Original Article

Effects of Incubation Temperature at 37 ˚C on Canine Sperm Quality Before Cooling to 5 ˚C

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ABSTRACT

Background: One of the most important aspects of artificial insemination in animals is the practical design of long-term preservation of sperm. Although there are many reports on canine sperm storage methods, an improved system for chilling canine spermatozoa is required for successful breeding programs in companion and working dog colonies.

Objectives: This study aimed to compare the effects of incubation temperature at 37 ˚C on dog sperm parameters before cooling.

Methods: Around 1-2 mL ejaculated sperm from the testes of 5 mature, healthy dogs were collected and divided into two groups: C: TRIS+egg yolk 20% (control, without incubation at 37 ˚C), T: TRIS+egg yolk 20% (treatment group, incubation at 37 ˚C for 10 minutes). Evaluation of cooled sperm was done by computer-assisted sperm analysis (CASA) for motility test, morphology, eosin-nigrosin vital staining and hypo-osmotic swelling test (HOST) after cooling.

Results: In the treatment group, total and progressive motility (PM), curvilinear velocity, straight line velocity, average path velocity (VAP), linearity (LIN), straightness (STR), the proportion of sperm with normal morphology, the proportion of viable sperm and the proportion of sperm positive for HOST were not affected by the interaction of group by time. However, they were lower in treatment than the control group ($P<0.05$) and they decreased throughout preservation ($P<0.001$).

Conclusion: Keeping sperm at a temperature of 37 ˚C and applying heat shock by bain-marie has highly adverse effects on the quality of ejaculated sperm.

Keywords: Incubation, Dog, Sperm parameter, Cooling, Computer-assisted sperm analysis (CASA)

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Introduction

Recently, reproductive biotechnologies have emerged and replaced conventional techniques. Advances in reproductive biotechnologies for future reproductive preservation are needed (Yousef et al., 2022). Preserving spermatozoa at a cool temperature reduces sperm metabolism while preserving sperm viability and reproductive potential (Mohajer et al., 2024; Mohajer et al., 2024). Preserving high-quality sperm is essential for successful canine reproductive outcomes, maintaining genetic diversity, improving breeding programs and facilitating artificial insemination. However, sperm cells are inherently vulnerable to various environmental stressors, including thermal fluctuations, which can profoundly impact their viability and functionality. Among these stressors, heat shock emerges as a critical factor that poses significant risks to sperm quality during handling, processing and storage. Heat shock is characterized by a transient exposure of cells to elevated temperatures, typically exceeding their physiological range, which triggers a cascade of biochemical and physiological responses (Paris et al., 2024).

Temperature regulation is crucial in studying sperm physiology due to the high sensitivity of sperm to thermal stress. The adverse effects of heat stress on animal reproductive functions have been studied extensively for many decades. Exposure of the sperm to 37 ˚C temperature affects the sperm number in the ejaculate and affects sperm parameters, such as motility, morphology, and plasma membrane integrity (Hansen, 2009).

Sperm motility is essential for successful fertilization, enabling sperm to move through the female reproductive tract and reach the egg. Heat stress can impair sperm motility by disrupting the structural integrity of flagella or affecting the production of ATP, which is essential for sperm motility (Kim et al., 2012; Sabés-Alsina et al., 2016).

Based on the studies, it has been determined that sperm morphology, which includes the size and shape of sperm cells, is essential for their function. Abnormalities in morphology can prevent sperm motility and their ability to penetrate the egg for fertilization. Damage caused by heat stress can lead to morphological defects, thereby reducing sperm quality (Bansal & Bilaspuri, 2010; Menkveld et al., 2011).

One of the other important aspects of sperm parameter evaluation is the issue of sperm viability in the ejaculated sample, which indicates their fertilization potential. High temperature can increase oxidative stress and induce apoptosis, thereby reducing sperm viability and overall fertility. Plasma membrane integrity is also critical for sperm function, as it maintains cell structure and regulates ion transport and signaling processes (Boni, 2019; Kassis et al., 2021).

Heat stress can compromise membrane integrity, lead to ion leakage and impaired sperm motility and viability (Holt et al., 2015; Shahat et al., 2020).

In the context of canine sperm, particularly sensitive to environmental changes, heat shock can induce structural and functional alterations that compromise their ability to fertilize ova and sustain reproductive success. The detrimental effects of heat shock on sperm quality before chilling are multifaceted and encompass various aspects of sperm physiology. Elevated temperatures can disrupt sperm membrane integrity, leading to leakage of intracellular components and compromising sperm viability. Moreover, heat shock can impair mitochondrial function, reducing ATP production essential for sperm motility and energy metabolism. Oxidative stress induced by heat shock further exacerbates cellular damage by promoting lipid peroxidation and DNA fragmentation, compromising sperm function and fertilization capacity (Huang et al., 2022).

Bain-Marie, or water bath, is a method widely used for precise temperature control in research settings, allowing researchers to study the effects of heat stress on various parameters of sperm, including motility, morphology, viability, and plasma membrane integrity (Taşdemir et al., 2013).

Advancements in reproductive biotechnology and sperm preservation techniques have underscored the importance of optimizing cooling protocols and minimizing heat shock during sperm handling. Rapid cooling methods aim to mitigate the adverse effects of heat shock by reducing the time sperm cells are exposed to damaging temperatures (Bencharif & Dordas-Perpinya, 2020). Advanced imaging techniques and computer-assisted sperm analysis (CASA) have provided valuable insights into the effects of heat shock on sperm motility parameters (e.g. total motility [TM], progressive motility [PM], curvilinear velocity [VCL], straight-line VCL, average path VCL, linearity [LIN] and straightness [STR]) (Gonçalves et al., 2021). So far, the effect of thermal shock on dog semen has not been studied. This study aimed to compare the impact of bain-marie thermal shock on dog sperm parameters before cooling.

Materials and Methods

Animals and semen collection

Five intact male mixed breed dogs aged 3-8 years (body weight=25-40 kg) were assigned for this study, and the semen of three dogs was pooled for each replicate of this experiment. Semen was collected using the manual technique by stimulating the dog's penis within the prepuce until a partial erection occurred. The prepuce was slid caudally behind the bulbus glandis, and the steady pressure on the penis was maintained until the dog ejaculated. The collected semen was assigned to the control and treatment groups. In the control group, the collected semen was kept at room temperature in the laboratory. In the treatment group, the collected semen was maintained at 37 ˚C for 10 min. During this period, the semen was evaluated using a light microscope for motility, and only semen samples with TM greater than 70% were used for further procedures. Afterward, the semen in both groups was centrifuged at 700×g, the supernatant was removed, and the semen was diluted by the ratio of 1:10 using a TRIS-base medium (containing 3.025 g TRIS, 1.7 g citric acid, 1.25 g fructose, 100 IU/mL penicillin, 100 µg /mL streptomycin and 20% egg yolk). Eventually, the diluted semen was transferred to the laboratory at 4-6 ˚C and was evaluated at the time of arrival to the laboratory (time point 0) as well as 24, 48, and 72 hours afterward. The number of biological replicates for each experimental group was 3 (n=3).

CASA analysis

At the laboratory, semen was diluted to the concentration of 2×10^7 sperm/mL using the TRIS-base medium supplemented with 1% egg yolk and then, it was analyzed using a system of CASA (sperm class analyzer [SCA] version 6.3.0.59; Microptic SL, Barcelona, Spain) connected to a microscope (Nikon, Japan) projecting an image on a monitor, and the photos were captured using a video camera (Caméra Digital Basler A312, Germany). The CASA analysis was configured with negative phase-contrast optics and at 50 frames per second, the minimum cell size of 5 μm^2 and the maximum cell size of 80 μm^2. The magnification of the microscope for the evaluation of semen samples was 10x and for each sample, more than three fields and at least 200 sperm were analyzed. TM was the proportion of sperm with >10 µm/s curvilinear VCL. PM was the proportion of sperm with >65 µm/s VCL and ≥80% STR. STR was calculated by dividing straight-line VSL by average path velocity (VAP) and LIN was calculated by dividing VSL by VCL (Akbarinejad et al., 2018).

Sperm morphology

Sperm morphology was assessed by preparation of a smear slide of the semen sample, which was further air-dried, and fixing it using sperm blue fixative for 10 minutes and next staining it with sperm blue stain (Microptic SL, Barcelona, Spain) for 10 minutes (Roshan et al., 2023). Morphological examination was implemented at 1000x magnification under oil immersion by a light microscope (Nikon, Japan) and a minimum of 200 spermatozoa were counted for each slide. Eventually, the percentage of sperms with normal morphology was calculated.

Sperm viability

Sperm viability was assessed by eosin–nigrosin staining. The sperm smears were prepared by mixing a drop of semen with two drops of eosin–nigrosin stain on a warm slide and spreading the mixture immediately with the aid of a second slide. Viability was assessed by counting 200 sperm with a light microscope (Nikon, Japan) under 1000× magnification. Sperms that showed partial or complete colorization were considered dead. Only sperms that showed strict stain exclusion were deemed alive (Roshan et al., 2023). The percentage of live sperm was considered as sperm viability.

Plasma membrane integrity

The integrity of the sperm plasma membrane was evaluated using a hypo-osmotic swelling test (HOST) solution prepared with 9 g fructose and 4.9 g sodium citrate per liter of distilled water (Roshan et al., 2023). In brief, 10 µL of semen was diluted with 100 µL of HOST solution and incubated at 37 ˚C for 60 minutes. After incubation, 20 µL of diluted semen was spread on a warm slide (37 ˚C) and covered with a cover slip. For evaluation, 200 sperm cells were enumerated under 1000x magnification using a phase-contrast microscope (Nikon, Japan). Sperm with swollen or coiled tails were recorded as intact, and those with curled tails were recorded as damaged. The proportion of intact sperm was considered plasma membrane integrity.

Statistical analysis

Data associated with sperm motility, morphology, viability, and plasma membrane integrity were analyzed using repeated measures models of mixed procedure. The LSMEANS statement was used to perform multiple comparisons. All analyses were conducted in SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA; SAS, 2013). Differences at $P<0.05$ were considered significant.

Results

The effects of incubation temperature at 37 ˚C on the parameters (motility, morphology, viability and plasma membrane integrity) of sperms were analyzed and the results are as follows.

Effects of incubation temperature at 37 ˚C on sperm motility

Total and PM were not affected by the interaction of the group by time (P>0.05). However, they were lower in the treatment than the control group (P<0.05) and they decreased throughout preservation (P<0.001; Figures 1A and 1B).

VCL, VSL and VAP were not influenced by the interaction of the group by time (P>0.05). VCL was greater in the control than in the treatment group (P=0.05), but VSL and VAP were comparable between the control and treatment group (P>0.05). In addition, VCL, VSL and VAP decreased over time regardless of the experimental group (P<0.0001; Figures 2C, 2D and 2E).

LIN and STR were not affected by the interaction of the group by time and the main effect of the group (P>0.05), but they decreased over time (P<0.001; Figures 3F and 3G).

Effects of incubation temperature at 37 ˚C on sperm morphology

The proportion of sperm with normal morphology was not impacted by the interaction effect of the group by time (P>0.05). Yet it was greater in the control than that in the treatment group (P=0.05) and it decreased over time (P<0.0001; Figure 4).

Effects of incubation temperature at 37 ˚C on sperm viability

The proportion of viable sperm was not influenced by the interaction effect of the group by time (P>0.05). Yet it was greater in the control group than that in the treatment group (P<0.001), and it decreased over time (P<0.0001; Figure 5).

Effects of incubation temperature at 37 ˚C on sperm positive for HOST

The proportion of sperm positive for HOST was not affected by the interaction effect of the group by time (P>0.05). Yet it was greater in the control than in the treatment group (P<0.05) and it decreased over time (P<0.0001; Figure 6).

Discussion

There is an increasing demand for sperm cooling to preserve male fertility and use in assisted reproductive technology. The results of this study showed that storing semen at 37 ˚C for 10 minutes had a negative effect on sperm health by reducing sperm motility, morphology, viability, and plasma membrane integrity, following previous studies (Payan-Carreira et al., 2011).

Based on the study of Leboeuf et al. (2000) it was determined that semen samples stored at 37 ˚C showed a significant decrease in PM after 12 hours of incubation, which showed an average value of less than 25% in all samples. The shorter lifespan observed in samples stored at 37 ˚C results from faster depletion of sperm metabolic ability, decreasing motile sperm count with increasing storage time, which is also consistent with the results of our study (Leboeuf et al., 2000).

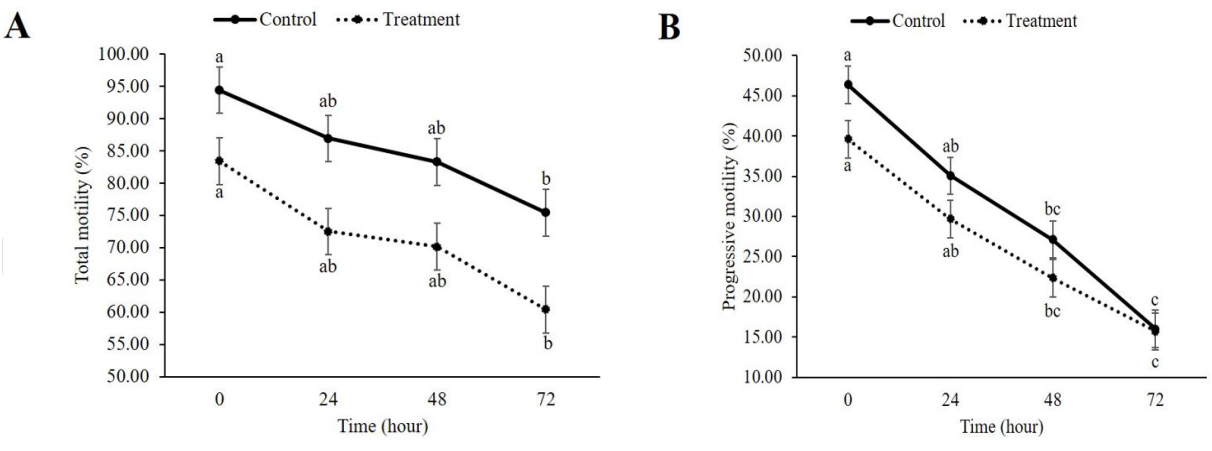

Figure 1. Effect of incubation temperature at 37 ˚C on A) Sperm total motility, and B) PM

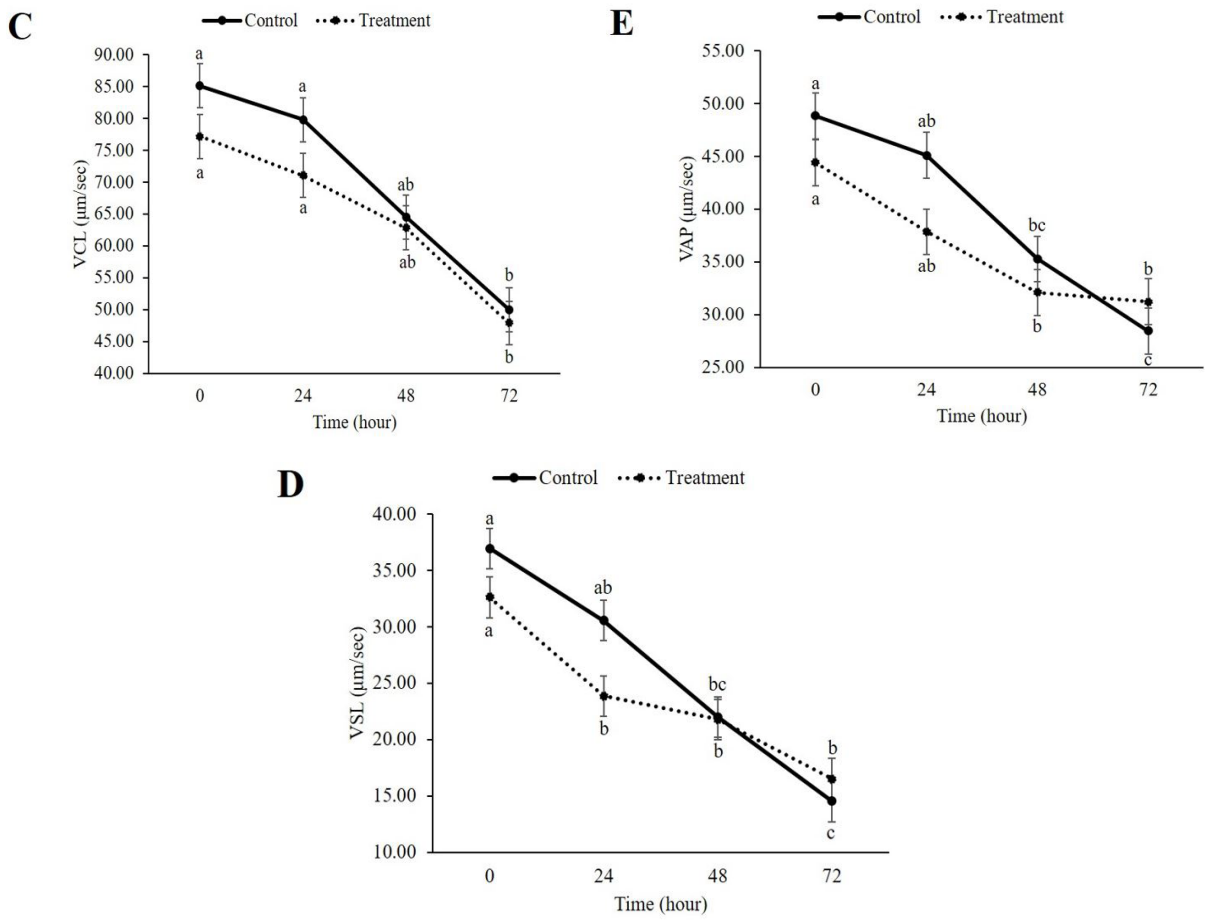

Figure 2. Effect of incubation temperature at 37 ˚C on C) Sperm curvilinear velocity, D) Straight-line velocity, and E) VAP

The study by Karimi Zarchi et al. (2020) showed that after 2 and 4 hours of incubation at room temperature, sperm PM and viability decreased significantly. Sperm DNA fragmentation increased considerably following 2 and 4 hours of incubation at room temperature and 37 ˚C, which is also consistent with the results of our study.

Ramon et al. (2014) study showed that semen samples incubated at 37 ˚C for 24 hours caused a significant decrease in PM, which is consistent with our study results. Based on the study conducted by Batista et al. (2012), it was found that the rate of progressive sperm motility varied from 40% to 50% for the first 8 hours of incubation at 37 ˚C. Still, after that, a rapid decrease in sperm motility was observed. The results of this study are consistent with our research. Indeed, the results of Batista et al. suggest that semen samples that have been incubated at 37 ˚C for 24 h show reduced motility associated with a high percentage of abnormal spermatozoa cells (Batista et al., 2012).

The results of Rasad et al. (2020) study showed that the incubation time (45, 60 and 75 minutes) at 37 ˚C significantly affected the longevity of the sexed sperm of Pasundan bull, which is also consistent with the results of our study.

According to the study of Rigau et al. (2001), dog sperm incubation for 60 minutes at 37-39 ˚C caused a faster decrease in sperm viability and, thus, less motility with increasing storage time, which is consistent with the results of the present study. Pinto et al. (1999) and Rijsselaere et al. (2002) found that the percentage of abnormal spermatozoa in dog semen samples stored at 37 ˚C for 5 min was non-uniform throughout the experimental period. Studies have reported similar percentages of sperm abnormalities in semen samples stored at 37 ˚C. Also, the percentage of abnormal sperm cells in samples stored at 37 ˚C increased significantly with incubation time. Furthermore, a direct relationship was observed between a higher percentage of abnormal tails and a faster decline in sperm motility over time in semen incu-

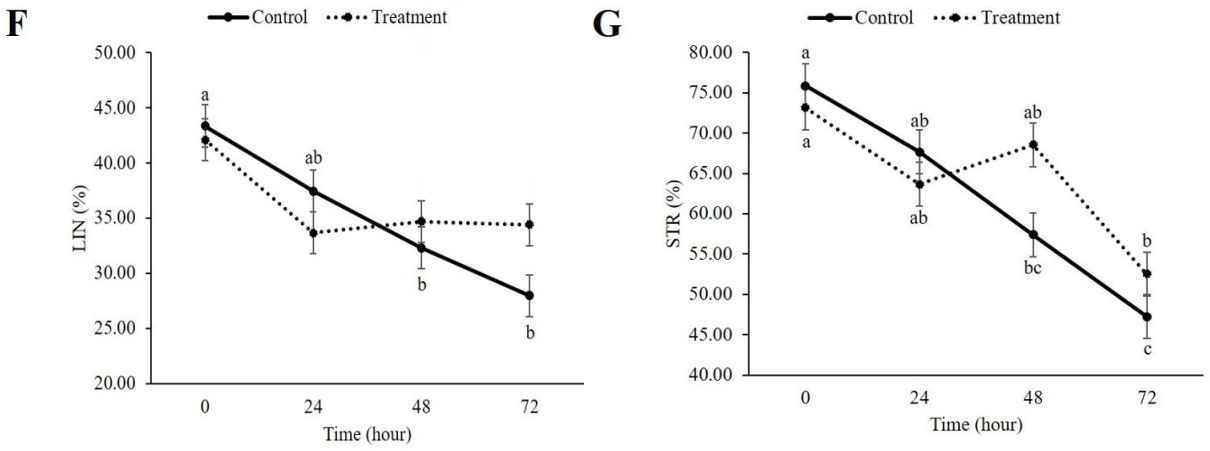

Figure 3. Effect of incubation temperature at 37 ˚C on F) Sperm LIN and G) STR

bated at 37 ˚C (Mascarenhas & de Paula, 2018; Sugai et al., 2023; Zabret, 2022).

Hahn et al. (2019) confirm the results of our study, which reveals that the viability, total and PM, and morphological abnormalities of sperm that were incubated at 37 ˚C for 3, 10, 15 and 30 minutes were significantly affected by the time passed.

According to a study, it was determined that semen samples stored at 15 ˚C for 9 days had higher sperm PM, survival time, sperm membrane integrity, acrosome integrity, level of sperm reactive oxygen species, superoxide dismutase activity, catalase enzyme activity, and ATP content as compared to the other two groups (20 ˚C and 25 ˚C), which is also consistent with the results of our study (Zhang et al., 2022).

Sperm motility is an important parameter influencing a male animal's fertility (Kathiravan et al., 2011). Temperature of preservation essentially influences semen motility. Tuli and Holtz (1995), recommend examining sperm motility in isothermal conditions of 36–38 ˚C. Verstegen et al. (2002) suggested 37 ˚C is an ideal temperature for semen evaluation. These recommendations do not seem to be applied to the raw semen of dogs, according to the results of our study. After assessing the measurements of the CASA system, there was a tendency that individual motility of the semen cells in the control group was higher than the treatment group. A significant influence of temperature was observed on the proportion of fast-motile sperm kept at 37 ˚C.

According to our results, canine semen can be stored without incubation at 37˚ after collection and during semen analysis without affecting overall semen quality. However, we only considered the effect of temperature

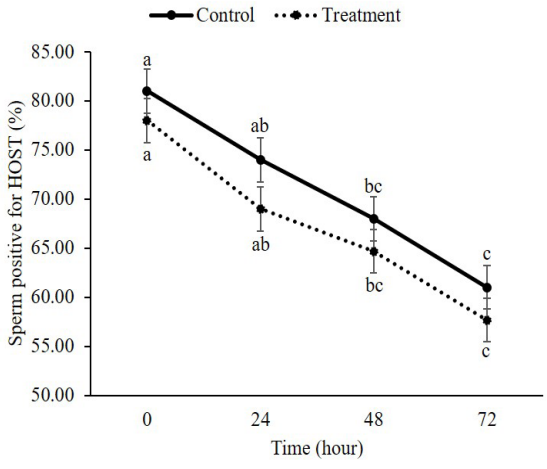

Figure 4. Effect of incubation temperature at 37 ˚C on sperm normal morphology

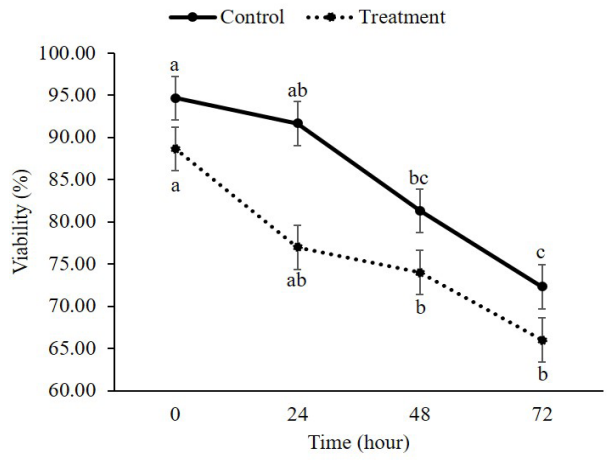

Figure 5. Effect of incubation temperature at 37 ˚C on sperm viability

37 ˚C for 10 min. Murphy et al. (2016) also demonstrated no effect of different storage temperatures (5 ˚C, 15 ˚C, 22 ˚C, 32 ˚C) on liquid bull semen viability. According to Busch (2007), undiluted semen of small ruminants kept at 30 ˚C post collection for 20–30 min can still be used for artificial insemination. This statement is comparable to the results of our work. The ejaculates kept at 37 ˚C were just below the minimum requirements.

Examination time significantly influenced semen motility. Especially fast motility was affected and decreased. Our results agreed with Busch (2007), who recommended that the extracted ejaculate be examined within 10 minutes of collection. The proportion of live sperm decreases with an increasing duration of storage time. Therefore, this parameter should generally be examined as soon as possible after semen collection.

The cooling (4–5 ˚C) of canine semen for 24 h is not associated with physical or functional changes in sperm (Kumi-Diaka & Badtram, 1994). Still, when the cooling time is prolonged, the sperm motility decreases.

On the other hand, the presence of different sperm subpopulations has been reported in canine semen samples (Dorado et al., 2011; Núñez-Martínez et al., 2006). These sperm subpopulations are not distributed uniformly among male dogs and could show different sensitivities to environmental changes, especially those involving temperature (Pignataro et al., 2020b). In addition, individual variability in post-freezing seminal quality

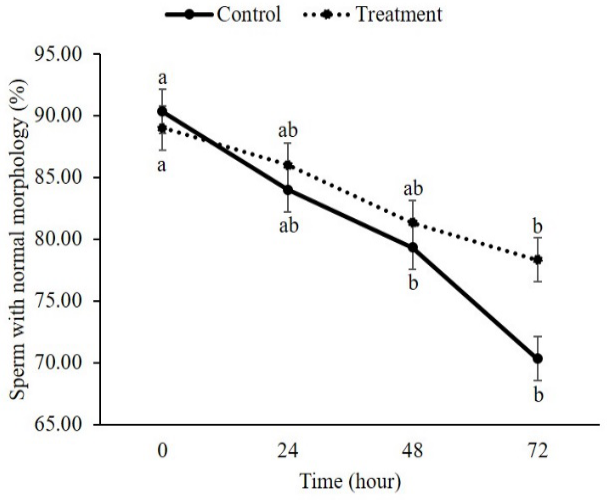

Figure 6. Effect of incubation temperature at 37 ˚C on sperm positive for HOST

has been reported (Eilts, 2005; Petrunkina et al., 2003), and therefore, the semen of individual dogs may respond differently to chilling. Therefore, we cannot rule out that the dogs showing better semen quality could also have sperm subpopulations with high longevity.

According to the study of Pignataro et al. (2020a), it was found that short-term storage of semen at 5 ˚C or 15 ˚C did not show any difference in motility, viability, and integrity of the membrane, which indicates better cold tolerance than heat.

Conclusion

The results of our study show that the various parameter values are higher in the samples, resulting in them not being incubated at 37 ˚C compared to the samples incubated at 37 ˚C for 10 minutes. This premise may be attributed to the irritability of sperm due to temperature conditions. As a result, improving the sperm characteristic requires further research in fresh and cold semen and its incubation at various temperatures and different times.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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The paper was extracted from the PhD dissertation of Javad Jafari, approved by Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Authors' contributions

Methodology and data curation: Vahid Akbarinejad; Investigation: Javad Jafari; Formal analysis: Vahid Akbarinejad; Writing the original draft: Javad Jafari, Vahid Akbarinejad, and Hamid Ghasemzadeh-nava, Conceptualization, resources, supervision, review and editing: Hamid Ghasemzadeh-nava, Vahid Akbarinejad, and Abdolhossein Shahverdi

Conflict of interest

The authors declared no conflict of interest.

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