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Frozen Boar Semen Can Be Successfully Stored at −80°C, Reducing Need for LN_2 Storage Containers

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ABSTRACT

This study explores alternative freezing approaches aimed at reducing or avoiding the use of liquid nitrogen (LN_2) to store boar semen. Sperm-rich fractions from three boars were pooled and frozen in 0.25- and 0.5-mL straws using four different protocols: a conventional method (Control; LN_2 vapours followed by immersion and storage in LN_2), FT1 (as Control but stored at −80°C), FT2 (LN_2 vapours followed by storage at −80°C), and FT3 (direct transfer from 5°C to −80°C plus storage at this temperature). Sperm quality and intracellular H_2O_2 production were evaluated at 30- and 150-min after thawing. Sperm motility and viability in FT1 and FT2 were similar to Control, whereas FT3 showed poorer sperm quality and higher intracellular H_2O_2 generation. Straw size also influenced cryosurvival, being lowest in 0.5 mL straws. In addition, sperm quality deteriorated alongside post-thaw incubation time. In contrast, storage time (one week vs. one month) did not show a clear effect on the evaluated parameters. In conclusion, these findings suggest that freezing protocols based on LN_2 vapour cooling, combined with storage at −80°C, could be a practical alternative for short-term storage of boar semen, reducing LN_2 use.

1 | Introduction

Sperm cryopreservation is a key assisted reproductive technology (ART) in pigs, as it allows genetic preservation, improves biosecurity and facilitates the exchange of germplasm worldwide. It also supports other ARTs, such as in vitro fertilisation, embryo production and genome editing (Knox 2011; Yeste et al. 2017). Despite these advantages, its practical use is still limited by factors such as the high cost of liquid nitrogen (LN_2), logistical challenges and handling risks. Moreover, boar sperm are particularly susceptible to cryopreservation due to a high proportion of polyunsaturated fatty acids in their plasma membrane, which makes sperm more vulnerable to cold shock and oxidative stress (Cerolini et al. 2000).

In recent years, alternative approaches have been explored, including freeze-drying in rodents (Kaneko 2015) and storage at ultra-low temperatures (−80°C) in species such as goat (Batista et al. 2008), dog (Pezo et al. 2017), and mouse (Raspa et al. 2018). Nevertheless, strategies that reduce or avoid LN_2 use in boar sperm remain limited, most likely because storage at −80°C (above the glass transition temperature) in combination with boar sperm plasma membrane composition may cause severe deterioration in quality and compromise long-term sperm storage (Mazur 1984). This study aimed to evaluate alternative freezing and storage protocols to reduce or eliminate LN_2 use, particularly for storage. Specifically, the effects of three freezing protocols combined with storage at −80°C on sperm quality and functionality were assessed.

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2 | Materials and Methods

All procedures performed in this study were approved by the Bioethics Committee of the University of Murcia (research code CBE-774/2025) and were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1 | Animals and Semen Collection

Three healthy, mature and fertile boars (two Duroc and one Landrace × Large White) were used. A total of 12 sperm-rich fractions (SRF; one per boar, four replicates) were collected using the gloved-hand method. Samples were extended 1:1 (v/v) in Beltsville Thawing Solution (BTS) and stored at 17°C for 24 h. Only ejaculates with $>200\times10^6$ sperm/mL, ≥ 75% total motility and ≥ 85% normal morphology were included.

2.2 | Cooling, Freezing and Thawing

The SRFs collected from each boar were pooled to avoid individual boar effects and cryopreserved following the protocol described by (Parrilla et al. 2025). Briefly, samples were centrifuged (2400× *g*, 3 min, 17°C) and pellets re-extended to 1500×10^6 sperm/mL in cooling extender [80% (v/v) β-lactose (310 mM), 20% egg yolk, 80 μg/mL kanamycin]. After cooling to 5°C for 120 min, samples were re-extended to 1000×10^6 sperm/mL in freezing extender [89.5% cooling extender supplemented with 9% (v/v) glycerol and 1.5% (v/v) Equex STM]. Semen was loaded into 0.25- and 0.5-mL straws and assigned to a Control [LN_2 vapours (10/20 min), plunged into LN_2 and stored in LN_2], and three treatment protocols FT1 [LN_2 vapours (10/20 min), plunged into LN_2 but stored at −80°C], FT2 [LN_2 vapours (10/20 min) and storage at −80°C], and FT3 [direct freezing to and storage at −80°C]. Prior to thawing at 37°C for 20 s, samples stored at −80°C were moved to LN_2, thus enabling proper comparison of post-thaw variables with Control samples which were thawed under the same conditions (37°C for 20 s). Additionally, the potential need to transfer semen samples from −80°C storage to liquid nitrogen for field application, was considered in choosing this thawing protocol of this thawing protocol for FT1, FT2 and FT3 samples. Two replicates per straw size and treatment were analysed at each time point. Samples were pooled by straw size and treatment, extended 1:1 (v/v) in BTS at 37°C, and maintained at 37°C in the dark until analysis.

2.3 | Sperm Assessment

Post-thaw sperm quality (motility and viability) and functionality (intracellular H_2O_2 generation in viable sperm) were evaluated at 30- and 150-min of incubation time as follows: as previously described (Li et al. 2018). Motility was assessed using a CASA system, including total (average path velocity ≥ 20 μm/s) and progressive motility (straight-line velocity ≥ 40 μm/s). Viability, acrosome integrity (H-42/PI/FITC-PNA), and intracellular H_2O_2 production (H-42/PI/CM-H_2DCFDA) were evaluated by flow cytometry (10,000 sperm/sample). Viable spermatozoa with intact acrosome were identified as H-42-positive and PI- and PNA-negative (live sperm). Intracellular H_2O_2 production in viable sperm were expressed as CM-H_2DCFDA mean fluorescence units per 10^4 live sperm (FU-H_2O_2).

2.4 | Statistical Analysis

Statistical analyses were performed using R software. For each response variable, linear mixed-effects models were fitted including treatment, straw size, storage time, post-thaw incubation time and their interactions as fixed effects, and replicate as a random intercept. This approach was selected because the experiment had a factorial structure and multiple observations were obtained within the same experimental replicate; therefore, linear mixed-effects models allowed the simultaneous assessment of the experimental factors while accounting for the non-independence of observations within replicate. Non-significant higher-order interactions were removed sequentially while preserving model hierarchy. Model assumptions were evaluated by visual inspection of residual diagnostic plots, including residuals versus fitted values and Q–Q plots. Fixed effects were assessed using Type III Wald χ^2 tests. When significant effects were detected, pairwise comparisons of estimated marginal means were performed with Tukey adjustment for multiple comparisons. Results are expressed as mean ± standard deviation, and statistical significance was set at $p<0.05$.

3 | Results

Results are presented in Figure 1 and Table 1. No significant interactions among treatment, straw size, post-thaw incubation time, and storage time were detected for any of the variables evaluated. Treatment, specifically FT3, affected all variables ($p<0.001$). Compared to the Control, FT1 and FT2 showed similar sperm motility and live-sperm values, whereas FT3 consistently showed lower values for both variables. FT3 also showed higher FU-H_2O_2 values than the other treatments, with no differences among the remaining groups, regardless of straw size, incubation time, or storage time. Straw size did not affect motility or FU-H_2O_2, but influenced live sperm ($p<0.001$), with lower values in 0.5 mL straws in most groups. Post-thaw incubation time negatively affected motility and live sperm ($p<0.001$), with lower values at 150 min, whereas FU-H_2O_2 remained unaffected. Storage time had no consistent effect on any of the variables evaluated.

4 | Discussion

To the best of our knowledge, this is the first study evaluating freezing protocols for boar semen that could reduce or eliminate LN_2 use combined with storage at −80°C in boar sperm. FT1 and FT2 maintained sperm quality comparable to the control, whereas FT3 showed impaired performance. However, values in FT3 remained within acceptable ranges reported for

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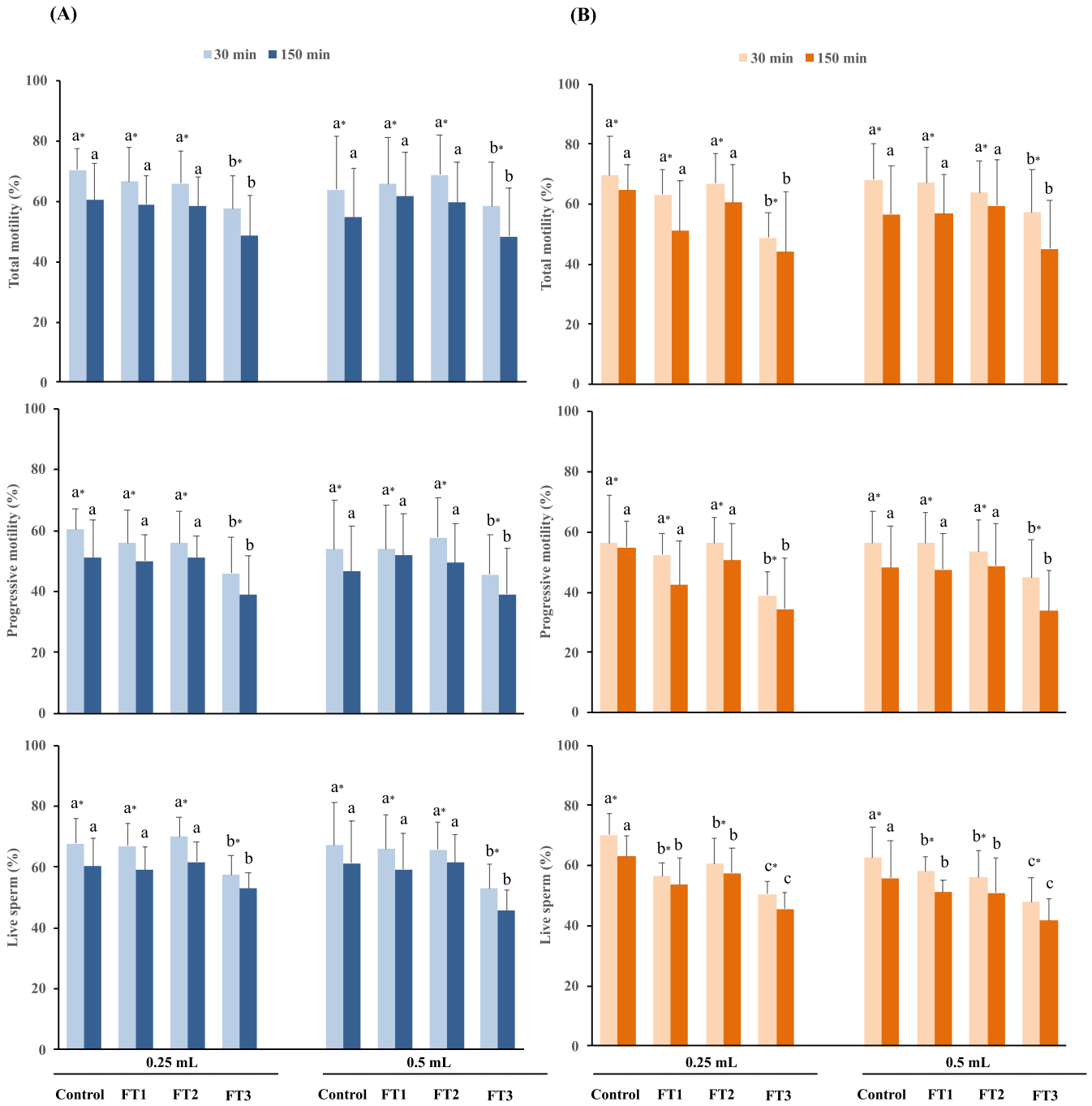


FIGURE 1 | Sperm quality parameters (means ± SD) evaluated at 30- and 150-min post-thaw after one week (A) and one month (B) of storage. Different lowercase letters within the same straw size (0.25- and 0.5-mL) and incubation time indicate significant differences among treatments (Control, FT1, FT2 and FT3) ($p < 0.001$). Asterisks within the same treatment and straw size indicate significant differences between incubation times ($p < 0.001$). Control [LN_2 vapours (10/20 min), plunged into LN_2 and storage in LN_2 containers; $n = 4$], FT1 [LN_2 vapours (10/20 min), plunged into LN_2 and storage at −80°C; $n = 4$], FT2 [LN_2 vapours (10/20 min) and storage at −80°C; $n = 4$], and FT3 [direct freezing to −80°C; $n = 4$].

conventional boar semen cryopreservation (Martinez et al. 2019; Pérez-Patiño et al. 2019), suggesting that reduced LN_2-based approaches may be a feasible alternative.

Treatment, specifically FT3, was the main factor affecting sperm performance. FT1 and FT2 were comparable to the control, indicating that LN_2 use during freezing can be reduced if an appropriate cooling strategy is applied. A reasonable explanation for this finding could be the fact that spermatozoa in FT1 and FT2 are subjected to rapid cooling rates which reduce cellular injury due to reduced solution and ice crystal effects (Morris et al. 2012). In contrast, the poorer results in FT3 suggest that avoiding LN_2 vapour cooling may lead to suboptimal cooling rates and increased cryodamage (Mazur 1984; Pezo et al. 2017). This is consistent with the higher intracellular H_2O_2 generation (FU-H_2O_2) observed in FT3, suggesting increased oxidative stress under these conditions. Elevated H_2O_2 production has been linked to membrane damage and reduced sperm function after thawing (Aitken and Baker 2004; Catalán et al. 2024).

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TABLE 1 | Intracellular H_2O_2 production in viable sperm (FU/10^4 live sperm; means ± SD) according to treatment, straw size and post-thaw incubation time in frozen–thawed boar semen.

	Intracellular H_2O_2 production in viable sperm (FU/10^4 live sperm)			
Straw size (mL)	0.25		0.5	
Incubation time (min)	30	150	30	150
Treatment (week of storage)				
Control	107.1 ± 28.0[a]	96.2 ± 35.2[a]	122.2 ± 66.5[a]	113.5 ± 41.0[a]
FT1	112.5 ± 27.7[a]	140.9 ± 76.7[a]	127.3 ± 45.8[a]	100.3 ± 36.9[a]
FT2	114.9 ± 32.3[a]	152.9 ± 65.9[a]	128.8 ± 56.4[a]	155.1 ± 87.4[a]
FT3	179.4 ± 75.4[b]	170.1 ± 83.9[b]	182.9 ± 90.0[b]	163.4 ± 78.6[b]
Treatment (month of storage)				
Control	107.4 ± 31.8[a]	100.7 ± 35.3[a]	123.5 ± 34.4[a]	141.5 ± 69.2[a]
FT1	152.3 ± 31.7[a]	145.1 ± 75.9[a]	133.7 ± 40.7[a]	132.8 ± 35.4[a]
FT2	124.0 ± 34.0[a]	129.5 ± 44.6[a]	125.8 ± 30.2[a]	139.0 ± 51.2[a]
FT3	187.9 ± 54.0[b]	168.0 ± 68.5[b]	185.3 ± 65.5[b]	176.9 ± 58.6[b]
Probability				
Treatment	< 0.001			
Straw size	NS			
Incubation time	NS			
Storage time	NS			

Note: Lowercase letters indicate significant differences among treatments within the same straw size, incubation time and storage period (week or month) ($p < 0.001$). Control [LN_2 vapours (10/20 min), plunged into LN2 and storage in LN_2 containers; $n = 4$], FT1 [LN_2 vapours (10/20 min), plunged into LN2 and storage at −80°C; $n = 4$], FT2 [LN_2 vapours (10/20 min) and storage at −80°C; $n = 4$], and FT3 [direct freezing to −80°C; $n = 4$]. Probability values for main effects are shown below.

Straw size also influenced sperm viability, which may be explained by differences in heat transfer during freezing. Larger volumes generally cool more slowly and less uniformly, increasing the risk of cryodamage (Jiang et al. 2007).

Sperm quality declined with longer post-thaw incubation time. This is a well-known effect in cryopreserved boar sperm and is usually associated with membrane instability and the accumulation of metabolic stress after thawing (Holt and Medrano 1997).

Storage time did not show a clear effect within the period studied, suggesting that storage at −80°C for up to one month does not markedly impair sperm quality. However, since this temperature is above the glass transition point, some residual molecular activity may still occur, potentially leading to gradual cellular damage over time (Mazur 1984).

In order to enable proper comparisons between Control and Treatments after thawing and further in an effort to approximate these protocols to a practical application, the experimental groups were thawed after exposing the straws to −196°C (LN_2), since most likely this temperature could be required for transport or longer storage periods before thawing. This implies a rapid cooling from an incomplete freezing state (−80°C) to a deeply frozen state (−196°C), which may modify ice crystal size and distribution, thereby damaging sperm membranes and impairing sperm quality (Bwanga et al. 1991). Future studies to better understand the effects of the warming step on sperm survival and to design alternative thawing procedures are needed.

In conclusion, FT1 and FT2 appear to be suitable alternatives to the Control conventional protocols, allowing a reduction in LN_2 use while maintaining sperm quality during short-term storage. Further work is needed to refine these approaches and improve post-thaw sperm function.

Author Contributions

A.L.-J., I.P. and E.A.M. conceived and designed the study. A.L.-J., X.T., M.G.-C., M.A.G., C.C. and I.P. performed the experiments. A.L.-J., I.P. and E.A.M. analysed and interpreted the data. A.L.-J. wrote the primary manuscript. H.R.-M., E.A.M. and I.P. revised, discussed and edited the manuscript. All authors revised and approved the manuscript for publication. H.R.-M., I.P. and E.A.M. obtained the funding to carry out the study.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Aitken, R. J., and M. A. Baker. 2004. "Oxidative Stress and Male Reproductive Biology." *Reproduction, Fertility, and Development* 16, no. 5: 581–588. https://doi.org/10.10371/RD03089.

Batista, M., T. Niño, D. Alamo, et al. 2008. "Successful Artificial Insemination Using Semen Frozen and Stored by an Ultrafreezer in the Majorera Goat Breed." *Theriogenology* 71: 1307–1315. https://doi.org/10.1016/j.theriogenology.2008.12.024.

Bwanga, C. O., H. Ekwall, and H. Rodriguez-Martinez. 1991. "Cryopreservation of Boar Semen. III: Ultrastructure of Boar Spermatozoa Frozen Ultra-Rapidly at Various Stages of Conventional Freezing and Thawing." *Acta Veterinaria Scandinavica* 32, no. 4: 463–471. https://doi.org/10.1186/BF03546946.

Catalán, J., I. Yánez-Ortiz, M. Torres-Garrido, et al. 2024. "Impact of Seminal Plasma Antioxidants on DNA Fragmentation and Lipid Peroxidation of Frozen–Thawed Horse Sperm." *Antioxidants* 13, no. 3: 322. https://doi.org/10.3390/antiox13030322.

Cerolini, S., A. Maldjian, P. Surai, and R. Noble. 2000. "Viability, Susceptibility to Peroxidation and Fatty Acid Composition of Boar Semen During Liquid Storage." *Animal Reproduction Science* 58, no. 1–2: 99–111. https://doi.org/10.1016/S0378-4320(99)00035-4.

Holt, W., and A. Medrano. 1997. "Assessment of Boar Sperm Function in Relation to Freezing and Storage." *Journal of Reproduction and Fertility. Supplement* 52: 213–222. https://doi.org/10.1530/biosciprocs.15.0015.

Jiang, Z. L., Q. W. Li, W. Y. Li, J. H. Hu, H. W. Zhao, and S. S. Zhang. 2007. "Effect of Low Density Lipoprotein on DNA Integrity of Freezing–Thawing Boar Sperm by Neutral Comet Assay." *Animal Reproduction Science* 99, no. 3–4: 401–407. https://doi.org/10.1016/J.ANIREPROSCI.2006.08.022.

Kaneko, T. 2015. "Simple Sperm Preservation by Freeze-Drying for Conserving Animal Strains." *Methods in Molecular Biology* 1239: 317–329. https://doi.org/10.1007/978-1-4939-1862-1_19.

Knox, R. V. 2011. "The Current Value of Frozen–Thawed Boar Semen for Commercial Companies." *Reproduction in Domestic Animals* 46, no. Suppl. 2: 4–6. https://doi.org/10.1111/J.1439-0531.2011.01822.X.

Li, J., I. Barranco, A. Tvarijonaviciute, et al. 2018. "Seminal Plasma Antioxidants Are Directly Involved in Boar Sperm Cryotolerance." *Theriogenology* 107: 27–35. https://doi.org/10.1016/J.THERIOGENOLOGY.2017.10.035.

Martinez, C. A., J. M. Cambra, C. Maside, et al. 2019. "High Pre-Freezing Sperm Dilution Improves Monospermy Without Affecting the Penetration Rate in Porcine IVF." *Theriogenology* 131: 162–168. https://doi.org/10.1016/J.THERIOGENOLOGY.2019.04.001.

Mazur, P. 1984. "Freezing of Living Cells: Mechanisms and Implications." *American Journal of Physiology* 247, no. 3 Pt 1. https://doi.org/10.1152/AJPCELL.1984.247.3.C125.

Morris, G. J., E. Acton, B. J. Murray, and F. Fonseca. 2012. "Freezing Injury: The Special Case of the Sperm Cell." *Cryobiology* 64: 71–80. https://doi.org/10.1016/j.cryobiol.2011.12.002.

Parrilla, I., M. A. Gil, C. Cuello, and E. A. Martinez. 2025. "Sperm Cryopreservation in Boars." *Methods in Molecular Biology (Clifton, N.J.)* 2897: 183–192. https://doi.org/10.1007/978-1-0716-4406-5_13.

Pérez-Patiño, C., J. Li, I. Barranco, et al. 2019. "The Proteome of Frozen-Thawed Pig Spermatozoa Is Dependent on the Ejaculate Fraction Source." *Scientific Reports* 9, no. 1: 705. https://doi.org/10.1038/s41598-018-36624-5.

Pezo, F., C. Cheuquemán, P. Salinas, and J. Risopatrón. 2017. "Freezing Dog Semen Using −80°C Ultra-Freezer: Sperm Function and In Vivo Fertility." *Theriogenology* 99: 36–40. https://doi.org/10.1016/J.THERIOGENOLOGY.2017.05.007.

Raspa, M., M. Fray, R. Paoletti, L. Montoliu, A. Giuliani, and F. Scavizzi. 2018. "A New, Simple and Efficient Liquid Nitrogen Free Method to Cryopreserve Mouse Spermatozoa at −80°C." *Theriogenology* 119: 52–59. https://doi.org/10.1016/j.theriogenology.2018.06.020.

Yeste, M., J. E. Rodríguez-Gil, and S. Bonet. 2017. "Artificial Insemination With Frozen-Thawed Boar Sperm." *Molecular Reproduction and Development* 84, no. 9: 802–813. https://doi.org/10.1002/MRD.22840.

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