Effect of different thawing temperatures on viability, kinematic parameters and progressive motility of frozen goat sperm

MOHAMED BESBACI*[1], AMINE ABDELLI[2], MOUNIR MEBARKI[#3], ABDELAZIZ LOUNAS[#1], SABRINA SELLALI[1], NORHANE SARAH KETFI[1], AMINA BOUGUELMANI1[1], IBRAHIM BELHENNICHE[1]

[1] Laboratory of Biotechnology in Animal Reproduction (LBRA), Institute of Veterinary Sciences, University of Blida 1, 09,000, Blida, Algeria.
[2] Department of Agricultural Sciences, Laboratory of Management and Valorization of Natural Resources and Quality Assurance (LGVRNAQ), University of Bouira, 10,000, Bouira, Algeria.
[3] Department of Veterinary Science, Veterinary Sciences and Agricultural Sciences Institute, University of Batna 1, Algeria.
[#] These two authors contributed equally to this work.

SUMMARY

Caprine artificial insemination (AI) is generally based on the use of frozen semen straws, cryopreserved in liquid nitrogen. Thawing straws requires appropriate methods. The aim of the current study was to determine the best among different practical methods of thawing on the total motility, progressive motility, vitality and kinematic parameters of caprine semen such as: (i) curvilinear velocity (VCL) ; (ii) average path velocity (VAP) ; (iii) straight line velocity (VSL) ; (iv) amplitude of lateral head displacement (ALH) ; (v) beat cross-frequency (BCF) ; (vi) wobble coefficient (WOB, %) ; (vii) linearity (LIN, %) ; and (viii) straightness (STR, %). All of these parameters were measured during a short-term incubation period, in order to adopt the most efficient method for preserving sperm qualities. We thawed goat semen straws by four (04) different methods: 37 °C for 40 seconds (s), 50 °C for 15 s, 70 °C for 5 s, and by using hot air (HA) at ±60 °C for 10 s. The frozen semen was analyzed by the Computer Assisted Semen Analyzer (CASA) system. Our findings highlighted the existence of a significant effect of the thawing method on the sperm mobility and vitality, and came up with the most reliable method. In conclusion, on one hand, the method of thawing at 50 °C/15 s gave better results concerning the total motility and vitality of goat frozen sperm. On the other hand, the 70 °C/5 s thawing method was observed to show better performances regarding progressive mobility and dynamic parameters. Very poor results were obtained after thawing with HA±60 °C/10 s. After two hours of post-thaw incubation at 50°C/15 s, higher proportions of spermatozoa vitality and spermatozoa with fast and progressive movement were observed. However, it is unknown whether this minor difference affects *in vivo* fertilizing ability of thawed caprine frozen semen straws.

KEY WORDS

Artificial insemination, thawing, CASA, mobility, vitality, goat.

INTRODUCTION

Caprine artificial insemination (AI) was used to disseminate the most valuable genes to improve the production of milk, hair and meat [1]. Cryopreservation, as a technique of goat semen storage, supports a genome resource bank for an indeterminate period of time [2]. The cryopreservation of mammalian sperm is a complex process that involves balancing many factors in order to obtain satisfying results. To ensure even minimal success, not only proper extender, semen dilution rate, cooling rate and thawing rate are required, but also a sophisticated knowledge of the species semen particularities is essential to maximize post-thaw recovery of sperm, and consequently its fertilizing ability [3]. Nevertheless, freezing and thawing processes induce detrimental effects on the ultra-structure, biochemistry, and functional integrity of the sperm [4], resulting in a reduction of motility, membrane integrity and fertilizing ability [3]. The technique of straws thawing is an important factor that affects semen quality parameters [5]. Usually, straws of frozen goat semen are thawed at 37 °C in a water bath for 12-30s [5,7]. Deka and Rao (1987) [7] used another slower protocol (5 °C/2 min in water bath) with demonstrated lesser efficiency, whereas Tuli et *al.* (1991) [5] observed significantly higher progressive motility on goat semen thawed at 70 °C/7 s, compared with the thawing rates of 37 °C/2 min and 40 °C/20 s.

Sperm progressive motility has conventionally been considered as a good indicator of motility and a key functional parameter, essential for fertilization [8]. Forward progressive motility is thought to be required for spermatozoa efficient passage through the uterotubal junction for oviductal reservoir colonization. Spermatozoa can be graded as progressively motile,

Corresponding Author:
Mohamed Besbaci (besbaci@univ-blida.dz; besbaci@gmail.com)

non-progressive (exhibiting only lateral head displacement), and immotile [9].

The objective of this study was to assess the rates of four thawing protocols, faster than the one usually recommended for straws of frozen goat semen. The four chosen thawing protocols were placing the straws into a water bath at i) 37 °C/40 s, ii) 50 °C/15 s, iii) 70 °C/5 s, and iv) using hot air (HA) ±60 °C/10 s.

MATERIALS AND METHODS

Goat frozen semen

Commercial goat semen straws, frozen at the National Center of Artificial Insemination and Genetic Amelioration **(CNIAAG)** - Algiers, were used for this study. In brief, the semen was diluted with a commercial extender (BioXcell®, IMV Technologies, France), packed into 0.25 ml plastic straws containing $20x10^6$ spermatozoa per straw. The semen was cooled gradually from 4°C to crystallization at 5cm from the surface of liquid nitrogen. After 15 min in static vapor, the straws were immersed in liquid nitrogen in which semen was conserved at -196°C.

Sperm preparation and post-thawing incubation

The semen straws used in this experimentation originated from the same ejaculations of two different goat breeds, Saanen and Alpine. In total, 24 (12 Saanen and 12 Alpine) goat semen straws cryopreserved in liquid nitrogen were used. They were divided into four batches of six straws for each thawing method; in each repetition, two straws (one of each breed) were assessed. The thawing of semen was done by immersing the semen straws in a water bath at different temperatures: (i) 37 °C/40s, (ii) 50 °C/15s, (iii) 70 °C/5 s, or by heating them with HA ±60 °C/10 s. The content of both straws was then mixed and diluted 1:1 with physiological saline buffer (NaCl, 0.9%). In each test, two straws were used-with three repetitions being performed for each thawing method.

Sperm motility variables

For each sample, two semen preparation drops and three microscopic fields were analyzed (approximately 600 spermatozoa) on a pre-warmed glass slide using Sperm Class Analyzer, and a light microscope, with a x10 negative phase objective at 37 °C. The percentage of motile spermatozoa was calculated, and progressive motility with kinematic characteristics were studied. For each spermatozoa, the following motion characteristics were measured in kinematic parameters: (i) curvilinear velocity (**VCL**): average velocity of motile sperm over the distance travelled, including all deviations of sperm head movement; (ii) average path velocity (**VAP**): average velocity over a smoothed path; (iii) straight line velocity (**VSL**): average velocity calculated using the straight line distance between the beginning and end of the sperm track; (iv) amplitude of lateral head displacement (**ALH**): mean values of the extreme side to side movement of the sperm head in each beat cycle; (v) beat cross-frequency (**BCF**): frequency in which the actual track crosses the smoothed track; (vi) wobble coefficient (**WOB**, %): oscillation measure of the actual path of the sperm head in its relationship with the VAP (VAP/VCL) x 100); (vii) linearity (**LIN**, %): percentage of linearly motile sperm as a ratio of (VSL/VCL) x 100; and (viii) straightness (**STR**, %): percentage of straight motile sperm as a ratio of (VSL/VAP) x 100. The motility analyses were carried out in two steps: T0 (directly after thawing) and T2 (after two hours of incubation at 37°C) for each thawing protocol.

Sperm viability assessment

Viability was assessed by counting 200 cells per field by photonic microscopy (CKX41; Olympus, Tokyo, Japan) at x 400 enlargement. A small drop (10 µL) of semen was placed on a pre-warmed slide, then mixed with a drop (10 µL) of each of eosin and nigrosine stains to prepare a thin and uniform smear (by spreading the mix with a slipping movement of a second slide). Spermatozoa with red coloration on the head were considered dead. For each thawing protocol, vitality analyses were performed in two stages: T0 (immediately after thawing) and T2 (after two hours of incubation at 37°C).

Statistical analysis

Repeated-measures analysis of variance (ANOVA) in R software (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria) via RStudio (version 1.1.383, RStudio Inc., Boston, MA) was performed for all measured variables. Post-hoc comparisons were computed using the lsmeans function from the lsmeans package in R. The results were represented by the last square means (LSM) ± standard error of mean (SEM). A probability (P) value less than 0.05 ($P < 0.05$) was considered as statistically significant.

RESULTS

Post-thaw sperm variables that consisted of total and progressive sperm motility, vitality and kinematic parameters during a short-term incubation period are shown in Tables 1 and 2.

The percentage of sperm motility according to straws thawing methods was significantly different ($P<0.001$). At the beginning of incubation (time=0h), the total motility for 37 °C/40 s and 50 °C/15 s methods was significantly higher compared to HA ±60 °C/10 s. However, there was no difference between 70 °C/5 s and HA ±60 °C/10s methods. After two hours of incubation, the highest values of total motility were obtained with straws thawed using 50 °C/15 s method. In contrast, the overall sperm progressive motility was unaffected by the thawing methods ($P=0.133$). Further, there was a significant effect of the thawing methods on the percentage of sperm vitality ($P=004$). Spermatozoa thawed using 50 °C/15 s and 70 °C/5 s methods yielded a higher percentage of vitality after tow-hour incubation ($P<0.05$) than those thawed using 37 °C/40 s and ha ±60 °C/10 s methods. Total sperm motility, progressive motility and vitality changed significantly over time. An interaction ($P= 0.015$) between method and time was observed only for motility (Table 1).

Similarly to the total motility, all the kinematic parameters were significantly influenced by the thawing methods ($P<0.01$). Immediately after thawing, 70°C/5 s method showed total higher values of kinematic parameters compared to the other methods. Indeed, our findings showed that the highest values of VAP, VSL, STR, WOB and LIN were obtained with spermatozoa thawed using 70 °C/5 s method ($p<0.05$). Two hours after thawing, VCL, VAP and ALH were higher in spermatozoa thawed using 70 °C/5 s method, whereas VSL, STR, WOB, LIN and BCF were higher in spermatozoa thawed using 70 °C/5 s, 50 °C/15

Table 1 - Percentage of sperm (±SE) motility, progressive motility and vitality of frozen goat semen straws thawed at 37 °C/40s, 50 °C/15s, 70 °C/5s and with hot air ±60 °C/10s, after 0 and 2 h of incubation at 37 °C.

		Incubation time (h)		SE	P-value		
	Thawing technique	0h	2h		Thawing technique	Time	Interaction
Motility (%)	37 °C/40s	**56.1 a**	**15.1 a**	4.75	<0.001	<0.001	0.015
	50 °C/15s	**65.0 a**	**59.3 b**				
	70 °C/5s	**52.5 ab**	**33.3 c**				
	HA±60 °C/10s	**39.6 b**	**16.3 a**				
Progressive motility (%)	37 °C/40s	**26.91 ab**	15.71	4.24	0.133	0.001	0.153
	50 °C/15s	**16.42 a**	14.79				
	70 °C/5s	**32.84 b**	16.83				
	HA±60 °C/10s	**20.73 ab**	11.69				
Vitality (%)	37 °C/40s	56.5	**34.2 a**	5.56	0.004	0.002	0.324
	50 °C/15s	64.3	**61.5 b**				
	70 °C/5s	64.2	**53.0 b**				
	HA±60 °C/10s	52.2	**32.7 a**				

a, b, c: Significant differences can be seen between different superscripts within the same row. h: hour. SE: standard error. HA: hot air.

s or HA ±60 °C/10 s methods (Table 2). The eight kinematic parameters expressing the velocity of sperm and their behavior while moving were relatively similar (as revealed by the statistical analysis).

DISCUSSION

Findings from this study indicate significant difference among applied thawing protocols, in terms of total post-thaw sperm motility, progressive motility, vitality and kinematic parameters during a short-term incubation period. Traditionally, a straw is thawed in a water bath at 37 °C for 12 to 30s [7]. This method seemed better (Average motility of 36.1% after 4 h incubation) than a slow thaw method (18.9% motility), in which the sperm straw was placed in a 5°C water bath for 2 min [7]. The increase of thawing temperature to 70 °C, and the thawing of sperm straw for only 7s resulted in a much higher progressive motility (36.9%) compared with straws that thaw at 37 °C during 2 min (31.5%) or at 40° C during 20s (32.4%) [5].

In terms of thawing rates, higher temperatures (50°C for 15 s and 70°C for 5 s) resulted in greater sperm quality than 37°C (for 40 s). Total and progressive sperm motility were consistently and significantly better for the fast thawing protocol. According to Malo et al. (2019) [10], total and progressive motility at T=0 h and T=1 h (P 0.001) was higher for 60°C thawing temperature compared to 37°C. Moreover, our findings are in line with those of Iaffaldano et al. (2016) [11]; according to which thawing turkey sperm at 50°C for 10 s was more proficient than 4°C for 5 min. The current study's observation of a positive relationship between sperm motility and thawing rate supports Ahmad's (1984) [12] contention that faster thawing protocols were associated with improved sperm motility and acrosomal integrity.

The modification of thawing protocols becomes more critical at temperatures superior than 37° C, as such elevated temperatures can result in huge sperm mortalities if improperly performed [5]. In a bull semen study, Aamdal and Andersen (1968) [13] reported that 75°C thaw temperature for 12s resulted in a 27.9% higher live sperm rate. Lahnsteiner (2000) [14] recommended thawing at elevated temperatures to avoid recrystallization, since warming damage generally occurs when sperm pass through the critical stage of -50 to -15 °C or -5 °C. Likewise, spermatozoa seem to suffer from osmotic stress, when the thawing time is insufficient for the excess cryoprotectants to flow out of the cell, and the sperm swells and lyses when the medium suddenly gets diluted due to the melting of extracellular ice. This study showed also that faster (50 °C for 15 s and 70 °C for 5 s) thawing protocols resulted in higher post-thaw viability. Conversely, the slowest thaws (37 °C for 40 s) resulted in the lowest post-thaw viability. This is in agreement with the previous studies [15,16], which revealed that post-thaw fertilizing ability of spermatozoa is greatly affected by thawing temperature and duration [17].

Remarkably, when the thaw is performed at 37° C, after two-hour incubation, sperm mobility decreased considerably. Yet, it was not the case for 50° C thawing temperature, and the mobility decrease was less significant at 70 °C. This indicates that spermatozoa suffered more from severe latent damage during slow thawing than during rapid thawing, and the thaw at 50° C was less damaging than at 70 °C [18].

The kinematic parameters significantly varied depending on the thaw method used. Spermatozoa showed faster and more active movement (higher VAP, VSL, STR, WOB and LIN) with 70° C thaw compared to 37° C, 50° C or HA. Such an increased flagellar activity may have caused early exhaustion of sperm, resulting in a significant kinematic parameters' decrease after incubation [18].

Further, the current study found that thawing at 70°C for 5 seconds significantly improved the sperm parameters defined by progressive motility and kinematic measurements such as linear motility, in comparison with the control rate obtained at 37°C for 40 seconds.

These results are in concordance with reports by Rastegarnia

Table 2 - Percentage of sperm (±SE) motility characteristics for frozen goat semen straws thawed at 37 ºC/40s, 50 ºC/15s, 70 ºC/5s and with hot air ±60 ºC/10s, after 0 and 2 h of incubation at 37 ºC.

Sperm motility parameters	Thawing technique	Incubation time (h)		SE	P-value		
		0h	2h		Thawing technique	Time	Interaction
VCL (m/s)	37 ºC/40s	**53.8 a**	**26.2 a**	0.795	<0.001	<0.001	<0.001
	50 ºC/15s	**43.2 b**	**45.0 b**	0.696			
	70 ºC/5s	**52.8 a**	**50.9 c**	0.827			
	HA±60 ºC/10s	**48.0 c**	**41.1 b**	0.763			
VAP (m/s)	37 ºC/40s	**37.3 a**	**13.5 a**	0.681	<0.001	<0.001	<0.001
	50 ºC/15s	**27.8 b**	**27.6 b**	0.596			
	70 ºC/5s	**39.6 c**	**30.3 c**	0.709			
	HA±60 ºC/10s	**32.8 d**	**22.1 d**	0.654			
VSL (m/s)	37 ºC/40s	**27.86 a**	**8.16 a**	0.612	<0.001	<0.001	<0.001
	50 ºC/15s	**18.91 b**	**18.95 b**	0.536			
	70 ºC/5s	**30.34 c**	**19.77 b**	0.637			
	89 ºC/10s	**23.36 d**	**14.09 c**	0.588			
STR (VSL/VAP)	37 ºC/40s	**0.57 a**	**0.47 a**	0.008	0.07	<0.001	<0.001
	50 ºC/15s	**0.56 a**	**0.56 b**	0.007			
	70 ºC/5s	**0.62 b**	**0.51 a**	0.008			
	HA ±60 ºC/10s	**0.57 a**	**0.49 a**	0.007			
WOB (VAP/VCL)	37 ºC/40s	**0.60 a**	**0.49 a**	0.005	<0.001	<0.001	<0.001
	50 ºC/15s	**0.60 a**	**0.54 b**	0.005			
	70 ºC/5s	**0.67 b**	**0.54 b**	0.006			
	HA ±60 ºC/10s	**0.61 a**	**0.50 a**	0.005			
LIN (VSL/VCL)	37 ºC/40s	**0.41 a**	**0.26 a**	0.007	<0.001	<0.001	<0.001
	50 ºC/15s	**0.39 b**	**0.35 b**	0.006			
	70 ºC/5s	**0.47 c**	**0.33 bc**	0.007			
	HA ±60 ºC/10s	**0.40 ab**	**0.30 ac**	0.007			
BCF (Hz)	37 ºC/40s	**5.98 a**	**4.00 a**	0.102	<0.001	0.727	<0.001
	50 ºC/15s	**5.01 b**	**5.76 b**	0.089			
	70 ºC/5s	**6.19 a**	**5.51 bc**	0.106			
	HA ±60 ºC/10s	**4.66 c**	**5.10 c**	0.098			
ALH (µm)	37 ºC/40s	**1.97 a**	**1.42 a**	0.027	0.03	0.11	<0.001
	50 ºC/15s	**1.89 bc**	**1.94 b**	0.023			
	70 ºC/5s	**1.85 b**	**2.16 c**	0.028			
	HA ±60 ºC/10s	**1.95 ac**	**1.93 b**	0.026			

a, b, c: Significant differences can be seen between different superscripts within the same row. h: hour. SE: standard error. HA: hot air.
VCL: curvilinear velocity. VAP: path velocity. VSL: straight line velocity. ALH: amplitude of lateral head displacement. BCF: beat cross-frequency. WOB: wobble coefficient. LIN: STR: straightness.

et al. (2013) [19] that stated the linear trend of increased sperm velocities (VSL and VAP) and LIN after thawing at 70 . The plasma membrane integrity, the acrosomal ridge, and the motility characteristics of buffalo spermatozoa are all significantly affected by freezing and thawing process (visual or computerized motility, and curvilinear velocity) [20]. The post-thaw decrease in curvilinear velocity may be brought about by cryoinjuries to spermatozoa's mitochondrial apparatus and axoneme [21].
Findings of the kinematic parameters in this study congruent with similar studies conducted in other species, in which such relationships have been considered. When thawing at 70 for 5 s, the velocity kinematics (VAP, VSL, and VCL) showed greater values. Nagy et al. (2015) [22] found that VAP was the most important value in fertility prediction. Furthermore, the role of VSL in fertility has been documented in humans [23], dogs [24], and donkeys [25]. Gillan et al. (2008) [26] hypothesized that VSL may play a role in sperm transport along the female reproductive tract. In mice, the importance of VCL for reservoir development at the utero-tubal junction was established [27]. The most often utilized parameters to stipulate hyperactive sperm are high VCL and ALH levels and low LIN values. In the current investigation, we found no changes in ALH. These results were found to be accordant with the study by Eriksson and Rodriguez-Martinez (2000) [28], whose findings indicated that faster thawing protocols resulted in better post-thawing quality for boar spermatozoa than slower ones, whereas Muio et al. (2008) [18] found no differences between fast and slow protocols for bull semen. As for buck sperm, different temperatures in thawing protocols have been tested by various authors [5,7].
Our results showed that after an *in vitro* incubation period of two hours, the kinematic parameters varied significantly, and indicated that, most frequently, the swimming speed of spermatozoa had decreased. It is worth underlining an exception to this rule for samples thawed at 50ºC, whose movement is faster and more gradual after incubation. Thus, our results suggested that thawing at 50ºC was less cyto-harmful than thawing at 70ºC, 35ºC or by HA.
In contrast, studies on rams [29], bulls [18], boars [28] and dogs [30] have shown better post thaw quality in faster thawing method then slower one.

CONCLUSION

Semen thawing method at 50°C gave better results regarding total motility and viability of goat sperm. The method of thawing at 70°C was more efficient in matter of progressive mobility and dynamic parameters of individual motility (VCL, VSL, VAP, LIN and STR); however, results were similar to those obtained by thawing at 50°C regarding viability. According to this study, the most effective method would be 70°C/5 s, and should be adopted for thawing goat semen straws.

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