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Effect of different thawing procedures on motility and kinetic parameters of frozen-thawed bull sperm

Stevan Mitković[1]*, Miodrag Jovanović[1], Aleksandra Jevtić[1], Branislav Vejnović[2], Slobodanka Vakanjac[3], Svetlana Nedić[3]

[1] *Department of Agriculture and Food Technology Studies, Toplica Academy of Applied Studies, 18400 Prokuplje, Serbia*
[2] *Department of Economics and Statistics, Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobođenja 18, 11000 Belgrade, Serbia*
[3] *Department of Reproduction, Fertility and Artificial Insemination, Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobođenja 18, 11000 Belgrade, Serbia*

**Corresponding author: stevan.mitkovic@gmail.com*

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ABSTRACT

This study aimed to evaluate the effect of thawing temperature for deep-frozen semen by analyzing sperm motility and kinetic parameters. Before computer-assisted semen analysis (CASA), semen straws (0.25 mL) were thawed in a water bath using three protocols: 38 °C for 20 seconds (Group A, $n = 6$), 55 °C for 10 seconds (Group B, $n = 6$), and 65 °C for 5 seconds (Group C, $n = 6$). While total and progressive sperm motility showed a tendency to increase with higher thawing temperatures and shorter periods (65 °C for 5 seconds, Group C) compared to Groups A and B, these differences did not reach statistical significance ($P > 0.05$). Notably, the percentage of locally motile spermatozoa was significantly higher when semen was thawed at 65 °C compared to 55 °C ($P < 0.05$). Similarly, kinetic parameters were generally higher in Group C, with a statistically significant increase in DAP compared to Group B ($P < 0.05$). Conversely, LIN values were lower in Groups B and C than in Group A, reaching statistical significance ($P < 0.05$). The cytomorphological evaluation revealed no significant differences between the three groups in terms of the percentage of pathologically altered spermatozoa, live spermatozoa, or the prevalence of head, midpiece, or tail defects ($P > 0.05$). These results suggest that rapid semen thawing at 65 °C for 5 seconds may optimize sperm motility and kinetic parameters. However, further research with larger sample sizes is needed to evaluate the fertilization capacity of semen during artificial insemination.

Keywords: deep-frozen semen, thawing temperature, CASA, kinetics

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ИЗВОД

Циљ рада је био да се испита ефикасност отапања дубоко замрзнутог семена при различитим температурама преко оцене параметара покретљивости и кинетике сперматозоида. Пре прегледа семена компјутерски асистираном анализом семена (CASA), пајете од 0.25 mL су отапане у воденом купатилу на температури 38 °C у трајању од 20 секунди (група А, $n = 6$), 55 °C током 10 секунди (група Б, $n = 6$) и на температури 65 °C током 5 секунди (група В, $n = 6$). Укупна и прогресивна покретљивост сперматозоида била је виша уколико је семе отапано на вишој температури краћи временски период (65 °C 5 секунди, група В) у поређењу са групама А и Б, али без статистичке значајности ($P > 0.05$). Проценат локално покретних сперматозоида био је значајно виши уколико је семе отапано на 65 °C у односу на семе отапано на 55 °C ($P < 0.05$). Такође, у нашем раду су утврђене више вредности параметара кинетике сперматозоида у групи В, али су статистички значајно више биле само вредности DAP у поређењу са групом Б ($P < 0.05$). Ниже вредности LIN утврђене су у група Б и В у поређењу са групом А, на нивоу статистичке значајности $P < 0.05$. Цитоморфолошким испитивањем је утврђено да нема разлике у процентуалној заступљености патолошки промењених сперматозоида, проценту живих сперматозоида, као ни процентуалне заступљености дефеката главе, средњег дела нити дефеката репа сперматозоида ($P > 0.05$). Иако је у нашем раду брзо отапање семена (5 секунди) на температури 65 °C дало најбоље резултате, неопходна су даља истраживања на већем броју узорака, као и фертилизациони капацитет семена приликом вештачког осемењавања.

Кључне речи: дубоко замрзнуто семе, температура отапања, CASA, кинетика

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1. Introduction

Deep freezing of bull semen has been a cornerstone of improved genetics and reproductive efficiency in the cattle industry for decades. However, the reduced fertilization capacity of frozen-thawed semen remains a significant challenge (Upadhyay et al., 2021). High sperm motility and viability are crucial for successful artificial insemination of cows, as evidenced by the strong positive correlation between post-thaw sperm viability and conception rates in cows (Correa et al., 1996). Some authors state that if you want to prevent the effects of heat stress of cows, you should not use a natural performance bull, as spermatogenesis will most likely be disrupted (Cincović et al., 2023). Both the freezing and thawing processes affect semen quality (Nur et al., 2003). Standard practice usually involves thawing semen straws at physiological temperatures (38 °C), although slow thawing is known to induce recrystallization and damage to sperm organelles (Sharafi et al., 2022). However, the potential benefits of rapid thawing at higher temperatures for shorter durations remain unclear, with conflicting results reported in the literature and limited post-thaw semen quality evaluation parameters. Recent research suggests that thawing at 39 °C, compared to 70 °C, may prolong sperm exposure to harmful sub-physiological temperatures within the straw (Nguyen et al., 2023). Therefore, this study aimed to evaluate sperm motility and kinetic parameters following thawing at temperatures higher than standard, using varied exposure times.

2. Materials and methods

Deep-frozen bull semen, stored in liquid nitrogen at –196 °C, was transported to the Laboratory of the Department of Reproduction, Fertility and Artificial Insemination to perform an objective examination of motility and kinetic parameters via computer-assisted sperm analysis (CASA) using the AndroVision system (Minitube, Germany). Before the examination, semen straws (0.25 mL) were thawed in a water bath using three protocols: 38 °C for 20 seconds (Group A, $n = 6$), 55 °C for 10 seconds (Group B, $n = 6$), and 65 °C for 5 seconds (Group C, $n = 6$). Following thawing, semen was transferred from the straws to Eppendorf tubes on a heated plate (Minitube, Tiefenbach, Germany), mixed thoroughly, and a 2.7 µL subsample was loaded into a Leja chamber (Leja, GN Nieuw Vennep, The Netherlands). Spermatozoa were analyzed across ten fields of view using a phase-contrast microscope (Motic BA310, Barcelona, Spain) equipped with a heated stage. Motility and kinetic parameters were automatically assessed using the AndroVision software (Minitube Manual 12500/0000, AndroVision, Germany). The following parameters were measured using the CASA system: sperm concentration, total and progressive motility, percentages of rapid, slow, circularly motile, locally motile, and immotile spermatozoa. Kinetic parameters included velocity curved line (VCL), velocity straight line (VSL), velocity average path (VAP), distance curvilinear line (DCL), distant straight line (DSL), distance average path (DAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), head activity (HAC), wobble (WOB), linearity (LIN) and straightness (STR).

2.1. Cytomorphological examination of spermatozoa

Spermatozoa cytomorphology was evaluated using eosin-nigrosin staining according to the standard protocol (Dott and Foster, 1972). A total of 100 spermatozoa were assessed to determine the percentages of live and dead spermatozoa, as well as the presence of pathological defects in the head, midpiece, and tail.

2.2. Statistical analysis

Data distribution was assessed for normality using the Shapiro-Wilk test. As the data were normally distributed (Shapiro-Wilk test, $p > 0.05$), one-way analysis of variance (ANOVA) was employed to compare the experimental groups. Post-hoc comparisons were performed using Tukey's test. Data are presented as mean value ± SD in tabular form. Statistical analyses were conducted using GraphPad Prism version 7 software (GraphPad, San Diego, CA, USA).

3. Results and Discussion

It is well-established that deep freezing and thawing of semen can reduce sperm motility and induce structural, biochemical, and physiological damage to spermatozoa (Senger, 1980). For decades, the recommended thawing temperature for deep-frozen bull semen has been around physiological temperature, typically 37–39 °C (Correa et al., 1996; Ömür, 2022). However, recent investigations have explored alternative thawing temperatures and durations to determine optimal conditions for getting the highest post-thaw percentages of sperm viability and motility (Dhami & Sahni, 1993; Lyashenko, 2015; Nguyen et al., 2023).

Table 1 presents the post-thaw semen quality parameters for the three temperature protocols. Sperm concentration was significantly higher ($P < 0.05$) in Group B (55 °C for 10 seconds) than in Group A (38 °C for 20 seconds).

Table 1.
Post-thaw deep-frozen bull semen quality parameters

	38 °C – 20 sec. (Group A)	**55 °C – 10 sec. (Group B)**	**65 °C – 5 sec. (Group C)**
Concentration of sp. (10^6/mL)	**103.50 ± 26.36**	**127.60 ± 7.23***	108.60 ± 3.57
Total mobility of sp. (%)	62.96 ± 9.35	66.05 ± 6.35	70.11 ± 3.91
Progressive mobility of sp. (%)	58.14 ± 10.33	59.84 ± 6.88	65.45 ± 4.45
Rapid sp. (%)	35.96 ± 9.46	36.05 ± 5.42	42.43 ± 3.94
Slow sp. (%)	22.03 ± 2.68	23.67 ± 1.49	22.81 ± 1.30
Circularly motile sp. (%)	0.13 ± 0.10	0.13 ± 0.02	0.22 ± 0.08
Locally motile sp. (%)	4.83 ± 1.49	**6.21 ± 0.69**	**4.66 ± 0.61**$^{\#}$
Immotile sp. (%)	37.04 ± 9.35	33.95 ± 6.35	29.89 ± 3.91

*Statistically significant difference: *P < 0.05 Group A vs Group B;* $^{\#}$*P < 0.05 Group B vs Group C*

Although not statistically significant ($P > 0.05$), both total and progressive sperm motility tended to be higher when semen was thawed at higher temperatures for shorter exposures (Group C, 65 °C for 5 seconds) compared to Groups A and B. Specifically, total sperm motility was highest in Group C; namely, it was 11.36% higher than in Group A and 6.15% higher than in Group B. Progressive motility followed a similar trend, with Group C exhibiting a 12.57% increase compared to Group A and a 9.37% increase compared to Group B. These findings align with that of Nguyen et al. (2023), who reported that rapid semen thawing at 70 °C, followed by stabilization at 39 °C, significantly improved motility, viability, and mitochondrial activity compared to standard thawing at 39 °C. This suggests that minimizing thawing time may enhance the number of spermatozoa capable of regaining maximal motility (Smirnov, 1982).

In this study, the percentage of rapid spermatozoa was approximately 18% higher in Group C than in Groups A and B, while the percentage of slow spermatozoa was about 4% lower in Group C. Notably, the percentage of circularly motile spermatozoa in Group C was 69.23% higher than in Groups A and B, though this increase reached no statistical significance ($P < 0.05$). The percentage of locally motile spermatozoa was significantly higher in the case of semen thawing at 65 °C than in semen thawing at 55 °C ($P < 0.05$). Although the percentage of immotile spermatozoa was 24% lower in Group C than in Group A, this difference was not statistically significant ($P > 0.05$). Similarly, Yilmaz et al. (2020) found no statistically significant difference in the percentage of rapid spermatozoa between semen thawed at 37 °C for 20 and 30 seconds and semen thawed at 60 °C for 8 seconds. Lyashenko (2015) observed improved sperm motility when semen was thawed at 65–70 °C for 6 to 7 seconds compared to standard thawing at 39 °C, attributing this to reduced recrystallization and hydration damage during rapid thawing. Similarly, other studies reported improved sperm motility when deep-frozen bull semen was thawed at higher temperatures, such as 60 °C and 80 °C (Narasimha et al., 1986; Dhami et al., 1996).

Table 2 presents the sperm kinetic parameters, assessed using CASA, for deep-frozen bull semen thawed at different temperatures. Sperm kinetic parameter evaluation found higher VCL values in Group C, with a 14.23% increase compared to Group A and an 11.43% increase compared to Group B, though these values showed no statistical difference ($P > 0.05$). Similarly, deep-frozen semen thawing at higher temperatures (65 °C for 5 seconds) yielded approximately 12% higher VSL and VAP values compared to groups where semen was thawed at lower temperatures. Shah et al. (2016) observed higher VCL and VSL values when thawing semen at 50 °C for 15 seconds and at 70 °C for 7 seconds compared to thawing semen at 37 °C for 30 seconds. These findings align with that of our study. DCL and DSL values were also higher in Group C than in Group B (16.26% and 22.52%, respectively), though these differences were not statistically significant ($P > 0.05$). However, DAP was significantly higher in Group C than in Group B ($P < 0.05$), with an 18% increase. ALH value was also 11.57% higher in Group C than in Groups A and B. Conversely, LIN was significantly lower in Groups B and C than in Group A ($P < 0.05$). Doležalová et al. (2017) tested four deep-frozen semen thawing methods: control group (38.5 °C for 30 seconds), slow thawing (30 °C for 50 seconds), medium thawing (50 °C for 15 seconds), and fast thawing (70 °C for 3 seconds) and reported significantly lower VSL and LIN values in the control group than in all other tested groups. Overall, our data suggest that higher thawing temperatures enhance sperm motility and kinetics, which aligns with the results of other studies (Nur et al., 2003; Rastegarnia et al., 2013).

Table 2.
Post-thaw sperm kinetic parameters

	38 ˚C – 20 sec. (Group A)	**55 ˚C – 10 sec. (Group B)**	**65 ˚C – 5 sec. (Group C)**
VCL (µm/s)	107.50 ± 21.78	110.20 ± 11.97	122.80 ± 8.91
VSL (µm/s)	36.17 ± 7.10	36.31 ± 3.62	40.31 ± 2.84
VAP (µm/s)	47.61 ± 8.73	48.72 ± 4.67	53.76 ± 3.55
DCL (µm)	35.54 ± 5.58	33.76 ± 3.43	39.25 ± 1.94
DSL (µm)	9.56 ± 1.90	8.26 ± 0.98	10.12 ± 0.61
DAP (µm)	14.07 ± 2.16	**13.00 ± 1.33**	**15.31 ± 0.75**$^{\#}$
ALH (µm)	1.21 ± 0.19	1.21 ± 0.11	1.35 ± 0.08
BCF (Hz)	10.33 ± 1.58	9.74 ± 0.89	10.85 ± 0.57
HAC (rad)	0.23 ± 0.05	0.23 ± 0.03	0.26 ± 0.02
WOB (VAP/VCL)	0.45 ± 0.01	0.44± 0.01	0.44 ± 0.01
LIN (VSL/VCL)	**0.34 ± 0.01**	**0.33 ± 0.00****	**0.33 ± 0.00***
STR (VSL/VAP)	0.76 ± 0.02	0.75 ± 0.01	0.75 ± 0.00

Statistically significant difference*: *P < 0.05 Group A vs Group C; **P < 0.01 Group A vs Group B;* $^{\#}$*P < 0.05 Group B vs Group C*

Table 3 presents the results of the cytomorphological examination of spermatozoa. No significant differences ($P > 0.05$) were observed between the three thawing protocols regarding the percentage of pathologically altered spermatozoa, live spermatozoa, or the prevalence of head, midpiece, or tail defects. This contrasts with findings reported by Yilmaz et al. (2020), who observed a higher percentage of pathologically altered spermatozoa when semen was thawed at 37 °C compared to 60 °C. It is well-established that sperm plasma membranes, acrosomal outer membranes, and mitochondrial membranes are highly susceptible to damage during freezing and thawing processes. These membranes, rich in unsaturated fatty acids, undergo phase transitions from liquid to gel during the deep-freezing process, leading to structural alterations (Watson, 1995). Furthermore, the high unsaturated fatty acid content renders spermatozoa vulnerable to oxidative stress, which occurs during both freezing and thawing (Holt, 2000). Oxidative stress and subsequent lipid peroxidation can compromise plasma membrane integrity, resulting in reduced sperm motility (Nur et al., 2006). In our study, the absence of significant differences in the percentages of live or pathologically altered spermatozoa suggests that thawing semen at temperatures higher than physiological does not significantly increase or decrease membrane damage.

Table 3.
Post-thaw sperm cytomorphology changes

	38 °C – 20 sec. (Group A)	**55 °C – 10 sec. (Group B)**	**65 °C – 5 sec. (Group C)**
Share of morphologically changed sp. (%)	8.33 ± 4.46	11.67 ± 1.97	10.33 ± 2.34
Living sp. (%)	70.00 ± 6.20	70.67 ± 7.76	73.67 ± 4.46
Dead sp. (%)	30.00 ± 6.20	29.33 ± 7.76	26.33 ± 4.46
Head defect in sp. (%)	5.67 ± 4.46	4.67 ± 2.42	4.67 ± 2.07
Midpiece defect in sp. (%)	2.00 ± 0.00	3.00 ± 1.16	2.00 ± 0.00
Tail defect in sp. (%)	3.00 ± 1.16	5.00 ± 3.52	5.00 ± 2.10

4. Conclusion

The findings of this study suggest that thawing semen at a higher temperature for a shorter duration (65 °C for 5 seconds) results in improved sperm motility and kinetics compared to standard physiological thawing temperatures. This enhanced bull semen motility and kinetic performance observed with rapid thawing at elevated temperatures may be attributed to reduced exposure to temperatures outside the physiological limits, consequently preserving mitochondrial activity in spermatozoa.

Declaration of competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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