Received: 11 March 2024 | Accepted: 17 May 2024

DOI: 10.1111/rda.14626

Reproduction in Domestic Animals WILEY

SHORT COMMUNICATION

High cooling rate of 60°C/min around ice nucleation during cryopreservation compromises chicken sperm viability

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Funding information
Consejo Superior de Investigaciones Científicas; Arquimea Agrotech, Grant/Award Number: CON22-225

Abstract

The present study compares two protocols for the cryopreservation of chicken semen. Both protocols had an initial low cooling rate in the first step, followed by higher cooling rates around ice nucleation (Protocol 1) or following the dissipation of the latent heat of fusion (Protocol 2) in the second step. Semen ejaculates obtained from 12 roosters were diluted with Rootex with 6% dimethylformamide and frozen following either Protocol 1 (from +5°C to −10°C at 5°C/min and from −10°C to −130°C at 60°C/min) or Protocol 2 (from +5°C to −35°C at 7°C/min and from −35°C to −140°C at 60°C/min). Compared with fresh semen, following both protocols, cryopreservation resulted in reduced post-thaw sperm quality ($p < .001$). Post-thaw percentage of sperm with an intact plasma membrane was greater using Protocol 2 than Protocol 1 ($p < .05$). The results suggest that high cooling rates around the time of ice nucleation are not recommendable.

KEYWORDS
chicken, cooling rate, freezing, sperm

1 | INTRODUCTION

During cryopreservation of semen, cold shock, osmotic shock and the production of reactive oxygen species damage cells and compromise sperm function (Partyka et al., 2011; Rakha et al., 2020). Therefore, the cooling protocol is important for sperm survival of rooster sperm (Madeddu et al., 2016). Higher cooling rates induce intracellular ice formation (Mazur, 1977), whereas too low cooling rates may cause excessive cell dehydration and expose cell membranes for longer periods of time to the pockets of hypertonic solutions, with possible deleterious effects from the generation of reactive oxygen species, and lipid and protein extraction (Katkov, 2012). The optimal cooling rate is a compromise between these opposing effects to decrease cryodamage and maximize sperm viability and functionality after thawing. However, there are no studies in rooster sperm that have addressed the effect of different cooling rates in the separate phases of the freezing protocol: prior to and after ice nucleation. In sperm from different mammal species, the application of a low initial cooling rate in the phase prior to ice nucleation, followed by high cooling rates around the time of ice nucleation, seems to improve sperm survival and functionality (Galarza et al., 2019).

The aim of the present study was to compare two different freezing protocols of rooster sperm that are based on a low cooling rate in the first cooling phase to minimize cold chock, followed by higher cooling rates around or following ice nucleation and ice growth.

2 | MATERIALS AND METHODS

2.1 | Experimental birds

Twelve White Prat roosters were used in this study. Birds were housed at the Research Station 'El Encín' (Madrid, Spain, 40°31'N). All birds were 1 year old and were fed a commercial feed containing 16% crude protein, 2700 kcal of metabolisable energy/kg, 3.5% Ca and 0.5% available P over the entire experimental period.

2.2 | Semen collection, management and freezing protocols

Semen samples were collected twice weekly in 15 mL graduated centrifuge tubes (Sterilin®) using the massage technique described by Burrows and Quinn (1937). Pools of semen were made during each semen recovery session. A total of 10 semen pools were used for each freezing method (a total of 20 semen pools). Each pool was immediately diluted 1:1 (v:v) at field temperature in a Rootex® (Arquimea Agrotech) medium and immediately cooled to 5°C for 45 min. Subsequently, diluted semen was further diluted with 1 volume of Rootex with 18% dimethylformamide (DMF) (dilution 1:2 (v:v); final DMF concentration 6%). Samples were kept for equilibration at 5°C for 15 min, loaded into 0.5 mL straws and frozen at two freezing rates before being stored in liquid nitrogen. Samples were frozen using the following two freezing protocols (Figure 1):

Protocol 1: from +5°C to −10°C at 5°C/min and from −10°C to −130°C at 60°C/min. Protocol 2: from +5°C to −35°C at 7°C/min and from −35°C to −140°C at 60°C/min. After 15 days, the straws were thawed in a water bath for 30 s at 5°C.

The temperatures of the freezing chamber and the straw's interior were recorded. In one dummy straw, the thermocouple of the biological freezer was introduced to a straw-containing freezing medium (Rootex-6% DMF) only. The thermocouple inside the dummy straw allows to register the occurrence of ice nucleation and to estimate the duration of the subsequent dissipation of the latent heat of fusion.

2.3 | Assessment of sperm variables

Sperm motility was assayed as previously described (Santiago-Moreno et al., 2012) using a computer-aided sperm analyses (CASA) system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) and employing Sperm Class Analyzer (SCA®) v.4.0. software (Microptic S.L., Barcelona, Spain). The percentage of motile spermatozoa, the percentage of progressive motility, the curvilinear velocity (VCL), the straight-line velocity (VSL) and the average path velocity (VAP) were analysed by CASA. Propidium iodide and SYBR-14 were used as fluorochromes in the examination of membrane integrity (Chalah & Brillard, 1998); 200 cells were examined using an epifluorescence microscope at 400× (wavelength: 450–490 nm) (Figure 2). Sperm was analysed in fresh samples, after equilibration, and in frozen-thawed samples.

2.4 | Statistical analyses

The effect of cooling rate on sperm variables was analysed by ANOVA. All statistical calculations were made using Statistica software for Windows v.10.0 MR1 (StatSoft Inc., Tulsa, OK, USA).

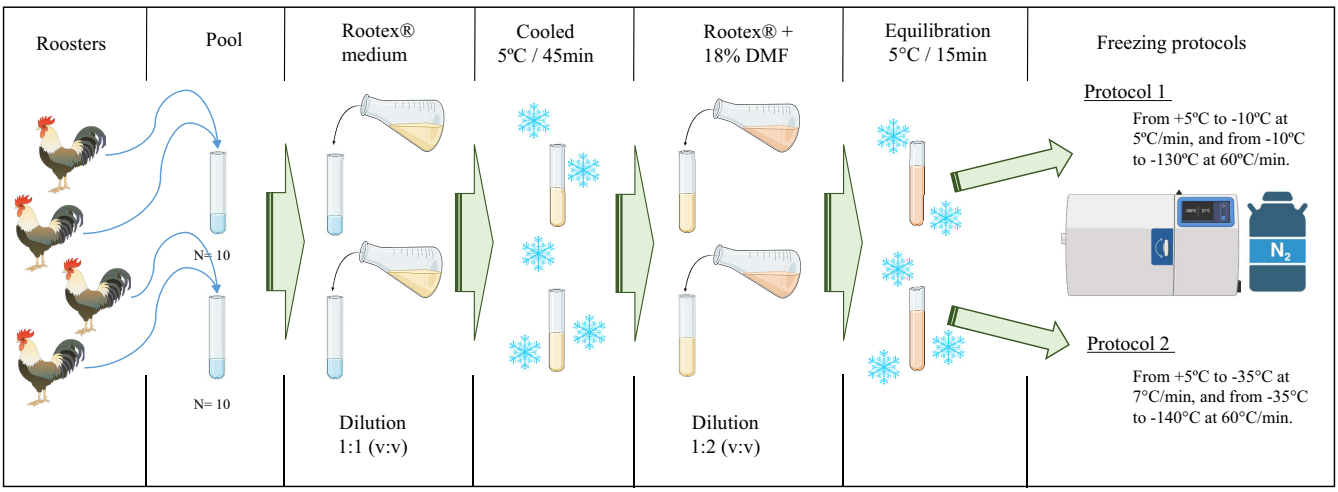

FIGURE 1 Experimental design diagram. Twenty pool sperm samples were diluted with Rootex® extender and 18% DMF (final DMF concentration = 6%). After 15 min at 5°C equilibration time, 10 pool samples were frozen using the freezing Protocol 1 (from +5°C to −10°C at 5°C/min and from −10°C to −130°C at 60°C/min) and other 10 samples using the freezing Protocol 2 (from +5°C to −35°C at 7°C/min and from −35°C to −140°C at 60°C/min).

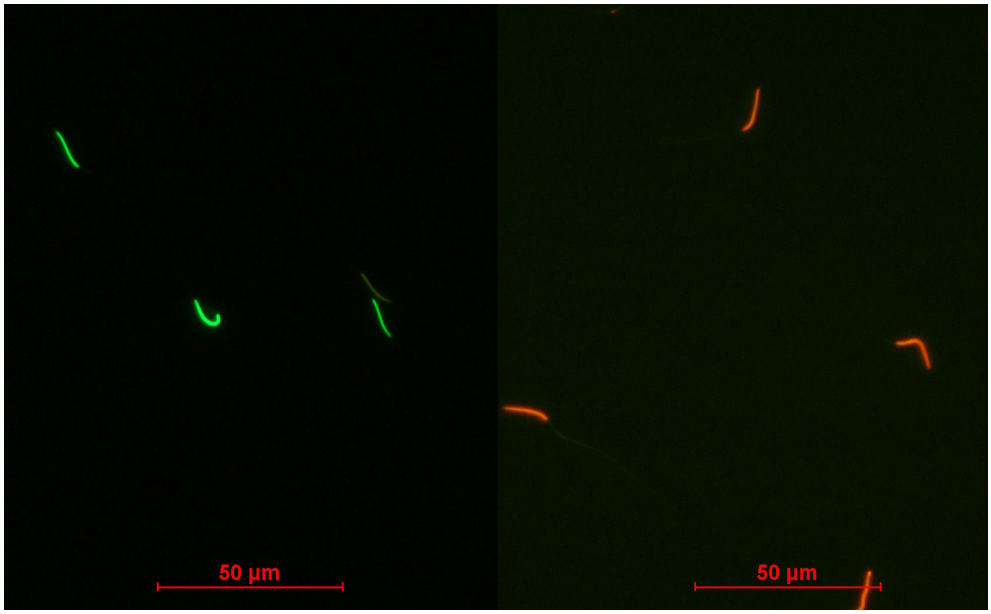

FIGURE 2 Sperm viability evaluated with propidium iodide and SYBR-14; sperm cells stained green were deemed to be live, whereas red coloured spermatozoa were considered dead (×400).

3 | RESULTS

The freezing curve used to cryopreserve the epididymal spermatozoa is depicted in Figure 3. For both protocols, ice nucleation (*seeding*) occurred at −10°C (at 3.8 min). In Protocol 1, ice nucleation occurred 20 s after the second-step cooling rate (from −10 to −130°C at 60°C/min), whereas in Protocol 2, ice nucleation occurred about 98 s before the second-step cooling rate (from −35°C to −140°C at 60°C/min). Equilibration time significantly reduced the proportion of motile sperm and the percentage of spermatozoa showing progressive motility ($p < .05$). The freezing-thawing process significantly reduced ($p < .001$) the values of all sperm variables recorded (Table 1). The proportion of viable spermatozoa was greater ($p < .05$) in spermatozoa that underwent Protocol 2 than in those that underwent Protocol 1.

4 | DISCUSSION

In the freezing protocols used in the present study, a low cooling rate was applied in the first cooling phase in order to minimize cold shock. Our findings indicate that initiating the second phase of the cooling process around ice nucleation (Protocol 1) had a negative effect on the post-thaw survival of rooster sperm. Indeed, Protocol 1 demonstrated the highest cryoinjury response, which may be attributed to the second-step cooling rate (from −10 to −130°C at 60°C/min) that could be too rapid when used on bird sperm. The spindle shape, very little cytoplasm and long flagellum of rooster spermatozoa may render them very susceptible to cryoinjury when a high cooling rate is produced around ice nucleation. This filiform characteristic of bird sperm might be responsible for the different response compared with mammal sperm (e.g. rams) for which a cooling rate of 60°C/min around the time of ice nucleation yields a better motility, plasma membrane and DNA integrities (Galarza et al., 2019). Although previous studies have recommended a rapid freezing rate of 50°C/min up to −140°C (Blesbois et al., 2007) or 59°C/min (Purdy et al., 2009), in the present work, the rapid freezing rate from −10°C to −130°C at 60°C/min was associated with poorer sperm characteristics than those obtained with Protocol 2. In Protocol 2, a low cooling rate (7°C/min) is maintained for a longer time (until −35°C); after ice nucleation, this allows for a better adaptation to the deleterious effects of unfrozen, extracellular, hypertonic solutions when ice growth occurs. Protocol 2 was previously used in Spanish chicken breeds using DMA as a cryoprotectant, providing good results after thawing (Santiago-Moreno et al., 2011). Our findings disagree with those of Madeddu et al. (2016) who recommend an initial high cooling rate to reach −25°C within 30 s using static LN2 vapour. However, a comparative study between vapour freezing of static LN2 and Protocol 2, using DMA as cryoprotectants, provided more desirable results for the latter method (Santiago-Moreno et al., 2011). In conclusion, the results suggest that high cooling rates around the time of ice nucleation are not recommendable.

AUTHOR CONTRIBUTIONS

Conceptualization and design: Julián Santiago-Moreno, Pablo Fernández-Alaez, Pedro García-Casado and Wendy M. Rauw. Material preparation and data collection: Rosalina Robledo, Esther Alba. Cristina Castaño, Olga Torres. Analysis and interpretation: Adolfo Toledano-Díaz, Rosalina Robledo and Berenice Bernal. Drafting and manuscript revision: Julián Santiago-Moreno, Adolfo Toledano-Díaz, Berenice Bernal and Wendy M. Rauw. All authors read and approved the final manuscript.

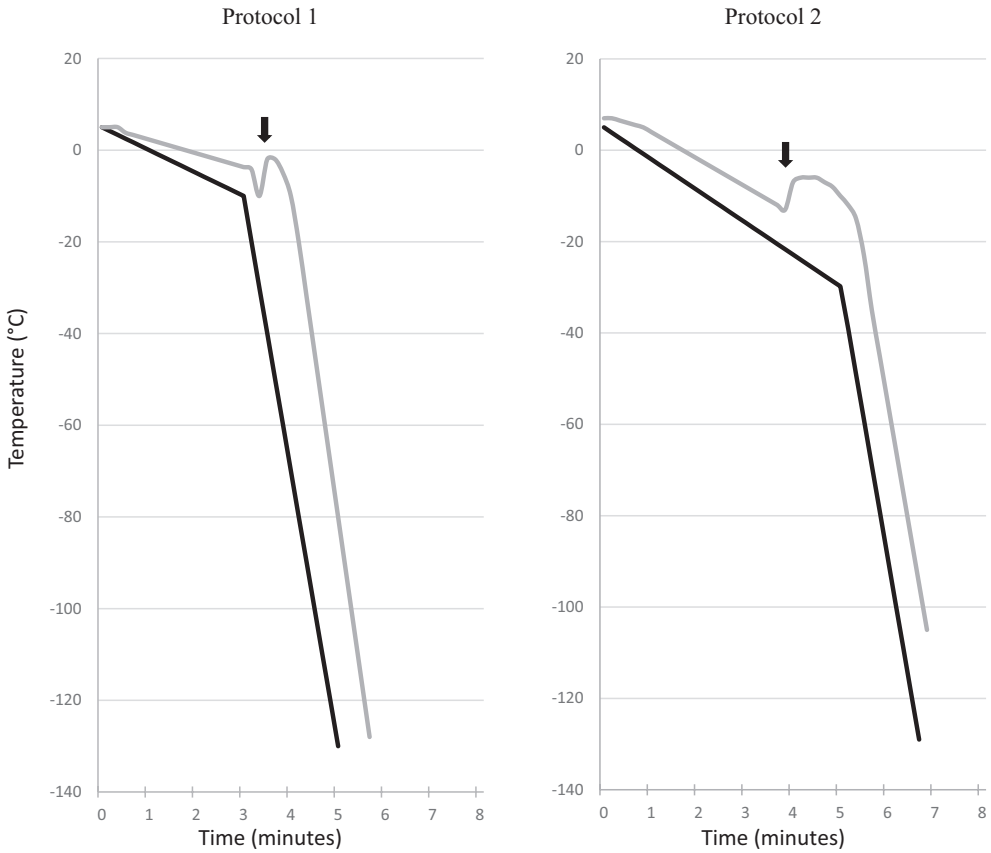

FIGURE 3 Time of ice nucleation (black arrow) as a function of the freezing curve. Protocol 1 freezing curve: from +5°C to −10°C at 5°C/min and from −10°C to −130°C at 60°C/min, Protocol 2: from +5°C to −35°C at 7°C/min and from −35°C to −140°C at 60°C/min. Black line: programmed chamber temperature; Grey line: actual sample temperature.

TABLE 1 Sperm variables (mean ± SE) in fresh samples, after equilibration 15 min at 5°C with cryoprotectant (DMF 6%), and after freeze-thawing, either Protocol 1 (from +5°C to −10°C at 5°C/min and from −10°C to −130°C at 60°C/min) or Protocol 2 (from +5°C to −35°C at 7°C/min and from −35°C to −140°C at 60°C/min).

	Fresh		Equilibrated		Thawed	
Sperm variable	Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 1	Protocol 2
Mot. sperm (%)	91.09 ± 1.79a	88.80 ± 1.86a	78.32 ± 2.87b	81.24 ± 2.69b	51.84 ± 2.14c	55.03 ± 2.71c
PM (%)	70.84 ± 5.00a	66.55 ± 4.74a	48.59 ± 4.56b	52.93 ± 3.57b	12.13 ± 1.50c	15.80 ± 2.24c
VCL (μm/s)	92.36 ± 6.07a	90.23 ± 5.21a	76.39 ± 458a	80.48 ± 3.96a	36.90 ± 2.02b	40.02 ± 2.43b
VSL (μm/s)	46.83 ± 3.41a	46.22 ± 4.03a	36.72 ± 3.60a	39.51 ± 2.76a	11.10 ± 1.34b	12.97 ± 1.31b
VAP (μm/s)	66.84 ± 4.91a	64.89 ± 4.29a	52.46 ± 3.85a	55.88 ± 3.03a	18.50 ± 1.90b	22.06 ± 2.00b
Viab (%)	80.14 ± 2.43a	77.45 ± 1.77a	77.29 ± 2.94a	75.45 ± 1.96a	24.30 ± 1.12b	36.80 ± 2.24c

Note: Within each row, different letters (a, b and c) indicate significant differences ($p < .05$).

Abbreviations: Mot. Sperm, percentage of motile spermatozoa; PM, progressive motility; VAP, average path velocity; VCL, curvilinear velocity; Viab, viable sperm; VSL, straight-line velocity.

ACKNOWLEDGEMENTS

This research was funded by CSIC-Arquimea Group S.A research contract CON22-225, and by INIA-CSIC program RGP2001-001, 'Conservación in vivo e in vitro de doce poblaciones de razas españolas tradicionales de gallinas'. We acknowledge support of the publication fee by the CSIC Open Access Support initiative through its Unit of Information Resource for Research.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Toledano-Díaz, A., Robledo, R., Alba, E., Fernández-Alaez, P., García-Casado, P., Bernal, B., Castaño, C., Torres, O., Rauw, W. M., & Santiago-Moreno, J. (2024). High cooling rate of 60°C/min around ice nucleation during cryopreservation compromises chicken sperm viability. *Reproduction in Domestic Animals*, *59(Suppl. 3)*, e14626. https://doi.org/10.1111/rda.14626