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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Effects of Slow and Rapid Thawing on Bull Semen Quality: An Evaluation via Thermal Resistance Test

Burcu ESIN[1]* **Cumali KAYA[1]**
[1]*University of Ondokuz Mayis, Department of Animal Reproduction and Artificial Insemination, Samsun 55200, Türkiye*

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*: https://orcid.org/0000-0002-5728-1478
: https://orcid.org/0000-0003-2215-2868

***Corresponding author's:**
Burcu ESIN
University of Ondokuz Mayis, Department of Animal Reproduction and Artificial Insemination, Samsun 55200, Türkiye
✉: burcuyalcin@omu.edu.tr

Abstract: This study aimed to comparatively evaluate the impact of two different thawing protocols-slow thawing at 37°C for 30 seconds and rapid thawing at 70°C for 6 seconds-on bull semen quality, with a particular focus on the thermal resistance of spermatozoa. In both groups, motility, progressive motility, kinematic parameters (VCL, VAP, VSL, STR, LIN, WOB, ALH, BCF), viability, and plasma membrane integrity were analyzed following thawing and after the thermal resistance test. Initially, no significant differences were found between groups in terms of motility and kinematic parameters; however, viability and membrane integrity were significantly higher in the rapid thawing group ($P<0.001$). By the end of the thermal resistance test, progressive motility, VAP, and VCL values were statistically higher in the slow thawing group ($P<0.001$). Moreover, a decline in motility and kinematic parameters was observed in both groups during incubation, which may be associate to thermal stress ($P<0.05$). These results demonstrate that while rapid thawing confers advantages in early post-thaw membrane preservation, slow thawing offers better maintenance of motility over extended periods. Therefore, the choice of thawing protocol should be guided by the specific requirements of the intended reproductive technique, as it can significantly influence the fertilizing potential of cryopreserved bull semen.

Keywords: bull, semen, thawing, thermal resistance test.

Yavaş ve Hızlı Çözümün Boğa Sperma Kalitesi Üzerine Etkileri: Termal Direnç Testi ile Değerlendirme

***Sorumlu yazar:**
Burcu ESIN
Ondokuz Mayıs Üniversitesi Hayvan Üreme ve Suni Tohumlama Bölümü, Samsun 55200, Türkiye
✉: burcuyalcin@omu.edu.tr

Öz: Bu çalışmada, spermatozoanın termal direnç testine odaklanarak, iki farklı çözme protokolünün (37°C'de 30 saniye boyunca yavaş çözdürme ve 70°C'de 6 saniye boyunca hızlı çözdürme) boğa sperması kalitesi üzerindeki etkisini karşılaştırmalı olarak değerlendirilmesi amaçlanmıştır. Her iki grupta da motilite, progresif hareketlilik, kinematik parametreler (VCL, VAP, VSL, STR, LIN, WOB, ALH, BCF), canlılık ve plazma membran bütünlüğü, çözdürme sonrasında ve termal direnç testinden sonra analiz edilmiştir. Çözüm sonrasında motilite ve kinematik parametreler açısından gruplar arasında önemli bir fark bulunmamıştır. Canlılık ve plazma membran bütünlüğü parametreleri hızlı çözdürme grubunda önemli ölçüde daha yüksek bulunmuştur ($P<0.001$). Termal direnç testinin sonunda, progresif motilite, VAP ve VCL değerleri ise yavaş çözdürme grubunda istatistiksel olarak daha yüksek elde edilmiştir ($P<0.001$). Ayrıca, inkübasyon sırasında her iki grupta da motilite ve kinematik parametrelerde bir azalma gözlemlenmiş ve bu termal stres ile ilişkilendirilmiştir ($P<0.05$). Bu sonuçlar, hızlı çözdürme yöntemi ile çözüm sonrası plazma membran bütünlüğünün korunmasında avantajlar sağlarken, yavaş çözdürme yönteminin uzun süreler boyunca hareketliliğin daha iyi korunmasını sağladığı ortaya konulmuştur. Bu nedenle dondurulmuş boğa spermasının döllenme potansiyelini önemli ölçüde etkileyebilen çözdürme yönteminin, kullanılacak olan üreme tekniğine özel seçilmesi önemlidir.

Anahtar kelimeler: boğa, sperma, çözdürme, termal direnç testi.

INTRODUCTION

For many years, the dairy and beef production industries have benefited greatly from the genetic advancement and effective reproduction that cryopreservation of bull semen has brought about. However, the primary issue still appears to be the limited fertilizability of frozen-thawed spermatozoa (Upadhyay et al., 2021). Although slow thawing of frozen spermatozoa appears to encourage recrystallisation and hence harm the organelles, it is advised that cryopreserved bull semen should be thawed at physiological temperature (Sharafi et al., 2022). The

quality of cryopreserved-thawed buffalo (Rastegarnia et al., 2013) and cattle spermatozoa (Lyashenko, 2015) has been improved by thawing at higher temperatures for shorter periods of time, but because of some mixed results (Yilmaz et al., 2019), a conclusive conclusion has not yet been reached and is still in the early stages of research.

Cryopreserved semen from domestic animals has mostly been thawed at around body temperature, 37-39 °C (Mehmood et al., 2017; Ogata et al., 2022). Since the harmful temperature zone in frozen spermatozoa often occurs at a critical level, between -60 and 0 °C, due to the harmful recrystallization of intracellular ice, it is necessary to pass through the dangerous range as soon as possible during thawing, as it passes from the solid phase to the liquid phase around the melting point. Cryopreservation and subsequent thawing of spermatozoa can compromise both functional and genetic integrity due to ice recrystallization and osmotic stress. These changes may negatively affect fertilization capacity, particularly in assisted reproductive techniques (Di Santo et al., 2012). In one study, when frozen semen was thawed in water at 70 °C, the temperature inside the straw increased significantly faster from -196 to 0, 15, 37 or 39 °C compared to thawing at 39 °C (Nguyen, 2023).

Rapid thawing may have positive effects because it reduces exposure to temperatures between -196 °C and the physiological temperature range, which in turn reduces damage to the biological membrane linked to mitochondrial health (Nguyen, 2023).

In order to examine thawing protocols for bull spermatozoa, the majority of previous research relied on subjective microscopic evaluations of post-thaw sperm motility, viability or morphology (Muiño, 2008). Currently, flow cytometry and CASA technologies can be used to provide more accurate and objective measurements of the structural and functional properties of spermatozoa. The application of new technologies available for semen analysis to the comparison of rapid and slow thawing protocols and to the re-evaluation of some controversial aspects of cryopreservation protocols may provide new and valuable information (Boe-Hansen and Stake, 2019).

Therefore, the aim of this study was to thaw frozen bull spermatozoa at different temperatures (37 and 70 °C) and then evaluate spermatological parameters, hypoosmotic swelling test (HOST) and Thermal Resistance Test (TRT).

MATERIAL AND METHOD

Sperm Samples and Experimental Design: In the research, to eliminate for potential individual variances, a total of 40 frozen sperm straws from the same Simmental bull's semen were used. The semen samples found in 0.25 ml straws were thawed in a water bath at 37°C for 30 seconds to form the slow thawing group (n=20), while the rapid thawing group (n=20) was formed by thawing at 70°C for 6-7 seconds.

Potential fertility prediction capacities of spermatozoa after slow and rapid thawing were evaluated by spermatological parameters. All analyses were evaluated after thawing and TRT.

Computer-Assisted Sperm Analysis – CASA: The spermatological parameters (motility (M), progressive motility (PM), and kinematic parameters) of frozen-thawed sperm were analyzed using the Computer-Aided Sperm Analyser (CASA) under a heated stage phase-contrast microscope (Nikon, Eclipse, Tokyo, Japan) with a 10x objective and a 60 frame/sec camera. (SCA®, Microptic, Barcelona, Spain). The CASA system measured and recorded the following values in at least five microscope fields: M (0–100%), PM (0–100%), viability, VCL (curvilinear velocity, µm/s), VAP (mean path velocity, µm/s), VSL (straight-line velocity, µm/s), STR (straightness, %), LIN (linearity, %), WOB (wobble, %), ALH (lateral head change, m), BCF (beat cross frequency, Hz).

Viability: Sperm viability was evaluated using the Eosin-Nigrosin staining method (2% Eosin solution prepared in 3% sodium citrate). In the staining process, 10 µl of thawed semen was applied to a slide and placed on a heating table. Then, 20 µl of the Eosin-Nigrosin stain was mixed with the semen, and a smear was prepared on the slide. The slide was examined under a phase-contrast microscope using a heating stage set to 37°C. 200 spermatozoa from different microscopic fields were analyzed with a 20x objective lens. Spermatozoa stained pink or red were considered non-viable due to disruption of plasma membrane integrity, while those that did not take up the dye were considered intact and live spermatozoa.

Hypoosmotic Swelling Test – HOST: Sperm functional membrane integrity was assessed using the HOS test. We incubated the tube containing 1.0 mL of hypoosmotic (7.35 g sodium citrate and 13.51 g fructose per 1:1, v/v of distilled water) solution with 10 µL of thoroughly mixed extended semen for one hour at 37 °C. (Padrik et al., 2012). Then 10 µL of the mixture was placed on a slide and a coverslip was applied. It was immediately evaluated under a phase contrast microscope at 400× magnification. Two hundred spermatozoa were counted in at least five different microscopic fields. Cell membranes with thickened or swollen tails, beginning in the mid- piece, were regarded as intact and functionally active.

Thermal Resistance Test – TRT: The semen of two thawed straws per group was transferred to a 1.5 mL microtube and kept in a water bath at 46°C for 30 min. After this period, the samples were spermatological analyzed by CASA (Bacinoglu et al., 2008).

Statistical analysis: Statistical analyses were conducted using IBM® SPSS® Statistics for Windows,

Version 21.0 (IBM Corp., Armonk, NY, USA). The normality of the data was initially assessed using the Shapiro-Wilk test. In addition to this statistical evaluation, visual inspections of Q-Q plots and histograms were performed to support the assessment of data distribution. Moreover, skewness and kurtosis values were examined to evaluate the symmetry and peakedness of the distributions. Based on these evaluations, VCL, VSL, STR, WOB, plasma membrane integrity were found to follow a normal distribution, whereas motility, progressive motility, VAP, LIN, ALH, BCF, and viability did not exhibit a normal distribution.

For comparisons between the two groups, variables exhibiting a normal distribution were analyzed using the Independent Samples t-test, following the evaluation of variance homogeneity by Levene's test. In cases where equal variances were assumed, standard t-test results were reported; otherwise, adjusted results were considered. Variables that did not exhibit a normal distribution were evaluated using the Mann-Whitney U test.

For within-group comparisons between measurements obtained at after thawing and TRT, variables following a normal distribution were analyzed using the Paired Samples t-test, whereas variables not following a normal distribution were evaluated using the Wilcoxon Signed-Rank test. For data that do not follow a normal distribution, the median was used as a measure of central tendency, and the interquartile range (IQR) was used to describe the distribution. This approach provides a more accurate and robust summary of the data distribution. A P-value of less than 0.05 was considered statistically significant, whereas P-values greater than 0.05 were interpreted as indicating no statistically significant difference.

RESULTS

A comprehensive summary of the spermatological parameters following different thawing protocols (slow and rapid thawing) at after thawing and thermo-resistance test is presented in Table 1.

Table1. Mean (±SD) semen parameters for bull semen samples slow thawed (ST) group at 37°C for 30 sec and rapid thawed (RT) group 70°C for 6 sec, after thawing and TRT, respectively.

Parameters	Groups	Semen samples thawed and incubation					
		Thawing			TRT		
		Mean±SD	Median	IR	Mean±SD	Median	IQR
Motility	**ST**	72.61±10.55^{a}	71.66	18.09	42.15±16.36*	42.32	26.16
	RT	73.35±11.32^{a}	70.18	21.53	29.16±13.32	26.60	26.06
Progressive motility	**ST**	48.99±9.69^{a}	45.50	18.97	21.81±16.02***	23.25	31.99
	RT	36.44±29.26^{b}	42.74	65.76	8.29±11.28	1.41	14.12
VCL	**ST**	88.79±13.10^{a}	-	-	50.27±24.94***	-	-
	RT	98.23±16.54^{a}	-	-	21.80±9.22	-	-
VAP	**ST**	61.79±9.05^{b}	61.87	13.55	41.03±24.20***	45.92	40.99
	RT	66.53±12.36^{b}	62.79	21.97	13.85±9.60	9.79	10.95
VSL	**ST**	48.22±7.99^{a}	-	-	31.26±20.96*	-	-
	RT	50.87±10.97^{a}	-	-	16.77±18.53	-	-
STR	**ST**	65.28±4.07	-	-	61.68±12.12	-	-
	RT	66.10±4.26	-	-	67.34±8.85	-	-
LIN	**ST**	46.61±5.98	46.60	10.96	47.14±15.03	46.33	19.15
	RT	46.29±8.28	49.55	13.73	45.37±16.01	44.30	20.05
WOB	**ST**	65.09±6.62	-	-	62.95±14.00	-	-
	RT	65.04±10.96	-	-	57.71±12.50	-	-
ALH	**ST**	3.21±0.54^{b}	3.07	0.86	1.75±0.52*	1.69	0.72
	RT	3.43±1.04^{a}	3.22	1.62	1.20±0.17	1.22	0.33
BCF	**ST**	6.84±1.08^{b}	6.68	1.88	4.45±1.76	4.69	3.01
	RT	7.30±1.64^{a}	6.65	2.22	3.34±1.72	2.57	2.91
Viability	**ST**	69.05±11.95^{b}	67.50	18.50	44.05±15.25*	46.50	26.00
	RT	79.25±7.93^{***b}	75.50	15.50	33.40±12.54	28.50	24.00
Plasma membrane integrity	**ST**	60.85±11.36^{b}	-	-	40.25±15.69	-	-
	RT	81.05±6.68^{**a}	-	-	36.20±11.15	-	-

*P<0.05, **P<0.01, ***P<0.001: significant differences between groups
^{c}P<0.05, ^{b}P<0.01, ^{a}P<0.001: significant differences between incubation times within groups

Comparisons between the two groups at after thawing revealed no significant differences in motility, progressive motility, VCL, VAP, VSL, STR, LIN, WOB, ALH, and BCF parameters (P>0.05 for all). However, significant differences were detected in positive viability (P<0.001***) and plasma membrane integrity (P<0.01**) values at after thawing, indicating that sperm viability and functional membrane integrity were initially different between the groups.

At after TRT, several parameters exhibited significant intergroup differences. Progressive motility, VAP, and VCL values were significantly higher in the slow thawing group compared to the rapid thawing group (P<0.001***), while motility, VSL, ALH, and viability values also differed significantly (P<0.05*). In contrast, STR, LIN, WOB, BCF, and plasma membrane integrity parameters did not show significant intergroup differences at after TRT (P>0.05), suggesting that these variables remained relatively stable between the evaluated conditions.

In the slow thawing group, significant reductions were observed in motility, progressive motility, VCL,

VAP, and VSL parameters following TRT ($P<0.001^a$ for motility, progressive motility, VCL, and VSL; $P<0.01^b$ for VAP). Similarly, ALH and BCF values significantly decreased after TRT ($P<0.01^b$), reflecting notable impairments in sperm kinematic properties over time. Sperm viability also deteriorated, as evidenced by a significant decrease in live eosin percentages ($P<0.01^b$). Moreover, plasma membrane integrity values demonstrated significant alterations between after thawing and TRT ($P<0.01^b$). In contrast, no significant changes were observed in STR, LIN, and WOB parameters ($P>0.05$), indicating relative stability in LIN and BCF indices within this group.

In the rapid thawing group, similar patterns of deterioration were observed. Motility, VCL, and VSL values markedly decreased after TRT ($P<0.001^a$), alongside significant reductions in VAP and progressive motility ($P<0.01^b$). Additionally, ALH and BCF values exhibited substantial decreases ($P<0.001^a$), indicating pronounced alterations in sperm movement dynamics over time. Regarding viability significantly decreased ($P<0.01^b$), consistent with a decline in overall sperm viability after TRT. Plasma membrane integrity values also showed highly significant changes ($P<0.001^a$), further supporting the evidence of compromised functional integrity. As with the slow thawing group, STR, LIN, and WOB parameters did not demonstrate significant differences between after thawing and TRT in the rapid thawing group ($P>0.05$).

DISCUSSION AND CONCLUSION

For many years, frozen spermatozoa have mostly been thawed at body temperature (Mehmood et al., 2017; Ogata et al., 2022; Ömür, 2022). Frozen spermatozoa are often thawed at body temperature to minimize ice recrystallization damage, which is crucial in preserving sperm function (Sharafi et al., 2022). Since the harmful temperature zone in frozen spermatozoa often occurs at a critical level, between -60 and 0 °C, due to the harmful recrystallization of intracellular ice, it is necessary to pass through the dangerous range as soon as possible during thawing, as it passes from the glassy phase to the liquid phase around the melting point. It has been standard procedure for many years to thaw frozen spermatozoa at body temperature (DeJarnette et al., 2000; Mehmood et al., 2017; Ogata et al., 2022). Due to the detrimental recrystallisation of intracellular ice, the hazardous temperature zone in frozen spermatozoa frequently occurs at a critical level, between -60 and 0 °C. As the spermatozoa thaw, it must pass through the dangerous range as quickly as possible, transitioning from the glassy phase to the liquid phase around the melting point. Moreover, recent cryobiology research emphasizes that rapid transition through this critical zone can limit irreversible damage to cellular membranes and organelles (Watson, 2000; Vutyavanich et al., 2010).

This study evaluated the fertility prediction potential of different thawing methods (37°C for 30s and 70°C for 6s) of bull semen by means of spermatological parameters, HOST and thermal resistance test. According to the results obtained, no significant differences were observed in many spermatological parameters, especially motility and progressive motility, between the two methods at thawing. However, significant advantages were found in the rapid thawing method (70°C/6s) in sperm viability and plasma membrane integrity parameters. Studies comparing different thawing temperatures report that rapid thawing protocols can yield significantly better motility recovery compared to slower ones. This has been attributed to the reduced exposure time within the critical temperature zone and minimized ice recrystallization (Calamera et al., 2010; Kumar et al., 2020). This finding shows that short-term thawing at high temperature is effective in preserving the cellular and membrane integrity of sperm. Similar findings are consistent with previous studies emphasizing that rapid thawing contributes to membrane stabilization (Nguyen et al., 2023).

After the TRT, i.e. in the later minutes of incubation (30th min), the superiority of the slow thawing method was observed among the kinetic parameters of sperm, especially in progressive motility, VAP and VCL values. This suggests that thawing at low temperatures allows sperm to maintain its kinetic and progressive motility for a longer time. These differences in the parameters after incubation support the view stated in the literature that slow thawing can better preserve the metabolic activity of spermatozoa (Zenteno et al., 2023).

During the incubation process, significant decreases in the motility and some kinematic parameters (VCL, VSL) and membrane functionality of spermatozoa are observed, emphasizing the importance of using spermatozoa in a short time after thawing. The observation of these decreases in both thawing methods reveals the general sensitivity of sperm to thermal and osmotic stresses. The significant decrease in movement dynamics, especially ALH and BCF, indicates that cellular energy sources are depleted over time and spermatozoon movement dynamics are impaired accordingly (Morrell et al., 2020; Güngör et al., 2021).

Post-thaw sperm viability and plasma membrane integrity are critical indicators of semen quality and fertilizing potential. In this study, both parameters showed significant deterioration after incubation regardless of the initial thawing protocol. Although viability and membrane integrity were initially higher in semen thawed at RT, these values declined more sharply following incubation

compared to ST, indicating a more fragile post-thaw profile. The observed reductions may be attributed to cumulative oxidative stress, membrane lipid phase transitions, and mitochondrial dysfunction, which are exacerbated during incubation (Purdy, 2006; Yeste, 2016). Spermatozoa are particularly vulnerable to cryo-induced damage due to the high proportion of polyunsaturated fatty acids in their membranes, leading to compromised integrity and viability during thawing and subsequent storage (Maldjian et al., 2005). While RT thawing may temporarily stabilize membranes by avoiding abrupt temperature gradients, the long-term resilience of sperm under incubation stress appears reduced, possibly due to accelerated metabolic exhaustion and ATP depletion (Martin-Hidalgo et al., 2019). These findings align with previous reports suggesting that post-thaw handling and incubation conditions are as critical as the thawing process itself in maintaining sperm functional competence.

In conclusion, the study findings reveal that rapid thawing is initially advantageous in terms of membrane integrity, but slow thawing is more effective in preserving kinetic properties in the long term. These findings support that the thawing method should be selected depending on the intended use of the sperm and that different thawing methods create significant effects in fertility estimation. In future studies, evaluating the relationships between stress markers at the molecular level and fertilization success will help to understand the effects of thawing methods on fertility more comprehensively.

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Data Availability: The datasets used and analyzed for this research are available from the corresponding author (BE) upon reasonable request.

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