sperm with intact acrosomal caps was evaluated using physiological saline solution and formalin, and observed under a phase-contrast microscope. *Status of sperm capacitation*: evaluated using chlortetracycline (CTC) fluorescence staining, CTC staining was used to differentiate sperm into three categories: F-pattern (non-capacitated), B-pattern (capacitated), and AR-pattern (acrosome-reacted), following the method described by [7]. *DNA fragmentation (Toluidine Blue):* The degree of chromatin fragmentation was assessed using toluidine blue staining. *DNA Fragmentation (Halomax®*): Sperm DNA fragmentation was further evaluated using the Halomax® kit according to the manufacturer's instructions [8]. *Sperm kinematics (Computerized Assisted Sperm Analysis)*: Parameters observed using CASA include total motile, progressive, straight line distance (DSL, μm), average path distance (DAP, μm), curvilinear distance (DCL, μm), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), lateral head amplitude (ALH, μm), beat cross frequency (BCF, Hz). *Bacterial Load*: Total bacterial count was performed on thawed semen samples by culturing on nutrient agar under sterile conditions and incubating at 37°C for 24 hours [9].

Bacterial Count. Frozen semen straws were retrieved from liquid nitrogen storage and thawed in a water bath at 37°C for 30 s. For bacterial analysis, all equipment and materials were sterilized. Semen samples were serially diluted in peptone water (10^{-1}, 10^{-2}, 10^{-3}). One milliliter from 10^{-2} and 10^{-3} dilutions was plated on sterile Petri dishes using the pour plate method with 10–15 mL of agar media. Plates were gently mixed to prevent uneven colony distribution, allowed to solidify, and incubated at 37°C for at least 24 h. Bacterial colonies were counted and expressed as colony-forming units per milliliter [9].

2.4 Statistical model and analysis

All data were analyzed using R Studio (version 4.4.3). Data normality was first assessed using the Shapiro–Wilk test. Considering the limited sample size, sperm quality parameters were analyzed using the Wilcoxon signed-rank test to minimize statistical bias and improve the validity of the analysis. Data are presented as mean ± standard deviation (SD), with statistical significance set at $p < 0.05$. Parameters with p-values between 0.01 and 0.05 were

considered to indicate a statistical trend. The bacterial load in semen was analyzed descriptively.

3 Results and discussion

The present study evaluated the semen quality and bacterial load of Holstein Friesian bull semen, both in a fresh state and after cryopreservation with two different extenders. The results are presented below, covering macroscopic and microscopic characteristics of fresh ejaculates, followed by post-thaw sperm quality parameters and bacterial contamination levels. Fresh ejaculates of Holstein Friesian bull exhibited acceptable macroscopic and microscopic characteristics. The semen volume averaged 7.00 mL with a milky-white appearance, a characteristic odor, and a thick consistency. The pH was 6.60, within the physiological range for bovine semen. Microscopic evaluation showed a mass motility score of 3+, with an individual progressive motility of 65.00% and viability of 77.62%. The proportion of abnormal sperm was low (8.40%). Sperm concentration was 1,808 × 10^6/mL, resulting in total motile sperm of 8,226.40 × 10^6. The membrane integrity was high, with hypo-osmotic swelling (HOS) test results of 73.50% and membrane integrity of 76.92%. DNA integrity was sufficient, with toluidine blue staining showing 8.46% fragmentation and Halomax® assay indicating 9.95% fragmented sperm. Capacitation status, as assessed by chlortetracycline (CTC) staining, showed 71.50% non-capacitated, 12.62% acrosome-reacted (AR), and 15.89% capacitated sperm.

These initial semen characteristics indicate that the samples met the standards required for cryopreservation. Following freezing and thawing, comparative evaluations were performed to assess the effects of two extenders, Tris aminomethane egg yolk and Trico egg yolk on post-thaw sperm quality and bacterial load. The post-thaw sperm quality and bacterial load of Holstein Friesian bull semen extended with Tris and Trico extenders are summarized in Table 1.

Table 1. Post-thaw sperm quality and bacterial load of Holstein Friesian bull semen extended with Tris aminomethane and Trico egg yolk extenders (Mean ± SD).

Parameter	T1 (Mean ± SD)	T2 (Mean ± SD)	P-value	Conclusion
Sperm Quality Parameters				
Motility (%)	48.20 ± 1.79	44.20 ± 1.92	0.027	Significant
Viability (%)	68.10 ± 1.26	65.22 ± 1.85	0.037	Significant
Abnormality (%)	5.72 ± 0.91	5.63 ± 0.88	1.000	Not significant
Concentration (10^6/straw)	27.06 ± 1.47	28.06 ± 1.09	0.295	Not significant
Total Motile Sperm (10^6/straw)	13.06 ± 1.14	12.40 ± 0.54	0.403	Not significant
Intact Acrosome (%)	67.93 ± 1.00	65.10 ± 1.98	0.037	Significant
Membrane Integrity (%)	67.92 ± 1.49	65.35 ± 1.02	0.037	Significant
CTC - F pattern (%)	70.36 ± 0.39	68.24 ± 1.35	0.125	Not Significant
CTC - AR (%)	11.39 ± 3.02	14.37 ± 2.68	0.1875	Not significant
CTC – B pattern (%)	18.24 ± 3.02	17.40 ± 2.04	0.625	Not significant
DNA Fragmentation (Toluidine Blue, %)	11.97 ± 1.45	12.71 ± 0.95	0.625	Not significant
DNA Fragmentation (Halomax, %)	11.18 ± 2.16	13.64 ± 0.50	0.250	Not significant
Sperm Kinematic Parameters				
Motile (%)	76.94 ± 6.07	74.70 ± 2.97	1.000	Not significant
Progressive (%)	46.66 ± 1.02	47.12 ± 1.88	0.284	Not significant
DAP (µm)	33.38 ± 1.44	30.07 ± 2.60	0.054	Not Significant (trend)
DSL (µm)	25.26 ± 1.13	25.29 ± 1.46	0.830	Not significant
DCL (µm)	62.22 ± 2.77	47.77 ± 6.45	0.010	Significant
VAP (µm/s)	84.31 ± 5.26	75.53 ± 4.28	0.054	Not significant
VSL (µm/s)	63.95 ± 2.07	63.51 ± 2.62	0.915	Not significant
VCL (µm/s)	155.06 ± 10.34	119.25 ± 15.54	0.010	Significant
STR (%)	77.00 ± 2.24	84.02 ± 4.05	0.087	Significant
LIN (%)	43.94 ± 2.46	56.22 ± 5.50	0.010	Significant
ALH (µm)	8.97 ± 0.92	6.36 ± 1.23	0.009	Highly Significant
BCF (Hz)	20.45 ± 1.55	23.66 ± 3.07	0.392	Not significant
WOB (%)	56.08 ± 1.11	65.40 ± 4.40	0.010	Significant
Bacterial Load (10^3 CFU/ml)				
E. coli spp.	0.7 ± 1.57	50.1 ± 101.96		
Mycoplasma spp.	92.5 ± 117.64	203.3 ± 94.86		
Staphylococcus spp.	47.1 ± 58.58	38.0 ± 26.66		
Pseudomonas spp.	0	0		

T1: semen was cryopreserved using a Tris aminomethane egg yolk extender; T2: semen was cryopreserved using a Trico egg yolk extender; CTC-F-pattern: non-capacitated; CTC-B pattern: capacitated; CTC-AR pattern: acrosome-reacted; CFU/ml: colony-forming units per mililiter.

Among the post-thaw sperm parameters evaluated, the motility percentage (48.20 ± 1.79% vs 44.20 ± 1.92%) and viability (68.10 ± 1.26% vs 65.22 ± 1.85%) were significantly higher ($p < 0.05$) in the Tris aminomethane extender compared to the Trico egg yolk extender. These results are in line with previous reports expressing that the advantages of Tris aminomethane egg yolk extender are due to its buffering capacity, pH stabilization, and the availability of energy substrates (lactose, raffinose, fructose) that support mitochondrial ATP production and maintain plasma membrane fluidity during cryopreservation [2, 10]. Similarly, the percentage of intact acrosomes (67.93 ± 1.00% vs 65.10 ± 1.98%) and membrane integrity (67.92 ± 1.49% vs 65.35 ± 1.02%) was significantly higher in the Tris aminomethane extender compared to the Trico egg yolk extender ($p < 0.05$). The results of this study indicate that Tris aminomethane extender provides better protection of the acrosome structure and helps maintain spermatozoa plasma membrane function and flagellar activity during cryopreservation.

The high buffering capacity of Tris aminomethane egg yolk extender stabilizes intracellular pH, thereby minimizing acidosis that can damage mitochondrial function and ATP production, both of which are crucial for maintaining motility. In addition, the presence of various energy sources (lactose, raffinose, and fructose) in the Tris diluent allows for a more sustainable supply of metabolic substrates during the freezing and thawing phases, thereby further supporting flagellar movement. Furthermore, membrane integrity is closely related to sperm resistance to cold shock and osmotic stress; Tris aminomethane egg yolk extender is protective in maintaining membrane plasma lipid and preventing phase transitions during cryopreservation, probably contributing to the trend of higher membrane integrity. Conversely, sperm abnormality, concentration, total motile sperm count, and DNA fragmentation (Toluidine Blue and Halomax® method) did not show significant differences. This indicates that both diluents are equally capable of maintaining chromatin stability and preventing major morphological damage. Figure 1. shows the viability of post-thaw sperm of Holstein Friesian bull semen extended with Tris aminomethane egg yolk and Trico egg yolk extenders.

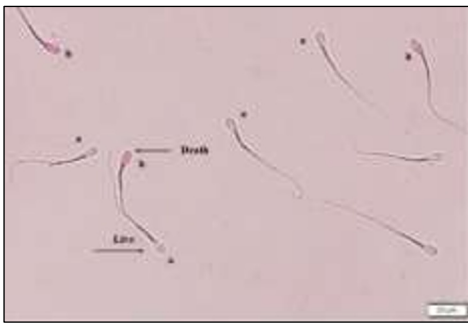

Figure 1. Viability of post-thaw sperm of Holstein Friesian bull.

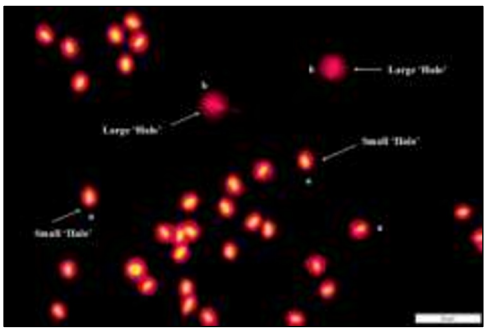

Figure 2. DNA Fragmentation of post-thaw sperm with Halomax®.

Analysis of capacitation status using chlortetracycline (CTC) staining showed that the distribution pattern of sperm capacitation did not differ significantly between extender groups. The results showed that the proportion of sperm with pattern F was slightly higher in the Tris aminomethane egg yolk group (70.36 ± 0.39%) compared to the Trico group (68.24 ± 1.35%). This trend indicates that Tris aminomethane extender is able to maintain sperm in a non-capacitated state for longer. This physiological condition is related to fertilization

readiness because sperm that are still in the non-capacitated stage have higher viability and higher fertilization capacity. Sperm will undergo the capacitation process after entering the female reproductive tract, where there is a suitable environment (changes in calcium ions, bicarbonate, and oviductal factors) to trigger the physiological changes necessary before fertilization. However, the difference in the proportion of pattern F (non-capacitated) is not statistically significant, meaning that the Trico egg yolk extender is also capable of preventing premature capacitation. Overall, these results show that both diluents are able to maintain the proportion of non-capacitated sperm, thereby allowing normal fertilization to occur.

The application of Tris aminomethane egg yolk extender was significantly higher in several parameters, including VCL (155.06 ± 10.34 μm/s vs. 119.25 ± 15.54 μm/s), DCL (62.22 ± 2.77 μm vs. 47.77 ± 6.45 μm), and ALH (8.97 ± 0.92 μm vs. 6.36 ± 1.23 μm) compared to the Trico egg yolk extender. These three parameters are the main indicators of hyperactive motility, which is characterized by asymmetrical flagellar movement with large amplitude and a crooked movement pattern. Hyperactive motility plays an important role in the final stage of fertilization, particularly in facilitating sperm penetration of the zona pellucida [11]. In addition, the increase in VAP (average path velocity) in Tris aminomethane egg yolk, although not significant ($p = 0.054$), reflects a higher cell energy status and is capable of supporting the continuity of progressive motility during the outing through the female reproductive tract. Hyperactivation is a necessary condition for zona pellucida penetration. The significant increase in VCL observed in the Tris aminomethane egg yolk group reflects higher average and curvilinear velocities, which are associated with increased mitochondrial activity and structural integrity of the spermatozoa tail [12]. Conversely, the Trico egg yolk extender showed significantly higher values for STR (77.00 ± 2.24% vs. 86.17 ± 4.05%), LIN (43.94 ± 2.46% vs. 56.22 ± 5.50%), and WOB (56.08 ± 1.11% vs. 65.40 ± 4.40%). These findings indicate that spermatozoa stored using Trico egg yolk extender exhibit more linear and stable movement. Linear and directed movement is important for guiding spermatozoa toward the ovum; however, excessively high STR and LIN values may indicate inadequate hyperactivation

The bacterial load of frozen semen showed considerable variation among bacterial species in both treatment groups (T1 and T2). Escherichia coli spp. exhibited a significant increase in the T2 (50.1 ± 101.96 × 10^3 CFU/ml) compared to T1 (0.7 ± 1.57 × 10^3 CFU/ml). This suggests a potential source of contamination or a lower effectiveness of the tris-egg yolk extender in inhibiting the growth of E. coli spp. Mycoplasma spp. was the most dominant bacterial species detected in both treatments. The average colony count in T1 was 92.5 ± 117.64 × 10^3 CFU/ml and increased significantly in T2 to 203.3 ± 94.86 × 10^3 CFU/ml. This addition indicates that the T2 may not be effective in suppressing the proliferation of Mycoplasma spp., or may even supply conditions that favor its growth. In contrast, Staphylococcus spp. showed a slight decrease in colony count in the T2 group (38.0 ± 26.66 × 10^3 CFU/ml) compared to T1 (47.1 ± 58.58 × 10^3 CFU/ml). However, the relatively high standard deviations observed in both groups indicate inconsistent bacterial distribution among samples, which could be controlled by individual sample variability or differences in semen collection and processing techniques. Pseudomonas spp. were not detected in either treatment group, indicating that this bacterium was either absent from the observed conditions and samples or effectively suppressed by both treatment protocols. According to [3] sources of contamination can be divided into two factors, namely biotic and abiotic. Biotic sources include the prepuce, skin, urogenital passageways, hair, respiratory fluids, and feces, as well as poor sanitary conditions and contaminations related to laboratory technicians. Abiotic contaminants originate from the laboratory environment, such as storage equipment, transportation, and sperm processing, processing laboratories, glassware, buffers, extenders, and straws.

Overall, trico egg yolk extender (T2) was associated with increased bacterial loads, particularly of E. coli spp. and Mycoplasma spp., which may serve as indicators of reduced semen quality due to contamination by these two bacterial species. It is essential to note that the antibiotic compositions of the two extenders differed, which may have influenced their effectiveness in suppressing bacteria. Tris aminomethane egg yolk extender included penicillin and streptomycin, both of which possess broad-spectrum antibacterial activity [3]. especially effective against Gram-positive and some Gram-negative bacteria. In contrast, the Trico extender included lincomycin and spectinomycin, which may hold a more limited or differing spectrum of antimicrobial action. These differences in antibiotic formulation could partially account for the higher variability and overall bacterial counts observed in the Trico group. Maintaining minimal bacterial contamination in semen is critical for preserving post-thaw sperm quality and fertility.

The presence of bacterial load in frozen semen samples has critical implications for sperm quality, viability, and the success rate of artificial insemination. The high levels of E. coli detected in T2 are potentially attributable to standard sources of contamination, such as feces or environmental factors. This bacterium is known to damage sperm motility and membrane integrity by releasing endotoxins and reactive oxygen species (ROS). Meanwhile, Mycoplasma spp. was the most dominant microorganism detected in both treatments, warranting particular awareness due to its pathogenic potential and resistance to commonly used antibiotics. Its proliferation in the T2 group reflects not only a lack of antimicrobial effectiveness in the extender but also the actuality of a microenvironment encouraging bacterial survival, possibly due to nutrient content or pH balance in the medium. The increased abundance of Mycoplasma spp. in frozen semen samples has previously been associated with egg yolk-based extenders under suboptimal processing conditions. The use of egg yolk in extenders may be a source of contamination leading to bacterial growth in semen. Further research is needed to test the effectiveness and efficiency of egg yolk trico extender in maintaining semen quality and membrane and DNA integrity, as well as inhibiting bacterial growth, as a substitute for egg yolk tris aminomethane extender with a larger number of samples and on different breeds of cattle to improve data accuracy and validation.

4 Conclusion

Both Tris aminomethane egg yolk and Trico egg yolk extenders maintained acceptable post-thaw sperm quality and bacterial control in Holstein Friesian bull semen. The Tris aminomethane egg yolk extender showed better performance in motility, viability, intact acrosome, and membrane integrity. Moreover, CASA analysis revealed that semen processed with Tris aminomethane exhibited significantly higher values in DCL, VCL, and ALH, indicating enhanced velocity, path curvature, and lateral head amplitude, traits associated with improved fertilizing capacity. Trico-based extenders resulted in significantly higher STR, LIN, and WOB values, suggesting a more linear response. The results showed that the Trico egg yolk extender is a promising and cost-effective alternative, showing comparable performance in several sperm quality parameters bacterial control but may benefit from further optimization to improve its cryoprotective potential, particularly in inhibiting bacterial growth.

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References

1. Juanpanich, T., Suttirojpattana, T., Parnpai, R. & Vutyavanich, T. (2022). The relationship between reactive oxygen species, DNA fragmentation, and sperm parameters in human sperm using simplified sucrose vitrification with or without triple antioxidant supplementation. Clinical and Experimental Reproductive Medicine. 49(2): 117–126. DOI: https://doi.org/10.5653/cerm.2021.05120.
2. Gupta, S., Kumar, A., Mahajan, A., Sharma, P., Sachan, V., Aggrawal, J., Yadav, S., Saxena, A. & Swain, D.K. (2022). Curcumin in a tris-based semen extender improves cryosurvival of Hariana bull spermatozoa. Andrologia. 54(1). DOI: https://doi.org/10.1111/and.14255.
3. Reda, A.A., Almaw, G., Abreha, S., Tadeg, W. & Tadesse, B. (2020). Bacteriospermia and sperm quality of cryopreserved bull semen used in artificial insemination of cows in South Wollo Zone, Ethiopia. Veterinary Medicine International. 2098315. DOI: https://doi.org/10.1155/2020/2098315.
4. Tvrdá, E., Lovíšek, D., Gálová, E., Schwarzová, M., Kováčiková, E., Kunová, S., Žiarovská, J. & Kačániová, M. (2022). Possible implications of bacteriospermia on the sperm quality, oxidative characteristics, and seminal cytokine network in normozoospermic men. International Journal of Molecular Sciences, 23(15): 8678. DOI: https://doi.org/10.3390/ijms23158678.
5. Hernández-Avilés, C., Love, C.C., Serafini, R., Ramírez-Agámez, L., Kelley, D.E., Martínez de Andino, E., Teague, S.R., LaCaze, K.A., Brinsko, S.P. & Varner, D.D. (2020). Inclusion of supplemental antibiotics (amikacin – penicillin) in a commercial extender for stallion semen: Effects on sperm quality, bacterial growth, and fertility following cooled storage. Theriogenology. 158: 209–217. DOI: https://doi.org/10.1016/j.theriogenology.2020.09.018
6. Yekti, A.P.A, Arif A.A, Utami P., Syah H.A, Pramudhita A.D, Rizqianti R.A, & Susilawati T. (2024). Quality and proportion of x and y sperm after sexing process using percoll density gradient centrifugation method on different gradients and diluents in belgian blue cross bull. Adv. Anim. Vet. Sci. 12(11): 2211-2220. DOI: https://doi.org/10.17582/journal.aavs/2024/12.11.2211.2220.
7. Benko, F., Arvay, J., Janˇco, I., Duraˇcka, M., Mohammadi-Sangcheshmeh A.,, Luk´a, N., Ivani, P. & Eva Tvrd. (2024). In vitro versus cryo-induced capacitation of bovine spermatozoa, part 3: Compositional and molecular changes to the plasma membrane. Cryobiology, 117. DOI: https://doi.org/10.1016/j.cryobiol.2024.104972.
8. Safa, F., Yekti, A.P.A., Utami, P., Syah, H.A., Febrianto, N., Rachmawati, A., Yuswati, A., Amaliya, A., Sulistyowati, D. & Susilawati, T. (2025). Sperm Quality, Kinematics, Membrane Integrity, and DNA Fragmentation of Frozen Sexed Semen in Holstein-Friesian Bulls. Tropical Animal Science Journal. 48(3): 199–210. DOI: https://doi.org/10.5398/tasj.2025.48.3.199.
9. Cojkic, A., Hansson, I. & Morrell, J.M. (2025). Bacterial survival below zero: Impact of storage time on bacterial viability in bull semen. Cryobiology. 119: 105233. DOI: https://doi.org/10.1016/j.cryobiol.2025.105233.
10. Yangngam Y., Chapanya S., Vongpralub T., Boonkum W., & Chankitisakul V. (2021). Effect of semen extender supplementation with sericin on post-thaw dairy bull sperm quality and lipid peroxidation. Czech J. Anim. Sci., 66: 13–20. DOI: https://doi.org/10.17221/104/2020-CJAS.

11. Dutta, S., Majzoub, A. & Agarwal, A. (2019). Oxidative stress and sperm function: A systematic review on evaluation and management. Arab Journal of Urology, 17(2), 87–97. DOI: https://doi.org/10.1080/2090598X.2019.1599624.
12. Utami P, Yekti APA, Simbolon CNA, Syah HA, Amaliya A, Siswoyo TA, Isnaini N, & Susilawati T. (2025). Analysis of kinetic parameters of sexed Holstein-Friesian bull spermatozoa using Percoll density gradient centrifugation with computer-assisted sperm analysis. Veterinary World. 18(2): 287–295. DOI: https://doi.org/10.14202/vetworld.2025.287-295.
13. Moreira, S.S.J., Santos, C.S., Castelo, T.S., Bezerra, L.G.P., Praxedes, É.C.G., Matos, T.M., Souza-Junior, J.B.F., Feijó, F.M.C., Comizzoli, P. & Silva, A.R. (2022). Investigating the need for antibiotic supplementation to the extender used for semen cryopreservation in collared peccaries. Frontiers in Veterinary Science. 9. DOI: https://doi.org/10.3389/fvets.2022.954921.