AMERICAN JOURNAL OF BIOMEDICINE AND PHARMACY
https://biojournals.us/index.php/AJBP
ISSN: 2997-7177

Article

Effects of Cooling Periods on Equine Semen Characteristics in Al-Zawraa Park

Roa'a N. Ahmed[1], Ali J. Al-Anifji2, H.M. Mohammed-Rashid3, Amal H. Khalaf4, L.A. Chiad5, A.A. Eessa6

Directorates of Animal Resource, Ministry of Agriculture, Baghdad, Iraq, [2] Parks of Afforestation, Dept. Al-Zawraa
*Correspondence: rnahmed13@gmail.com

Citation: Ahmed R. N. et al. Effects of Cooling Periods on Equine Semen Characteristics in Al-Zawraa Park. American Journal of Biomedicine and Pharmacy 2024, 6(2), 59-66.

Received: 25th Jan 2026
Revised: 28th Jan 2026
Accepted: 15th Feb 2026
Published: 22th Feb 2026

Abstract: Chilling stallion semen in equine reproduction prolongs sperm viability and makes artificial insemination easier, cooling causes physiological changes that can impact membrane integrity, acrosome stability, and overall semen quality; monitoring these parameters over time is crucial to comprehending the limits of semen usability during storage. This research aimed to evaluate the time-dependent changes in semen quality of stallions during chilled storage using a Tris-hydroxymethyl-aminomethan-based extender at Al-Zawraa Park, Baghdad. Twelve semen samples were collected from two stallions from October 2024 to April 2025. Ejaculates were collected, diluted, and stored at 5 °C for five consecutive days. Daily assessments included sperm viability, morphological abnormalities, plasma membrane integrity (HOST), and acrosomal status; the results demonstrated a progressive decline in semen quality over time. On day 1, semen samples showed high viability with minimal abnormalities, while plasma membrane and acrosomal structures were well preserved. From day 2 to day 3, a marked reduction in membrane integrity was observed accompanied by increasing morphological defects. By day 5, viability had dropped markedly, membrane integrity was severely impaired, and acrosomal status revealed limited fertilization potential. These findings confirm that cold storage, even with the protective effect of Tris extender, leads to cumulative structural and functional damage in spermatozoa. Stallion semen preserved at 5 °C with Tris-hydroxymethyl-aminomethan extender maintains acceptable quality only for a limited period, with substantial decline beyond 48–72 hours. This research highlights the importance of functional assays such as HOST and acrosomal evaluation for predicting fertility potential and suggests that extender modification or antioxidant supplementation may be required to prolong semen usability.

Keywords: Semen, Stallions, Cooling, Acrosome

Introduction

Artificial insemination (AI) is one of the most valuable reproductive biotechnologies in equine breeding programs, as it enables genetic improvement, disease control, and efficient management of stallions, among semen preservation techniques, liquid storage at low temperatures (4–5 °C) is widely applied for short-term use. However, chilled semen is prone to a time-dependent decline in sperm

quality due to alterations in plasma membrane structure, loss of acrosomal integrity, and decreased motility [1].

The plasma membrane and acrosome don't handle cold storage well—they're especially vulnerable to temperature stress, which cuts down fertilizing ability [2]. Several studies have shown that progressive motility and viability decrease significantly within 48–72 hours of storage, with variations depending on extender composition, stallion age, and environmental factors [3] [4]. Additionally, oxidative stress and calcium imbalance have been implicated as critical contributors to sperm deterioration during storage [1].

Preservation of acrosomal integrity is crucial because premature acrosome reactions can impair the ability of spermatozoa to penetrate the oocyte [5]. Research comparing chilled and frozen semen indicates that chilling is less damaging to the acrosome, yet still results in gradual deterioration with time [6]. Furthermore, supplementation of extenders with antioxidants has been explored as a strategy to mitigate oxidative damage and extend semen shelf life [7]. Oxidative stress is one of the main factors contributing to the decline of sperm quality during chilled storage, supplementation of extenders or diets with natural antioxidants has been explored to mitigate this effect [8]. In equine artificial insemination programs, the use of properly formulated extenders allows semen to be cooled and stored for up to 48 hours without significant loss of fertility, facilitating the transportation and use of semen over long distances [9].

Given these challenges, there is a continuous need to evaluate semen quality parameters during storage to determine the optimal time frame for insemination and to guide improvements in extenders and handling procedures [3].

There is a lack of biological or physiological studies on horses in Iraq, particularly those related to semen evaluation, cooling and freezing [10][11][12][13][14]. Since no previous studies have addressed this issue locally

The present study was aimed to evaluate some of characteristics of chilled stallion semen subjected to various cooling period (5 °C)

Materials and Methods

1- Animal:

This study utilized clinically healthy and fertile (n=2, age =12±2years, weight=480±20 Kg) housed at Al-Zawraa Park, with no history of reproductive disorders, the animals were fed a balanced diet formulated to meet the nutrient requirements, the ration consisted of 70% roughage (alfalfa hay and barley straw) and 30% concentrate mixture containing 14% crude protein, 2.5% fat, and approximately 12 MJ/kg metabolizable energy, the concentrate was composed of crushed barley, wheat bran, soybean meal, and a commercial vitamin-mineral premix. Fresh water and trace-mineral salt blocks were available.

2- Experimental design:

Twelve semen samples were collected from two healthy adult stallions using an artificial vagina. The collected semen was immediately transferred to the laboratory in a water bath maintained at 37°C for initial evaluation. A time-dependent, within-sample evaluation of chilled stallion semen stored at 5 °C for five consecutive days. Semen is diluted with a Tris-based extender (1:1). Daily assessments (Day 0 = immediately after dilution, Day 1, Day 2, Day

3, Day 4) include: sperm viability (%),abnormalities , plasma membrane integrity, and acrosomal integrity(%).

3- Reagents and materials:

The semen was diluted using a TRIS-egg yolk-based extender prepared as follows:

- 2.40 g TRIS
- 1.25 g citric acid
- 0.45 g glucose
- 22 ml egg yolk
- 4 ml glycerol
- Deionized water added to a final volume of 100 ml
- Antibiotics added: Gentamicin (0.6 mg) and Lincomycin (0.08 mg)

The extender was mixed thoroughly and sterilized by filtration before use. All chemicals were of analytical grade.

4- Semen collection and initial evaluation:

- Collection: Collect ejaculates using artificial vagina; immediately place semen in a (37 °C) container.
- Initial assessment: volume, concentration, morphology and record time of collection.

5- Chilling Protocol:

Following dilution at a 1:1 ratio, the semen samples were gradually cooled to 5°C over a period of two hours and stored in a refrigerator, assessments of semen quality were conducted at Day 0 (immediately after dilution and cooling to 5 °C), Day 1 (24 h), Day 2 (48 h), Day 3 (72 h), Day 4 (96 h) post-chilling to evaluate the effect of cold storage over time; viability, and morphology. Sperm membrane integrity was assessed using the hypoosmotic swelling test (HOST).

6- Laboratory assays:

- **Sperm viability:**

  Ten µl of semen was mixed with 10 µl eosin-nigrosin stain, smeared, and air-dried. Two hundred sperm were counted under ×1000 magnification. Live sperm excluded eosin, while dead sperm stained pink [15].

- **Morphology (head & tail abnormalities):**

  Fixed smears were stained with eosin-nigrosin stain and examined under ×1000 oil immersion. Two hundred sperm were evaluated per slide, classifying abnormalities as head or tail defects [16].

- **Hypo-osmotic swelling test (HOST):**

  One hundred µl semen was mixed with 1 ml HOST solution was prepared by dissolving 0.872g of Fructose and 0.474g of Sodium Citrate in 100 ml of distilled water incubated at 37 °C for 30–60 min [17]. Two hundred sperm were examined under ×400 magnification, and the percentage of swollen tails was recorded as intact membrane.

- **Acrosomal integrity:**

  Samples were stained Using Giemsa method for light microscopy, two hundred sperm were counted per slide and categorized as intact or damaged acrosome [18].

7- Statistical Analysis:

The collected data were analyzed using one-way ANOVA to determine the effect of storage time on semen quality parameters, including sperm viability, plasma membrane integrity, and acrosomal status. The results were expressed as mean ± standard deviation (SD). Differences among means were considered statistically significant at $P < 0.05$, all statistical analyses were performed using SPSS software version 26 [19].

Results

The semen quality parameters of two stallions after cooling, measured at multiple time points or conditions (labeled 1 to 5). The parameters include: Live (%), Abnormalities (AB), Tail, Head, HOST (%) (Hypo-Osmotic Swelling Test – membrane integrity) and Acrosome (%) (acrosomal integrity).

Table (1): the semen quality parameters of stallion (1).

Parameter	0 hr	24 hr	48 hr	72 hr	96 hr
Live (%)	72.04 ±0.94 ab	75.62 ±0.7 a	70.81 ±0.4 b	66.38 ±1.83 bc	63.19 ±0.66 c
Tail Abn. (%)	9.8 ±0.56 bc	9.67 ±0.9 c	13.32 ±1.17 b	14.53 ±0.99 ab	14.82 ±1.62 a
Head Abn. (%)	7.04 ±0.94 b	6.15 ±0.78 c	6.84 ±1.41 bc	10.57 ±1.27 a	8.13 ±0.68 ab
HOST (%)	83.58 ±1.37 a	80.75 ±0.64 ab	70.83 ±0.37 b	64.2 ±0.45 bc	59.24 ±1.57 c
Acrosome (%)	77.24 ±1.13 a	71.73 ±0.39 ab	67.88 ±0.7 b	63.28 ±0.7 bc	52.87 ±0.22 c

Table (2): the semen quality parameters of stallion (2).

Parameter	0 hr	24 hr	48 hr	72 hr	96 hr
Live (%)	70.68 ±0.73 b	76.35 ±0.29 a	71.62 ±0.82 ab	66.34 ±0.65 bc	59.47 ±0.24 c
Tail Abn. (%)	10.2 ±0.44 bc	10.15 ±0.2 c	13.93 ±1.26 b	13.97 ±1.14 ab	17.02 ±0.08 a

Head Abn. (%)	7.58 ±0.47 bc	6.08 ±0.73 c	8.63 ±0.54 b	9.93 ±0.48 ab	14.63 ±0.37 a
HOST (%)	80.69 ±0.83 ab	80.69 ±0.45 a	72.09 ±0.72 b	59.48 ±0.49 bc	52.6 ±0.5 c
Acrosome (%)	78.97 ±0.72 a	72.35 ±0.49 ab	68.17 ±0.09 b	62.87 ±0.56 bc	43.28 ±0.52 c

Daily evaluation of chilled semen samples revealed clear time-dependent changes in all quality parameters. On day 1, semen diluted with the Tris extender showed high sperm viability (>70%), with relatively low morphological abnormalities and well-preserved plasma membrane and acrosome integrity. By day 2, a reduction in live sperm percentage was observed, although most values still remained within acceptable ranges for insemination.

From day 3 onwards, sperm viability declined significantly, accompanied by an increase in morphological defects, particularly tail bending and head deformities. The HOST test results demonstrated reduced swelling response, indicating progressive deterioration of membrane integrity. Similarly, acrosome integrity showed increased percentages of damaged acrosomes compared to earlier days.

By day 5, semen quality had markedly deteriorated. Viability fell below 30–40%, membrane integrity was severely compromised, and acrosome status indicated a limited fertilization potential. Morphological abnormalities reached their peak, reflecting cumulative cold shock and oxidative damage. These results demonstrate that chilled stallion semen undergoes progressive functional decline with storage time, despite the protective role of the Tris extender.

Three mares were inseminated with semen that had been chilled and stored for 24 hours from stallion 1 that exhibited better semen quality. Pregnancy diagnosis was performed via trans-rectal ultrasonography approximately 14-16 days post insemination. All three mares were confirmed pregnant, indicating that the chilled semen retained sufficient fertilizing capacity after 24 hours of storage.

Discussion

The present findings demonstrated that storage time has a significant influence on the functional integrity of stallion spermatozoa preserved at 5 °C in a Tris-egg yolk extender. The gradual decline in sperm viability, plasma membrane integrity and acrosome status observed after 48 h indicates that prolonged chilling adversely affects sperm functionality, likely due to membrane destabilization and oxidative damage accumulated during cold storage. These results are consistent with the general pattern reported for stallion semen under similar conditions [18][20].

The temporary stabilization or slight improvement in viability detected after the first 24 h may be attributed to metabolic down-regulation following gradual cooling, a controlled reduction in metabolic activity can decrease reactive oxygen species (ROS) formation and help maintain cellular integrity during the initial cooling phase [21]. However, as storage time increases beyond 48 h, cumulative

oxidative stress, lipid peroxidation, and ATP depletion compromise mitochondrial function and sperm motility, leading to progressive loss of membrane integrity and viability [20][22].

Damage to the plasma membrane and acrosome during storage reflects the sensitivity of these structures to temperature-induced stress, membrane lipids in stallion spermatozoa have a high proportion of polyunsaturated fatty acids, which makes them particularly vulnerable to peroxidative injury during cooling and rewarming [1]. Loss of acrosome integrity can directly impair fertilizing capacity, as premature acrosome reactions hinder oocyte penetration and binding [5].

The current outcomes reinforce the importance of optimizing both extender composition and storage duration. Tris-based extenders containing egg yolk have proven effective in protecting sperm membranes against cold shock by supplying phospholipids and low-density lipoproteins that stabilize the membrane bilayer [23]. Nevertheless, deterioration after 72 h suggests that the antioxidant capacity of the extender becomes insufficient to neutralize accumulated ROS over time, future improvements may include the addition of potent antioxidants such as hydroxytyrosol or vitamin E to extend semen shelf life without compromising fertility potential [24].

The findings confirm that chilled semen from stallions can maintain acceptable quality for up to 48 h under controlled storage at 5 °C, after which a noticeable decline occurs in all major functional parameters. Continuous monitoring of viability, membrane integrity, and acrosomal status is therefore essential to determine the optimal time frame for artificial insemination and to enhance semen handling protocols in equine reproduction.

Conclusion

Chilled storage of stallion semen using a Tris-based extender results in time-dependent decline in sperm viability, increased morphological abnormalities, reduced membrane integrity (HOST), and compromised acrosomal integrity over five days. While the Tris extender offers protection, semen quality declines progressively.

These observations should be taken into account in breeding and semen transport planning. It is therefore recommended that insemination using chilled semen chiefly within 24-48 hours after collection to maintain acceptable quality and that further research should focus on improving extender formulations with antioxidant supplementation and controlled cooling protocols to extend semen shelf life without compromising fertilizing ability.

Acknowledgements: The authors express their sincere appreciation to the staff of Al-Zawraa Park for their support during sample collection and handling. The authors also extend their gratitude to the laboratory personnel for their assistance in performing the semen evaluations and providing the necessary facilities to complete this research.

Authors Contributions: All authors had significant contribution in achievement an design, analysis and writing this work.

Conflict of interest: No conflict of interest is disclosed by the authors.

Funding Sources: No fund was received by Author.

REFERENCES

[1] M. Umair et al., "In vitro aging of stallion spermatozoa during prolonged storage at 5°C," Cytometry Part A, vol. 103, no. 6, pp. 461–538, 2023, doi: 10.1002/cyto.a.24689.

[2] I. Ortiz, P. Manjunath, D. Chapman, and J. Garcia, "Flow-cytometric analysis of membrane integrity of stallion spermatozoa: Assessment of sperm plasma membrane and acrosome status," J. Assist. Reprod. Genet., vol. 38, no. 3, pp. 597–606, 2021, doi: 10.1007/s10815-020-02028-7.

[3] C. Hernández-Avilés, L. Ramírez-Agámez, D. D. Varner, and C. C. Love, "Factors affecting the analysis and interpretation of sperm quality in frozen/thawed stallion semen," Theriogenology, vol. 218, pp. 35–44, 2024, doi: 10.1016/j.theriogenology.2023.12.013.

[4] A. Dziekońska, A. Szczepańska, and A. Wysokińska, "Effect of season on the characteristics of Warmblood stallion spermatozoa stored in a liquid state at 5 °C," Animals, vol. 15, no. 7, p. 1035, 2025, doi: 10.3390/ani15071035.

[5] H. Breitbart, "Mechanisms that protect mammalian sperm from the spontaneous acrosome reaction and preserve fertilization ability," Int. J. Mol. Sci., vol. 24, no. 23, p. 17005, 2023, doi: 10.3390/ijms242317005.

[6] M. Bugno-Poniewierska et al., "Influence of cryopreservation on the acrosome reaction in Hucul stallion spermatozoa," Animals, vol. 15, no. 13, p. 1915, 2025, doi: 10.3390/ani15131915.

[7] F. Martínez-Pastor, M. F. Riesco, and M. R. Fernández-Santos, "Impact of the antioxidant hydroxytyrosol on the quality of stallion spermatozoa after cooling and freezing," Reprod. Domest. Anim., vol. 59, no. 2, pp. 221–230, 2024, doi: 10.1155/2024/6558480.

[8] A. S. Mohsin and S. M. Eidan, "Effect of adding antioxidants to tris extender on cooling and post-cryopreservative semen characteristics of Holstein bulls. 2. Salvia officinalis L aqueous extract," Biochem. Cell. Arch., vol. 19, no. 1, pp. 1157–1164, 2019, doi: 10.35124/bca.2019.19.1.1157.

[9] M. Vidament, "French field results (1985–2005) on factors affecting fertility of frozen stallion semen," Anim. Reprod. Sci., vol. 89, no. 1–4, pp. 115–136, 2005, doi: 10.1016/j.anireprosci.2005.07.003.

[10] B. J. Mohamed, "Effect of endurance exercise on sweat constituents of athletic Iraqi Arabian horses," Iraqi J. Vet. Sci., vol. 31, no. 2, pp. 95–100, 2017, doi: 10.33899/ijvs.2017.145604.

[11] M. M. Ismail et al., "Some physiological and chemical properties of horse and cow blood samples under effect of some different types of anticoagulants," Iraqi J. Vet. Med., vol. 42, no. 1, pp. 7–11, 2018, doi: 10.30539/iraqijvm.v42i1.23.

[12] W. Z. Turki and N. N. Al-Anbari, "Genetic diversity in original Arabian-WAHO, local and thoroughbred horses in Iraq from ACTN3-EXON19 gene polymorphism," IOP Conf. Ser.: Earth Environ. Sci., vol. 1214, p. 012033, 2023, doi: 10.1088/1755-1315/1214/1/012033.

[13] M. A. Abed, T. A. Abdulkareem, and W. A. Saleh, "Effect of reproductive stages on some blood metabolites in the show and racehorse mares in Iraq," Iraqi J. Agric. Sci., vol. 56, Special Issue, pp. 111–122, 2025, doi: 10.36103/s2gw6m75.

[14] M. A. Abed and T. A. Abdulkareem, "Blood biomarkers of show and racing equine stallions in Iraq: A comparative study," Iraqi J. Agric. Sci., vol. 56, no. 1, pp. 347–357, 2025, doi: 10.36103/g3ndj544.

[15] F. J. Peña, B. A. Ball, and E. L. Squires, "A new method for evaluating stallion sperm viability and mitochondrial membrane potential in fixed semen samples," Cytometry Part B Clin. Cytom., vol. 94, no. 2, pp. 302–311, 2018.

[16] S. Egyptien, S. Deleuze, J. Ledeck, and J. Ponthier, "Sperm quality assessment in stallions: How to choose relevant assays to answer clinical questions," Animals, vol. 13, no. 19, p. 3123, 2023, doi: 10.3390/ani13193123.

[17] D. M. Neild et al., "The HOST test and its relationship to fertility in the stallion," Andrologia, vol. 31, no. 3, pp. 155–161, 1999, doi: 10.1111/j.1439-0272.1999.tb02847.x.

[18] C. Hernández-Avilés, L. Ramírez-Agámez, D. D. Varner, and C. C. Love, "The stallion sperm acrosome: Considerations from a research and clinical perspective," Theriogenology, vol. 196, pp. 121–149, 2023, doi: 10.1016/j.theriogenology.2022.12.019.

[19] L. N. Muhammad et al., "Guidelines for repeated measures statistical analysis within the context of basic science," Front. Physiol., vol. 14, pp. 115–129, 2023, doi: 10.3389/fphys.2023.115.

[20] F. J. Peña, C. Ortega-Ferrusola, and J. Moreno-Fernández, "New insights into sperm physiology: Lessons from stallions," Reprod. Domest. Anim., vol. 55, Suppl. 4, pp. 62–71, 2020, doi: 10.1111/rda.13680.

[21] C. C. Love, S. P. Brinsko, and D. D. Varner, "Effect of cooling and storage on stallion sperm function," Theriogenology, vol. 133, pp. 152–160, 2019, doi: 10.1016/j.theriogenology.2019.06.007.

[22] C. Ortega-Ferrusola, J. Moreno-Fernández, and F. J. Peña, "Redox homeostasis in stallion spermatozoa: Role of mitochondria and strategies for improvement," Antioxidants, vol. 10, no. 3, p. 478, 2021, doi: 10.3390/antiox10030478.

[23] S. Naz et al., "Comparison of Tris egg yolk-based, Triladyl® and Optixcell extenders for stallion semen cryopreservation," Andrology, vol. 6, no. 6, pp. 908–917, 2018, doi: 10.1111/and.13063.

[24] "Protective effect of hydroxytyrosol on cooled stallion semen quality," J. Equine Vet. Sci., vol. 130, p. 105754, 2024, doi: 10.1016/j.jevs.2024.105754.