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COMPARISON BETWEEN RAPID AND SLOW CRYOPRESERVATION PROTOCOLS FOR RAM SEMEN

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Abstract: Information regarding adequate freezing protocols of ram semen considering freezing distance and time during cryopreservation has not been adequately reported. Therefore, this study aimed to compare two freezing protocols for Najdi ram's semen. In the rapid freezing protocol, the straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. While in the slow freezing protocol, the straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes. The semen was collected from five rams and extended with tris egg yolk glycerol cryodiluent. The extended semen was chilled slowly to 5^0C within two hours and equilibrated for two hours before being frozen on the liquid nitrogen vapor and cryopreserved at -196^0C. There was no significant ($P > 0.005$) effect of the freezing protocol on the sperm's total motility, plasma membrane integrity, DNA integrity, and abnormalities. However, the vitality, fast progressive motility, straight-line velocity, average pathway velocity, linearity, and wobble were significantly higher in rapid freezing than in slow freezing protocol. In conclusion, cryopreservation of ram semen using rapid freezing (5 cm for 15 mins) protocol was better than slow freezing (8 cm for 20 mins) protocol regarding post-thawing semen quality.

Key words: freezing protocol; cryopreservation; egg yolk; ram; semen

Introduction

Cryopreservation of semen is one of the essential technologies in animal research for enhancing productivity and reproductive efficiency (1). Semen cryopreservation helps to prolong sperm livability and vitality by slowing metabolism and preventing bacterial development, reducing the accumulation of metabolic byproducts (2), (3). Cryopreservation of mammalian sperm is a complex procedure involving balancing several elements to produce the best results. Diluents, dilution, cooling, freezing, and thawing procedures all have a role in the success of ram semen cryopreservation (4), (5). Compared to other species, ram spermatozoa have a low intramembrane cholesterol-to-phospholipid ratio. Therefore, cold-shock sensitivity in ram spermatozoa is higher than in other species (6).

The freezing protocol aims to gradually lower the semen temperature from 5 to −196°C to avoid sperm damage. The freezing rate regulates the extent and rate of sperm dehydration and ice crystal formation. These two main variables affect sperm freezing success; ice crystal formation is more dangerous than sperm dehydration. In the

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slow freezing protocol, sperm exposes to damage because of high solute concentrations for a long period which results in cell dehydration and volume contraction; however, minimum intracellular ice crystal formation has occurred. In the rapid freezing protocol, sperm exposes to damage because of intracellular ice crystal formation; however, minimum dehydration has occurred. Therefore, the optimum freezing protocol must be slow enough to minimize intracellular ice crystal formation and rapid enough to minimize sperm dehydration (7).

The acrosome, nucleus, mitochondria, axoneme, and plasma membrane are affected by rapid temperature variations because of the creation and dissolution of ice during the freezing and thawing processes (8), (9). Temperature changes during cooling and freezing cause stress on sperm membranes, resulting in lipid phase shifts and a difference in the functional condition of sperm membranes. Cold shock damage occurs when sperm is rapidly cooled from 30 to 4 °C (10). The phase of supercooling (0°C to −5°C) and the development of ice crystals (−6°C to −15°C) are the two main temperature ranges where sperm are damaged during freezing (11). Temperatures between 5° and −15°C are known to cause a significant phase change (12), and this could be the perfect temperature range for temperature-dependent damage. The sperm contents remain unfrozen at −10°C; while, the exterior surrounding media freezes between −5 and −10°C. Therefore, water flows out of the sperms osmotically and freezes outside (13). For the first time, Polge (14) and Mazur (15) reported that the crucial temperature range in which the most sperm destruction occurs is between −15 and −30°C and extended to −60°C and −80°C. Transferring the semen from +5°C to −100°C within 7 min at a rate of −15°C/min is recommended (16). Sperm freezing at a rate of 15–60°C/min is recommended and resulting in a good survival rate. A freezing rate of ≥ 20°C/min for ram is effective and recommended.

Semen is diluted with a cryoprotectant extender to minimize ice crystal formation and to avoid sperm damage (17). The proper freezing protocol aims to minimize intracellular ice crystal formation; therefore, it must be slow enough to allow water to leave the cells yet fast enough to prevent severe cell dehydration and the solu-tion effect (18). Therefore, this study aimed to know the best freezing protocol for Najdi ram's semen according to the freezing distance and time using the liquid nitrogen vapor (rapid freez-ing of 5 cm for 15 mins and slow freezing of 8 cm for 20 mins).

Materials and methods

Management of Animals

The experiment was conducted in the fall using five mature Najdi rams aged 2-4 years with an average body condition score of 3 at the Experimental Farm, Department of Animal Production, King Saud University, Riyadh, Saudi Arabia (latitude 24° 48′ N and longitude 46° 31′ E). The rams were sheltered in a covered yard within an open-sided barn. The ram's daily energy and protein needs were met with commercial mixed pellets (14.5 percent CP; 2.78 Mcal ME kg-1DM). All rams were found to be viable, and assessment of their ejaculates using computer-assisted sperm analysis (CASA) revealed normal parameters. The King Saud University Research Ethics Committee (REC) authorized the current project with Ethical Reference No: KSU-SE-21-33. This authorization was based on the advice of the Research Ethics Sub-Committee (minute number 7 and date 22/04/2021), as well as a suitable risk-to-benefit ratio and a study design that minimizes risks.

Preparation of extender

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA). This study prepared and utilized a Tris-hydroxymethyl-aminomethane, monohydrated citric acid, and D-fructose extender reported in previous studies (19)pigeon (P. Briefly, 250 mM of Tris, 88.5 mM of citric acid, and 69.38 mM of fructose were dissolved in 100 mL of distilled water to create the buffer. The buffer was then supplemented with 18 % chicken egg yolk and 8 % glycerol. Gentamicin was administered at a dosage of 13.3 mg mL^{-1}.

Collection and evaluation of semen

The artificial vagina was used to collect semen samples twice weekly from each ram (20). The semen samples were assessed macroscopically and microscopically using a sperm class analyzer (SCA®; version 4.0.0.5, Microptic S.L., Barcelona, Spain). For further processing, only ejaculates that met the following parameters were used: 1.5 ml volume, score 4 on mass activity grade scores, 80% progressive motility, and sperm concentration of 2×10^9 ml^{-1}). The ejaculates were pooled to exclude individual ram differences. Seven pooled ejaculates were used in this study.

Semen Extension and Freezing

The pooled ejaculates were gradually diluted 1:4 with tris egg yolk glycerol diluent. The diluted semen was gradually chilled for less than two hours, from 30 to 5°C. The diluted and cooled semen was placed into 0.25 ml straws using a semiautomatic filling and capping machine (minitube GmBH, Tiefenbach, Germany) and left at 5^0C for 2 hours to equilibrate the glycerol according to (21). After reaching equilibrium, the straws were frozen in liquid nitrogen vapor using two different protocols. On the rapid freezing protocol, the straws were hung at 5 cm over the surface of liquid nitrogen for 15 minutes. While on the slow freezing protocol, the straws were hung at 8 cm over the surface of liquid nitrogen for 20 minutes. The straws were then preserved at -196 °C after submerging in liquid nitrogen.

Semen Evaluation

The semen samples were evaluated on two occasions. The first was after achieving equilibrium (before freezing) and the second was at least 48 hours after cryopreservation. The frozen straws were thawed for thirty seconds in a 37 °C water bath. The following parameters of the sperm were evaluated (16), (21)

Sperm cell motility

SCA® was used to estimate the speed of each spermatozoon in three ways including the average pathway velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL). The sperms were classified as rapidly progressive, moderately progressive, sluggish, or static. For evaluating progressiveness on a relative scale, the straightness (STR=VSL/VAP), linearity (LIN=VSL/VCL), and wobble (WOB=VAP/VCL) metrics were calculated and reported as percentages. The amplitude of the lateral head displacement (ALH) and the transverse beat frequency (BCF) were also measured. The sperms velocities were categorized to rapid (VCL >75 µm/s), medium (45 < VCL < 75 µm/s), slow (10 < VCL < 45 µm/s) or static (VCL < 10 µm/s). The sperm presenting movement with a STR index ≥80% was considered progressive motile. The results were categorized as rapid progressive (A), slow progressive (B), non-progressive (C) or static (D) according to WHO 4th edition.

Livability (Vitality)

Using a FluoVit kit, fluorescence microscope, and SCA vitality software (Microptic S.L., Barcelona, Spain), the percentages of live/dead spermatozoa were determined.

Sperm morphology assessment and morphometry

The dry semen smears were stained for 1–2 min with New Rapid SpermBlue® fixative/stain (Microptic S.L., Barcelona, Spain). The stained smears were viewed with SCA® under a microscope at 600x magnification (33). Morphometries of sperms were measured including head length (µm), head width (µm), head area (µm^2), head perimeter (µm), acrosome (%), elongation, ellipticity, regularity, rugosity, midpiece width (µm), midpiece area (µm^2), distance (µm), and angle (°).

Plasma membrane integrity of spermatozoa

A hypo-osmotic swelling test was used to determine the functional integrity of the plasma membrane of spermatozoa via incubating 10 µl of semen with 100 µl of a 190 mOsM hypo-osmotic solution for 40 minutes at 37 °C in a 1.5-ml Eppendorf tube. On a warm slide, 100 µL of the liquid was distributed with a cover slide and examined under a microscope at 400x magnification. At least 200 sperms were evaluated. The percentage of spermatozoa with inflated and curled tails was calculated.

Sperm DNA fragmentation

The Halotech DNA kit was used to determine the degree of DNA fragmentation in ram sperm.

The samples of sperm were diluted to a concentration between 5 and 10x10^{6} mL-1. The agarose gel from the kit was incubated at 90-100°C for 5 minutes to fuse the agarose and then at 37°C for 5 minutes in an adjustable water bath. 25μ L of semen sample was added to the Eppendorf tube, along with the gel, and thoroughly mixed. Twenty microliters of the solution were deposited on a super-coated slide from the package, then placed on a cool surface and covered with a 22x22-mm coverslip. Slides were refrigerated for 5 minutes at 4 °C to produce a micro-gel containing an implanted sperm. Carefully removing the coverslips, the slides were immersed for 7 minutes in the previously prepared acid solution (80 L HCl in 10 mL of distilled water), according to (22). The slides were then transferred to a tray containing a lysing solution from the kit and incubated for 25 minutes, followed by moistening with distilled water and dehydrating in increasing ethanol concentrations for two minutes (70, 90, and 100 percent). Slides were stained with Giemsa or Wright's stain, washed with tap water, and dried at 25°C. Each slide was viewed at 100x magnification using a light microscope, and 200 spermatozoa were counted. A spermatozoon without fragmented DNA was placed in an agarose matrix and treated with lysing solutions to deproteinize the nucleus and form dispersed DNA halos. Halos refer to DNA loops loosely attached to the remaining nuclear structure (nucleus). Sperm nuclei with fragmented DNA produced small or no halos of scattered DNA, while nuclei without fragmented DNA shed DNA, forming large halos.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) to compare between two freezing protocols. The data were presented as mean ± standard error. Differences were considered significant at $P < 0.05$.

Results

The effects of using different freezing protocols on the motility grades of post-thawed Najdi ram sperms post thawing is presented in figure 2. The grade A (fast progressive) motility was significantly (P 0.05) higher in the rapid freezing protocol than in the slow freezing protocol. While grades B, C, and D motilities were not significantly ($P > 0.05$) different in the two protocols.

Effects of different freezing protocols on the vitality, plasma membrane integrity, and DNA integrity of the post-thawed Najdi ram sperms are presented in Figure 3. Plasma membrane integrity and DNA integrity of the post-thawed Najdi ram sperm were not significantly ($P > 0.05$) different in the two protocols. While, vitality was significantly ($P \leq 0.05$) higher in the rapid freezing protocol than in the slow freezing protocol.

The effects of the freezing protocol on post-thawed abnormalities and morphometry of Najdi ram sperms are presented in Table 2. The all-sperm morphometric parameters and sperm abnormalities of post-thawed Najdi ram semen were not significantly ($P > 0.05$) differed between the two protocols, rapid (5 cm in 15 mins) and slow (8 cm in 20 mins).

Discussion

Our results revealed that the percentages of fast-progressive motility, VSL, VAP, LIN, WOB, and vitality were significantly higher in the rapid freezing protocol than in the slow freezing protocol. However, the ALH was significantly higher in the slow freezing protocol than in the rapid freezing protocol. The velocities (rapid, medium, slow and static), grades B, C, and D motilities, all-sperm morphometric parameters, sperm abnormalities, plasma membrane integrity and DNA integrity of post-thawed Najdi ram semen were not significantly differed between the two protocols. These results agree with previous researches who concluded that slow freezing appears to be the most crucial aspect of the ram semen preservation procedure (23, 24, 25). Our results agree with previous researches which reported that Freezing semen straws 4–5 cm above liquid nitrogen for 4–5 min resulted in satisfactory post-thawing quality (26, 27).

The decreasing of post-thawed semen quality after using slow freezing protocol in our experiment can be explained by the sever sperm dehydration. While, in rapid freezing rates acceptable sperm dehydration and intracellular ice crystals were obtained. Our explanation agrees

Table 1: Effects of using different freezing protocols on the degree of motility and kinetic parameters of post-thawed Najdi ram sperms

parameter	Freezing protocol	
	Rapid*	Slow$^{\#}$
Total motility (%)	65.07±1.09	64.10±1.06
Total progressive (%)	24.15±0.96	22.79±0.94
Fast progressive (%)	12.63±0.60^{a}	10.66±0.59^{b}
Slow progressive (%)	11.52±0.81	12.14±0.79
Curvilinear velocity (VCL; µm s−1)	50.75±0.34	50.05±0.32
Straight-line velocity (VSL; µm s−1)	30.05±0.29^{a}	28.49±0.27^{b}
Average pathway velocity VAP (µm s−1)	39.19±0.31^{a}	37.84±0.29^{b}
Lateral head displacement (ALH; µm)	2.49±0.01^{b}	2.56±0.01^{a}
Beat frequency (BCF; Hz)	3.67±0.02	3.64±0.02
Linearity (LIN=VSL/VCL) (%)	48.01±0.22^{a}	46.98±0.20^{b}
Straightness (STR=VSL/VAP) (%)	63.49±0.22	63.12±0.20
Wobble (WOB=VAP/VCL). (%)	70.23±0.15^{a}	69.13±0.14^{b}

a, b mean ± the standard error that carrying different superscripts in the same row differs at $P < 0.05$. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes.

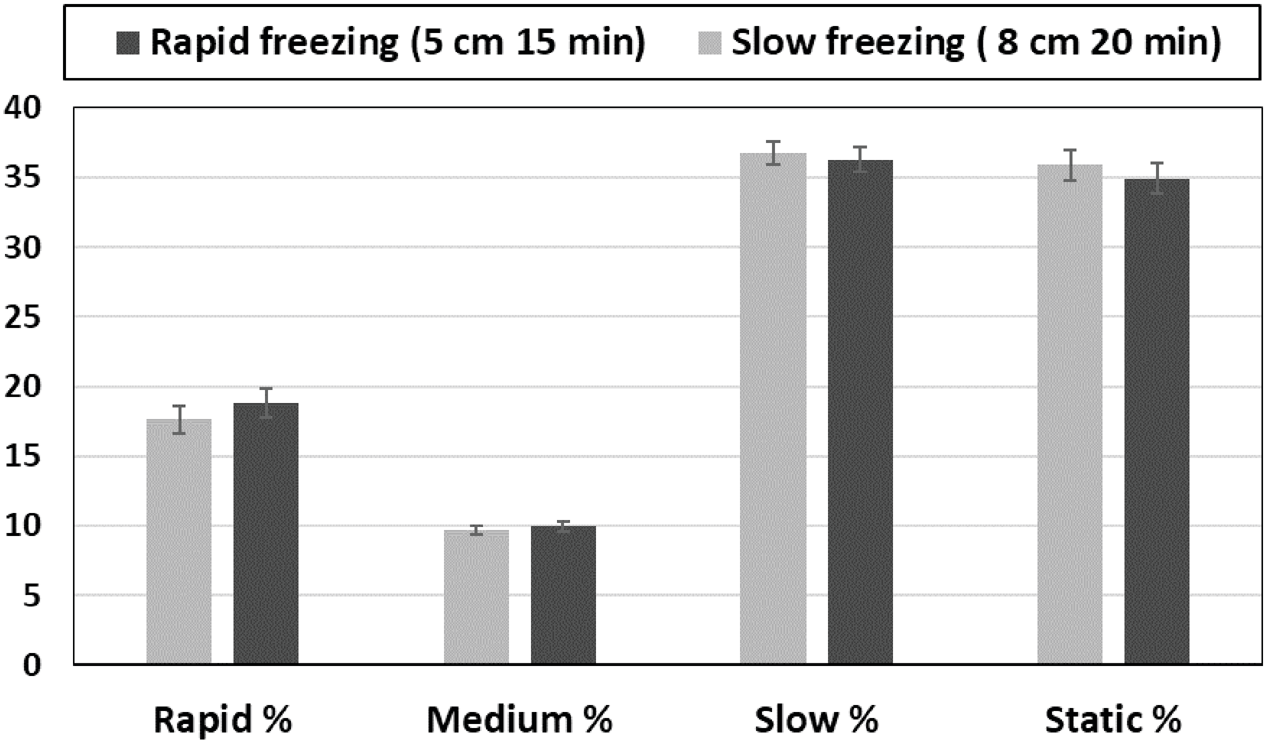

Figure 1: Effects of different freezing protocols on the percentages of the post-thawing velocity of Najdi ram sperm (mean ± standard error)

*The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes. The sperms velocities were categorized to rapid (VCL >75 µm/s), medium (45 < VCL < 75 µm/s), slow (10 < VCL < 45 µm/s) or static (VCL < 10 µm/s).

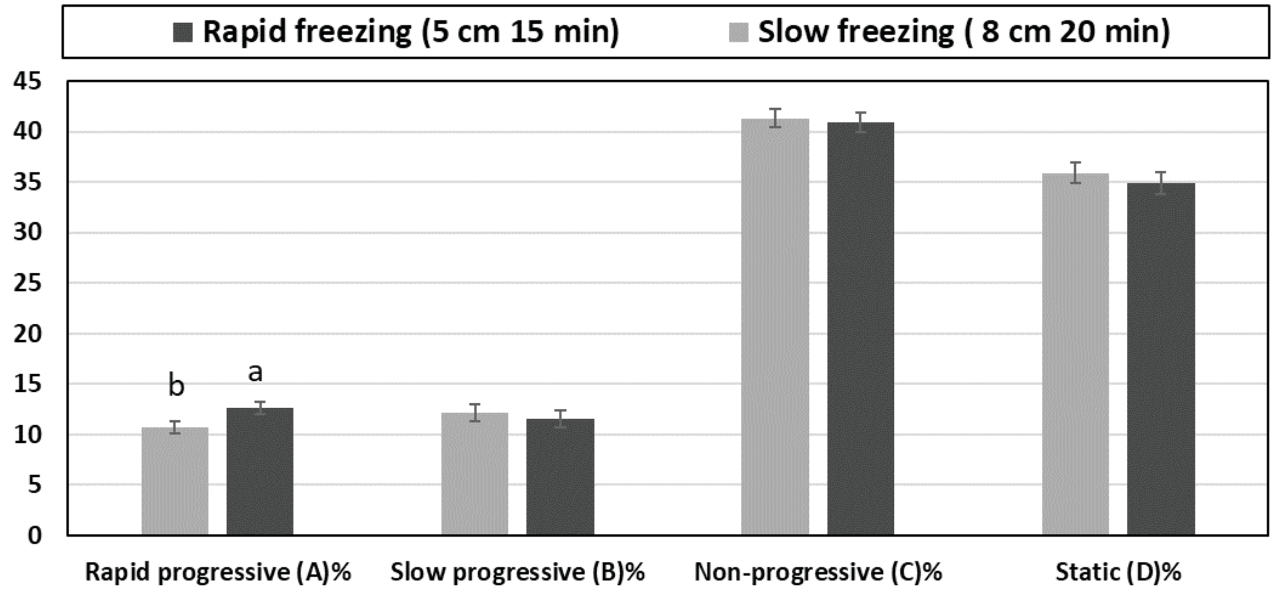

Figure 2: Effects of different freezing protocols on the percentages of the post-thawing motility grades (A, B, C, and D) of the Najdi ram sperms

a,b Means ± standard error carrying different superscripts within the same parameter differed at P<0.05. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes. The motility results were categorized as rapid progressive (A), slow progres-sive (B), non-progressive (C) or static (D) according to WHO 4th edition.

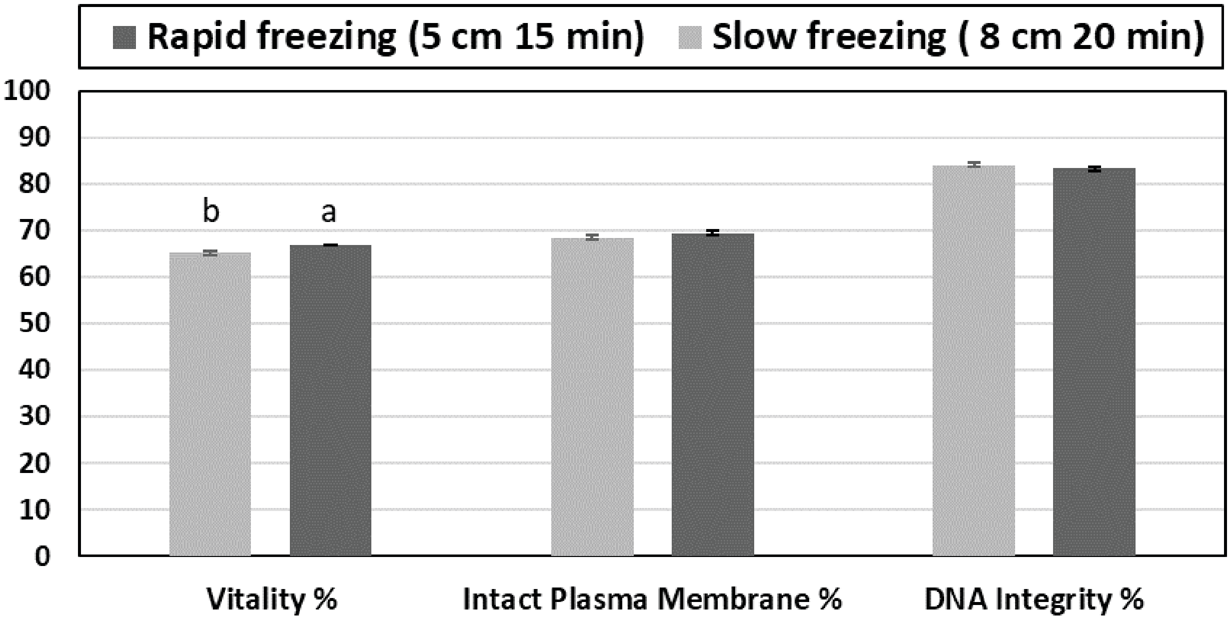

Figure 3: Effects of different freezing protocols on the percentages of post-thawing vitality, plasma membrane integrity and DNA integrity of Najdi ram sperms

a,b Means ± standard error carrying different superscripts within the same parameter differed at P<0.05. *The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. #The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes.

Table 2: Effects of different freezing protocols on the percentage of sperm abnormalities after thawing and measurement of the morphology of the thawed Najdi ram spermatozoa

parameter	Freezing protocol	
	Rapid[*]	Slow[#]
Sperm Abnormalities (%)	16.80±0.37	16.09±0.37
Head length (µm)	10.79±0.44	10.91±0.44
Head width (µm)	5.12±0.12	4.94±0.12
Head area (µm^2)	41.72±1.39	42.50±1.39
Head perimeter (µm)	28.72±0.90	29.36±0.90
Acrosome (%)	77.27±3.44	74.21±3.44
Elongation	0.34±0.02	0.37±0.02
Ellipticity	2.12±0.09	2.25±0.09
Regularity	1.04±0.02	1.00±0.02
Rugosity	0.66±0.02	0.64±0.02
Midpiece width (µm)	1.78±0.25	1.86±0.25
Midpiece area (µm^2)	4.55±0.52	5.48±0.52
Distance (µm)	1.10±0.24	0.98±0.24
Angle (*)	20.45±5.55	20.52±5.55

[a,b] Means ± standard error carrying different superscript letters within the same row differed at $P < 0.05$. [*]The straws were frozen at 5 cm over the surface of liquid nitrogen for 15 minutes. [#]The straws were frozen at 8 cm over the surface of liquid nitrogen for 20 minutes.

with the explanation of previous research which reported that Rapid freezing leads to intracellular water crystallization, which might induce fewer cell damage than slow freezing, which produces severe dehydration (28).

In a manual freezing, the semen straws are placed on horizontal rack and frozen for 8–10 minutes in the vapor of liquid nitrogen at 4–6 cm above the level of liquid nitrogen (−75°C to −125°C). When the temperature drops below 5°C and nears to −10°C, the intracellular water freezes, putting sperms at risk of ice crystals formation.

Chemineau et al. (29) reported that 0.25 ml straws should be frozen 16 cm above liquid nitrogen for 2 minutes before being lowered to 4 cm for 3 minutes before being plunged into liquid nitrogen for storage. Pontbriand et al. (30) found that temperature variations of 6-24°C/min and 10-100°C/ min were acceptable, suggesting that ram sperm can tolerate a wide range of freezing rates. The temperature dropped at a controlled and programmed rate while utilizing an automatic freezing machine, from 4 to −5°C at 20°C/min, −5 to −110°C at 55°C/min, and −110 to −140°C at 35°C/min (31). Sperm cells are often frozen at a high rate (15–60°C/min), resulting in the best post-thawing results (31). Semen is slowly chilled at a rate of 0.1°C/min from water-bath temperature to 5° C and then frozen at a rate of 10–60° C/ min to temperatures as lower as −80°C before being stored in liquid nitrogen (32). Before plunging into liquid nitrogen, the final temperature should be reduced to at least −130° C, regardless of whether the freezing is done slowly or quickly, to stop all metabolic processes, including thermally driven chemical changes (33).

Post-thaw sperm motility, mitochondrial function, membrane integrity, and viability decreased after cryopreservation and thawing. Cryopreservation of ram sperm alters choline glycerophospholipids (34), (35). After cryopreservation, glycerophospholipids containing 22:6n-3 disappear (36). Cryopreservation impairs mitochondrial activity and damages mitochondria by removing two essential lipids (cardiolipin with 18:2n-6 and phosphatidylethanolamine with 20:4n-6) (36). After cryopreservation, sperm membrane lipids lose sterols, and sphingomyelin species with long-chain PUFA decrease (36). These lipid alterations influence most post-thawing parameters.

Conclusion

The two freezing protocols can be used to freeze ram semen. However, rapid freezing (5 cm for 15 minutes) was better than slow freezing (8 cm for 20 minutes) in ram semen cryopreservation. Beside saving time, rapid freezing protocol can improve the post-thawed ram sperm vitality, fast progressive motility, straight-line velocity, average pathway velocity and linearity.

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