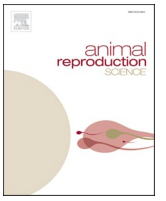
Animal Reproduction Science 250 (2023) 107209

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



Extension of the equilibration period up to 24 h maintains the post-thawing quality of Holstein bull semen frozen with OPTIXcell®

Amer Salman [a], Estela Fernández-Alegre [a,b], Rubén Francisco-Vázquez [c], Rubén Gómez-Martín [c], Alejandro Fernández-Fernández [c], Héctor Areán-Dablanca [c], Juan Carlos Domínguez [a,d], J. Ramiro González-Montaña [a,d], J.Néstor Caamaño [e], Felipe Martínez-Pastor [a,f,*]

[a] *INDEGSAL, Universidad de León, León, Spain*
[b] *Bianor Biotech SL, León, Spain*
[c] *Xenética Fontao, Lugo, Spain*
[d] *Department of Medicine, Surgery and Veterinary Anatomy, Universidad de León, Spain*
[e] *Selección y Reproducción Animal-SERIDA, Principado de Asturias, Gijón, Spain*
[f] *Department of Molecular Biology (Cell Biology), Universidad de León, León, Spain*

ARTICLE INFO

Keywords:
Bull
OPTIXcell
Equilibration time
Sperm cryopreservation
Sperm quality
Fertility

ABSTRACT

Semen cryopreservation in bovine livestock is well established, but logistics often require deviations from standard protocols. Extending the equilibration time to the following day is convenient in many situations. To improve our knowledge of the effects of this modification, we studied the post-thawing and post-incubation (4 h, 38 °C) sperm quality after freezing with 4 or 24-h extension in the OPTIXcell extender by using an ample panel of analyses: CASA for motility; flow cytometry for viability, physiology, oxidative stress, and chromatin parameters (DNA fragmentation, chromatin compaction, and thiol groups status); and spectrometry for malondialdehyde production. Semen was obtained from 12 Holstein bulls. The 24-h equilibration time showed few significant effects, with only a tiny decrease in progressive motility and a positive impact on chromatin structure. The incubation removed some of these effects, with the pattern for chromatin compaction remaining the same. No detrimental oxidative stress or increase in apoptotic or capacitation markers was detected. Additionally, the individual bull interacted with the effects of the incubation and the equilibration, especially regarding the chromatin status. Whereas this interaction did not critically affect sperm quality, it could be relevant in practice. Bull fertility as non-return rates (NRR56) was associated with some sperm parameters (especially with an improved chromatin structure) but not in the 4-h post-thawing analysis. Our study supports that extending the equilibration time by at least 24-h is feasible for bull semen freezing with the OPTIXcell extender.

* Correspondence to: Department of Molecular Biology (Cell Biology), Facultad de CC. Biológicas y Ambientales, University of León, Campus de Vegazana, 24071 León, Spain.
E-mail address: felipe.martinez@unileon.es (F. Martínez-Pastor).

https://doi.org/10.1016/j.anireprosci.2023.107209
Received 19 December 2022; Received in revised form 27 February 2023; Accepted 27 February 2023
Available online 1 March 2023

1. Introduction

The application of artificial insemination (AI) has increased in cattle breeding, becoming fundamental in the dairy industry (DeJarnette et al., 2004). Cryopreservation aims to indefinitely maintain the viability and functionality of spermatozoa at freezing temperatures. However, freezing and thawing deteriorate sperm quality (Woods et al., 2004). To protect cells from cryoinjury, a significant step in freezing protocols is the addition of cryoprotectants, notable glycerol in most mammals (Sieme et al., 2016). One of the essential steps in the process of semen cryopreservation is the equilibration phase, which typically begins after extending and cooling the semen sample. The spermatozoa are generally left for several hours, enabling permeable cryoprotectants and other osmotically active components to equilibrate with the intracellular compartment and membranes to adapt to the new environment (Bailey et al., 2000).

The equilibration period is essential for bull sperm cryopreservation (Leite et al., 2010). However, there is no agreement on what equilibration period is best for obtaining high-quality semen after thawing (Fleisch et al., 2017; Leite et al., 2010). The extension of the equilibration time —from four to eighteen, twenty-four, and seventy-two hours— improved post-thawing bull semen quality while not affecting field fertility (Fleisch et al., 2017; Foote and Kaprotht, 2002). Similarly, some studies on buffalo offer positive results for extending the equilibration period but with some conflicting results (Shahverdi et al., 2014). For instance, Herold et al. (2006) did not find an effect of modifying the equilibration time between 2 and 9 h for buffalo semen. In contrast, Shah et al. (2016) showed that a 4-h equilibration yielded better post-thawing quality than 2-h or 6-h. A study with ram semen (Paul et al., 2020) showed that extending the equilibration time improved post-thawing sperm quality, possibly by reducing the membrane cholesterol efflux.

Extending the equilibration period while preserving post-thawing sperm quality is interesting for commercial AI centers. This strategy could help with the aim of optimizing the production of semen doses. Centers processing high numbers of ejaculates find it challenging to handle all semen collected within the same working day. In these conditions, it would be more practical to prepare the sperm doses and freeze them the following morning (Fleisch et al., 2017; Foote and Kaprotht, 2002; Muiño et al., 2007; Shahverdi et al., 2014). A longer equilibration time is practical when semen collection is spatially separated from the freezing facility (Foote and Kaprotht, 2002).

The extender could be relevant while modifying the cryopreservation protocols. Most studies approaching prolonged equilibration periods were carried out using egg yolk or milk-based extenders (Almeida et al., 2017; Foote and Kaprotht, 2002; Shah et al., 2016). Some studies considered the interaction of the extender and the equilibration time in Gyr cattle, *Bos indicus* (Leite et al., 2010), buffalo, *Bubalus bubalis* (Shahverdi et al., 2014), and commercial cattle, *Bos taurus* (Fleisch et al., 2017). Leite et al. (2010) and Shahverdi et al. (2014) used an egg yolk-based Tris extender and the BioXcell extender (free of animal components), but only for up to 4 h of equilibration time. Interestingly, the egg yolk-based extender was considered superior for Gyr cattle, with a significant interaction extender × equilibration time. Still, it was the opposite for buffalo (with better results for BioXcell and no interaction). Fleisch et al. (2017) included egg yolk-based Triladyl and Tris and animal components-free Andromed and OPTIXcell and tested for up to 72 h for equilibration time. No relevant interactions were found, and the effects of extending the equilibration time were positive (assessing sperm motility, acrosomal status, mitochondrial potential, and DNA fragmentation). Moreover, a field fertility trial with the Tris extender found no differences between 4 and 72 h.

In this study, we aimed to investigate the effects of using an equilibration time of 4 or 24 h in dairy cattle (Frisian-Holstein) using the egg yolk-free commercial OPTIXcell extender. Since most studies considered a limited number of sperm quality parameters, our design pursued a complete characterization using CASA (computer-assisted sperm analysis), flow cytometry, and mainly including the study of the chromatin structure, a critical aspect for sperm fertility (Narud et al., 2021).

2. Materials and methods

2.1. Experimental design

Semen from 12 Holstein bulls (three ejaculates per bull) was extended with OPTIXcell and equilibrated for 4 or 24 h after cooling. After each equilibration time, semen was packaged in 0.25 ml French straws and cryopreserved. Samples were tested after thawing and four hours of incubation at 38 °C. Immediately after thawing and 4 h at 38 °C, motility was assessed by CASA, and sperm physiology and chromatin structure were estimated using flow cytometry. Oxidative stress parameters were evaluated by flow cytometry and spectrometry (malondialdehyde formation).

2.2. Reagents and media

General reagents, and when not stated, were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The freezing extender OPTIXcell was obtained from IMV (L′Aigle, France). Consumables and solutions for flow cytometry were purchased from Beckman Coulter (Brea, CA, USA) and Thermo Fisher (Waltham, MA, USA). Fluorescent probes for flow cytometry were purchased from Sigma-Aldrich or Thermo Fisher and are described in the corresponding section.

2.3. Animals, semen collection, and field fertility estimation

Semen was obtained by artificial vagina (45 °C) from trained animals subjected to a routine collection for semen freezing and AI. Twelve adult Holstein bulls (1–2 years old) housed in half-open barns in natural environment were used in this study. The collection

and cryopreservation were carried out by trained personnel from Xenética Fontao (Lugo, Spain; certified as an AI center following Spanish and European regulations: Reales Decretos 2256/1994, 2129/2008, 841/2011), and the researchers involved in the experiments did not contact with any animals.

Xenética Fontao provided information on the animals and non-return rates at day 56 post-AI (NRR). The NRR data was obtained from the farms' records after AI with cryopreserved doses (4-h equilibration time protocol).

2.4. Initial semen assessment and cryopreservation

Ejaculates were analyzed after collection for minimum quality requirements (>1000 $\times 10^6$ ml^{-1}, >85% motility, >80% normal morphology). The motility of the fresh samples was assessed by diluting the samples with PBS (20 $\times 10^6$ cells/ml) and loading a 20-μm Leja chamber (IMV). The samples were assessed at 37 °C at × 10 (negative contrast) for total and progressive motility in an IVOS II computer-assisted sperm analysis (CASA) system (IMV). For viability, the samples were stained in PBS (10^6 cells/ml) with the LIVE/DEAD Sperm Viability kit (SYBR-14 100 nM and propidium iodide, PI, 5 μg/ml, 15 min at 37 °C). The samples were run through a FACSCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ), acquiring at least 5000 spermatozoa with the CellQuest 5 software. The probes were excited with a 488-nm laser, detecting the green emission of SYBR-14 with a photodetector behind a 530/30 filter and the red emission of PI using a 650LP filter. Spermatozoa were gating as fluorescence-positive events, with viability defined as $SYBR\text{-}14^+/PI^-$.

The sperm doses were prepared with the OPTIXcell extender (IMV) at 160 × 10^6 cells/ml, slowly cooled to 5 °C for 2 h (~0,2 K/min), packed in 0.25-ml straws, and equilibrated for 4 or 24 h. The doses were frozen using a programmable freezer (Digit-cool; IMV). The freezing followed a standard curve for bovine semen, as described by Muiño et al. (2008a): − 5 K/min from + 4 °C to − 10 °C, − 40 K/min from − 10 °C to − 100 °C, and − 20 K/min from − 100 °C to − 140 °C, and then stored in liquid nitrogen. The sperm doses were shipped to the reproduction laboratory at the University of León for analysis.

Thawing was performed in a water bath at 37 °C (30 s). Two straws per sample were pooled and analyzed by CASA and flow cytometry directly after thawing and post-incubation (4 h at 38 °C, as a stress test) (Salman et al., 2021).

2.5. Determination of sperm motility by CASA-mot (computer-assisted sperm analysis, motility)

Motility was assessed in a 20-μm Makler chamber. Sperm samples were extended in PBS 0.5% BSA to reach 25 × 10^6 cells/ml. The CASA system was composed of a Nikon E400 microscope (Tokyo, Japan) with a thermostatic stage (37 °C), a × 10 negative phase contrast objective, a Basler A312fc video camera (Basler Vision Components, Ahrensburg, Germany), and a computer with the ISAS® V.1.019 software (Proiser R+D, Valencia, Spain). At least three fields and 200 cells/field were captured per sample at 53 frames/s. The CASA provided the proportions of motile (total motility, MOT) and progressive spermatozoa (PROG, STR>80%), and the kinematic parameters (from motile cells): Curvilinear path velocity (VCL), straight path velocity (VSL), average path velocity (VAP), linearity index (LIN), straightness index (STR), wobble index (WOB), amplitude of the lateral movement of the head (ALH), and beat-cross frequency (BCF).

2.6. Flow cytometry analysis

Flow cytometry analyses of the functional parameters of bull sperm were carried out using a CyAn ADP flow cytometer (Beckman Coulter, Inc., Brea, USA) fitted with three diode lasers (violet at 405 nm, blue at 488 nm, and red at 635 nm), as described previously for bull spermatozoa (Salman et al., 2021). Briefly, the sperm samples were incubated in 300 μl of PBS 0.5% BSA with different fluorescent probes combinations at 2 × 10^6 cells/ml (38 °C for 15 min in the dark): 4.5 μM Hoechst 33342 (H342), 100 nM YO-PRO-1 (YP), 2 μM Merocyanine 540 (M540), 3 μM propidium iodide (PI), and 100 nM MitoTracker deep red (MT), for debris identification, apoptosis-like and capacitation-like membrane changes, viability, and mitochondrial activity, respectively (H342/PI/YP/M540/MT); and 4.5 μM Hoechst 33258 (H258), 5 μM CM-H_2DCFDA (CFDA), 1 μM MitoSOX (MSX), and 1 μg/ml peanut agglutinin (PNA) Alexa 647, for viability, cytoplasmic ROS, mitochondrial superoxide production, and acrosomal integrity (H258/CFDA/MSX/PNA). Fluorescence was collected in the cytometer by the different photodetectors provided with emission filters: In the violet line, 450/50 nm (blue fluorescence: H342 and H258); in the blue line, 530/40 nm (green fluorescence: YP and CFDA), 575/25 nm for orange fluorescence (orange: M540), 613/20 nm for red fluorescence (red: PI and MSX); and in the red line, 665/20 nm for red fluorescence (PNA and MT). Sample acquisition was controlled by using the Summit V4.3.02 software. After an FSC height/area and forward/side scatter (FSC/SSC) gating, debris was discarded as $H342^-$ events for the first combination and as $H258^-$ (very low fluorescence) in the second one. The acquisition was stopped after reaching 5000 spermatozoa. The data obtained by cytometry were processed using Weasel v. 3.2 (Frank Battye, Melbourne, Australia). Compensation was applied for PI (−4% YP) and M540 (−5% YP), not required for other probe combinations. The supplementary material shows representative panels from Weasel in Figs. S1 and S2. Results were expressed as proportions except for cytoplasmic ROS: Live spermatozoa (PI^-); Showing apoptotic features (YP^+/PI^-); Apoptotic (YP^+) within the viable population (ratio of PI^-); Total acrosomal damage (PNA^+); Acrosomal damage (PNA^+) within the viable population (ratio of $H258^-$); Capacitated ($M540^+$; this probe reveals increased membrane disorder, a possible proxy for capacitation) within the non-apoptotic population (ratio of YP^-); Active mitochondria (YP^-/MT^+); Active mitochondria (MT^+) within the non-apoptotic population (ratio of YP^-); Cytoplasmic ROS as median fluorescence value of CFDA in the viable population ($H258^-$); And high mitochondrial $O_2^{\bullet-}$ production within the viable population (ratio of $H258^-$).

2.7. Sperm chromatin analysis

The SCSA (Sperm Chromatin Structure Assay) was used to assess sperm DNA integrity and chromatin compaction as described previously (Salman et al., 2021). Briefly, spermatozoa were extended at 2×10^6 cells/ml in TNE (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4), and 200 μl were mixed with 400 μl of acid-detergent solution (0.1% Triton X-100, 150 mM NaCl, 80 mM HCl, pH 1.2). After 30 s, 1.2 ml of acridine orange (AO) solution was added (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM Na_2EDTA, 0.15 M NaCl, 6 μg/ml AO). The sample was run through a FACSCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) before 5 min, acquiring at least 5000 spermatozoa with the CellQuest 5 software. The AO was excited with a 488-nm laser, detecting the green and red emission with photodetectors behind 530/30 and 650LP filters, respectively. Flow cytometry files (FSC v.2) were analyzed in the R statistical environment, obtaining the DNA fragmentation index (DFI) for each spermatozoon (red to total fluorescence ratio). We got the standard deviation of the DFI (SD-DFI), the proportion with DFI> 250 (%DFI), and chromatin compaction from the green fluorescence (%HDS as the low-compaction population).

2.8. Disulfide bridges analysis

Monobromobimane (mBBr) is a fluorescent probe with high reactivity to free thiol groups. Samples were diluted in TNE at 10^6 cells/ml and pipetted in two parallel black 96-well plates. One of the plates was used as a reference (positive) by reducing disulfide bridges to free thiols by adding DTT (1 mM). After 10 min at 37 °C, the samples were washed and resuspended. Both plates were incubated with mBBr fluorochrome (500 μM; 10 min, 37 °C), washed with PBS, and stained with PI (3 μM). The plates were kept at 4 °C in the dark for 24 h and analyzed in a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany), reading 10 000 spermatozoa per sample. Propidium iodide was excited with the blue line (488 nm) and detected with a photodetector behind a 655–700 nm filter (used for gating in sperm events), and mBBr was excited with the violet line (405 nm). Its emission was detected using a 450/50 nm filter (labeled thiol groups). Data were saved in flow cytometry standard (FCS) files and processed using the R statistical environment. Disulfide bridge abundance was estimated by subtracting total thiols (DTT-treated) from free thiols and dividing by 2 (Zubkova et al., 2005). In addition, mBBr-low, mBBr-moderate, and mBBr-high sperm populations (with few, intermediate, or high relative numbers of sulfhydryl groups) were estimated using appropriate thresholds from standard samples.

2.9. Assessment of lipid peroxidation

The susceptibility of the spermatozoa to lipoperoxidation was assessed using the Bioxitech® MDA-586 kit (Aoxre Bio-Sciences, Burlingame, CA, USA) to detect malondialdehyde (MDA) (Mata-Campuzano et al., 2012). First, the samples were diluted to 10^7 cells/ml in PBS with 40 μM $FeSO_4$ and 200 μM Na-ascorbate (freshly prepared) to induce MDA release. After 30 min at 37 °C, 60 μl of the sample were vortexed with 192 μl of the kit R1 reactive and 3 μl of probucol (120 mM), then vortexing after adding 45 μl of the R2 reactive and incubated at 45 °C for 1 h. The tubes were centrifuged, and the supernatant was read for absorbance (586 nm) in a 96-well flat bottom transparent plate (Nunc, Roskilde, Denmark) using a Synergy HT plate reader (BIO-TEK, Winooski, Vermont, USA). A standard curve from known quantities of MDA was included in each plate to calculate MDA concentration (μmol of MDA per 10^8 spermatozoa).

2.10. Statistical analysis

Statistical analyses were conducted in the R statistical environment (R Core Team, 2021). Normality was checked using the Shapiro-Wilk test, using a Box-Cox transformation if appropriate. The data were analyzed using linear mixed-effects models (LME) with equilibration time and incubation as fixed effects and the male as a random effect. For CASA-mot data, sperm subpopulations were obtained with a two-step cluster analysis described previously (Fernández-Alegre et al., 2022) and expanded in the supplementary material. Since the experimental design enabled the study of the bulls' influence on the response to different treatments, an additional analysis was carried out by generalized linear models (GLM), including the equilibration time, incubation, the bull effects, and their pairwise interactions. Finally, the relationships between variables and bull fertility (non-return rates, NRR) were studied by Pearson correlations. The level of significance was set at $P \leq 0.05$.

3. Results

3.1. Sperm quality of the fresh samples

Semen samples were obtained from the 12 Holstein bulls, with three ejaculates per bull. The mean and standard deviations (SD) for the total and progressive motilities assessed by CASA-mot were 68.0%± 15.9% and 13.5% ± 5.6, respectively. The sperm viability assessed by flow cytometry with the SYBR-14/PI combination was 71.0% ± 14.0.

3.2. Effects of the equilibration time on sperm quality

The amount of change of post-thawing semen parameters compared to the fresh samples was small, with mean ± SD of 53.8% ± 15.4 for total motility and 68.0% ± 18.7 for viability (PI^-), and even increased to 17.7% ± 8.5 for progressive motility (overall results

for both equilibration times). The extension of the equilibration time from 4 to 24 h induced small changes in the post-thawing sperm quality. Total motility (Fig. 1a) was not significantly affected, whereas progressive motility (Fig. 1b) was lower post-thawing when the samples were equilibrated for 24 h ($P = 0.004$), but differences equalized after the post-thawing incubation. Both trends were evident in the kinematic parameters provided by the CASA-mot system (Fig. 2), with VCL and ALH (Figs. 2a and 2g) not being affected and the other parameters showing higher values post-thawing for the 4-h equilibration. In all cases, differences were not significant ($P > 0.05$) after the post-thawing incubation. No differences were detected for the sperm subpopulations obtained from the cluster analysis (Table 1, Fig. 3).

The flow cytometry assessment of sperm physiology (Fig. 4) showed good sperm quality in most cases, with no differences between both equilibration times, except for the capacitation ratio (Fig. 4f), slightly higher post-thawing for the 4-h equilibration (P=0.025). In addition, the samples showed good resistance to the post-thawing incubation, with significant changes only for viability, total acrosomal damage, and total mitochondrial activity (Figs. 4a, 4d, and 4g). Oxidative stress parameters showed minimal free radical production in all cases (Fig. 5), not varying with equilibration time or after the post-thawing incubation.

The analysis of the sperm chromatin structure showed some small but significant changes between the equilibration times. The standard deviation for the DFI parameter was higher in the samples processed after the 24-h equilibration. However, this effect was noticeable only after the post-thawing incubation (Fig. 6a). Contrarily, the %DFI (DNA fragmentation) was lower for the 24-h equilibration if analyzed just after thawing (Fig. 6b). Chromatin compaction assessed by AO stainability (%HDS) was lower in the 24-h equilibrated samples both after thawing and after the incubation (Fig. 6c). Considering the abundance of free thiols, the proportions of the sperm populations showing low and moderate mBBr fluorescence swapped between equilibration and sampling times (Fig. 7a and b). The proportion of spermatozoa with low mBBr fluorescence was significantly higher both in the 24-h equilibration time and after the post-thawing incubation, compared to the 4-h equilibration and the post-thawing analysis, respectively. The opposite was observed for the moderate-mBBr population. The population of high-mBBr fluorescence was small and with no significant changes (Fig. 7c). The amount of disulfide bridges estimated by mBBr (Fig. 7d) was significantly higher in the samples processed with 4 h of equilibration time both post-thawing and after incubation.

The GLM analysis (Table S1 in the supplementary material) showed that the bull effect was significant for all variables (also confirmed by extracting the variance components from the random part of the LME models). This reflected the post-thawing variability in sperm quality, despite being bulls of proven fertility and tested for acceptable sperm quality. The effect of the incubation depended on the bull (significant interaction), suggesting not only a variable post-thawing quality between bulls but also a different post-thawing resilience. This interaction was found for some motility variables (especially those related to sperm progressivity) and all those connected to chromatin integrity and structure. The most relevant result was that the effect of the equilibration time depended on the male for the chromatin-related variables (except the proportion of the population with low mBBr fluorescence).

3.3. Association of quality variables and equilibration-time changes with field fertility

Non-return rates (NRR, day 56 post-AI) were provided for each bull, with a median of 50.8% and 47.5–53.65% as the 1st and 3rd quartiles. A simple correlation study was performed to establish possible associations with the quality variables in each experimental group. Interestingly, the sperm linearity in the fresh sample correlated negatively with NRR (LIN: −0.66, $P = 0.005$; STR: −0.72, $P = 0.008$). No significant correlations were found in the analyses of the 4-h equilibration samples when assessed just after thawing. However, some variables obtained after the incubation correlated with NRR, namely the capacitation ratio (−0.48, $P = 0.003$), the high superoxide production ratio (−0.51, $P = 0.001$), and the chromatin variables SD-DFI (−0.51, $P = 0.002$) and high-mBBr fluorescence (−0.58, $P = 0.023$). The samples processed with a 24-h equilibration period showed some correlations in the post-thawing assessment involving chromatin-status parameters, with SD-DFI (−0.46, $P = 0.005$), %HDS (−0.39, $P = 0.018$), and high-mBBr

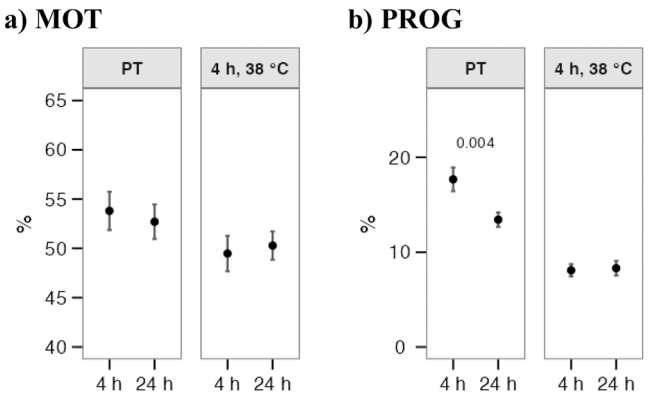


Fig. 1. Effect of the extension of equilibration time from 4 h to 24 h using the OPTIXcell extender on CASA-mot (computer-assisted sperm analysis, motility) variables (n = 12 bulls). The plots show mean ± SEM for total motility (MOT) and progressive motility (PROG), directly post-thawing (PT) and after 4-h incubation at 38 °C. Insets show P values for the effects of equilibration time within each sampling time. The overall effect of the incubation was P=0.030 for MOT and P<0.001 for PROG.

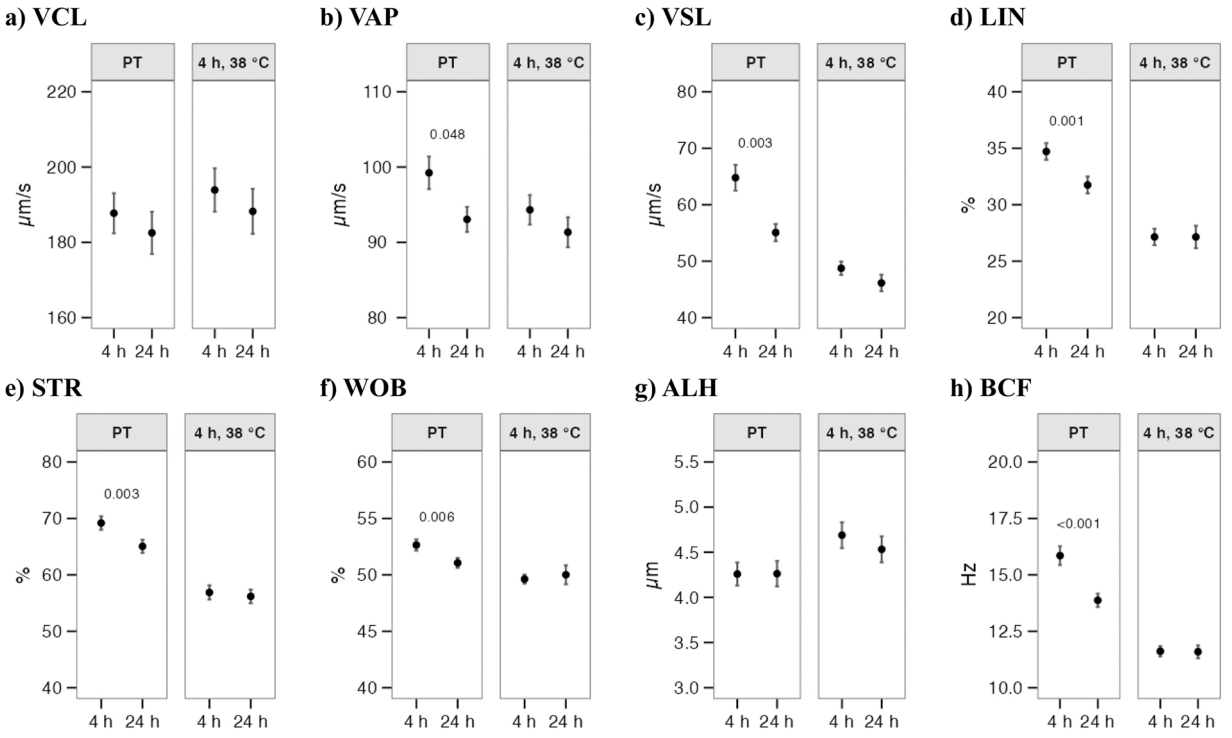


Fig. 2. Effect of the extension of equilibration time from 4 h to 24 h using the OPTIXcell extender on the CASA-mot kinematic variables (n = 12 bulls). The plots show mean ± SEM directly post-thawing (PT) and after a 4-h incubation (4 h at 38 °C). Insets show *P* values for the effects of equilibration time within each sampling time. The overall effect of the incubation was $P<0.05$ for ALH and $P<0.001$ for VSL, LIN, STR, WOB, and BCF.

Table 1
Descriptive statistics (median ± median absolute deviation) for the three subpopulations found in the motility data after a two-step clustering. A total of 219,735 spermatozoa (n = 12 bulls) were clustered, resulting in three subpopulations defined by their median motility parameters as Rapid (fast and lineal trajectories), Slow (low velocity and linearity), and Hyper (very fast, low linearity, high ALH, resembling hyperactivated swimming). The statistics were calculated from the total dataset after clustering, with the last column showing the overall proportion of each subpopulation in the initial dataset. For subsequent analyses, only the proportion of each subpopulation in each sample was used.

Cluster	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	%
Rapid	121.4 ± 56.4	70.1 ± 28	83.9 ± 28.1	57.3 ± 15.8	86.8 ± 9.9	68.6 ± 13.5	2.5 ± 1.3	18.6 ± 6.9	16.5
Slow	58.4 ± 25	10 ± 6.1	28.7 ± 12.3	17.4 ± 11.3	37.7 ± 21.9	48.8 ± 11.5	1.8 ± 0.6	6.0 ± 3	15.2
Hyper	216.2 ± 82.3	49.2 ± 32.8	98.2 ± 34.5	26.7 ± 12.2	58 ± 24.4	47.7 ± 8.2	5.2 ± 2.1	13.0 ± 5.4	68.3

fluorescence (−0.74, $P = 0.002$). After the 4-h incubation, the number of significant correlations increased, with the apoptotic ratio (−0.38, $P = 0.024$), the active mitochondria ratio (0.46, $P = 0.005$), the high superoxide production ratio (−0.36, $P = 0.032$), SD-DFI (−0.47, $P = 0.004$) and high-mBBr fluorescence (−0.77, $P = 0.023$).

To further explore these relationships, we determined the change between the 4 and the 24-h equilibration times in both incubation times and then the correlations with NRR. In the post-thawing analysis, only the change of %HDS and high-mBBr fluorescence correlated significantly with NRR (−0.53, $P = 0.001$; 0.53, $P = 0.042$, respectively). After the incubation, the change in some of the physiology and chromatin parameters correlated with NRR: Apoptotic cells (−0.37, $P = 0.028$), apoptotic ratio (−0.34, $P = 0.004$), the high superoxide production ratio (−0.35, $P = 0.026$), %HDS (−0.37, $P = 0.026$), low-mBBr fluorescence (−0.63, $P = 0.012$), high-mBBr fluorescence (−0.63, $P = 0.012$), and disulfide bridges (−0.59, $P = 0.021$).

4. Discussion

This study contributes to better assessing the suitability of extended equilibration times for freezing bull semen from commercial breeds, adding knowledge on some physiological and chromatin parameters. Many authors (Bailey et al., 2000; Leite et al., 2010) have

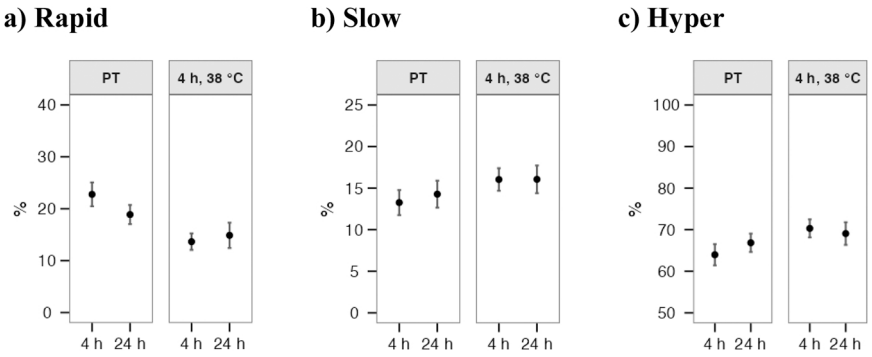


Fig. 3. Effect of the extension of equilibration time from 4 h to 24 h, using the OPTIXcell extender, on the motility subpopulations (Table 1; n = 12 bulls). The plots show mean ± SEM for the Rapid, Slow, and Hyper (hyperactive-like) subpopulations, directly post-thawing (PT) and after a 4-h incubation (4 h at 38 °C). Insets show *P* values for the effects of equilibration time. The overall effect of the incubation was *P*=0.001 for Rapid and *P*>0.05 for Slow and Hyper.

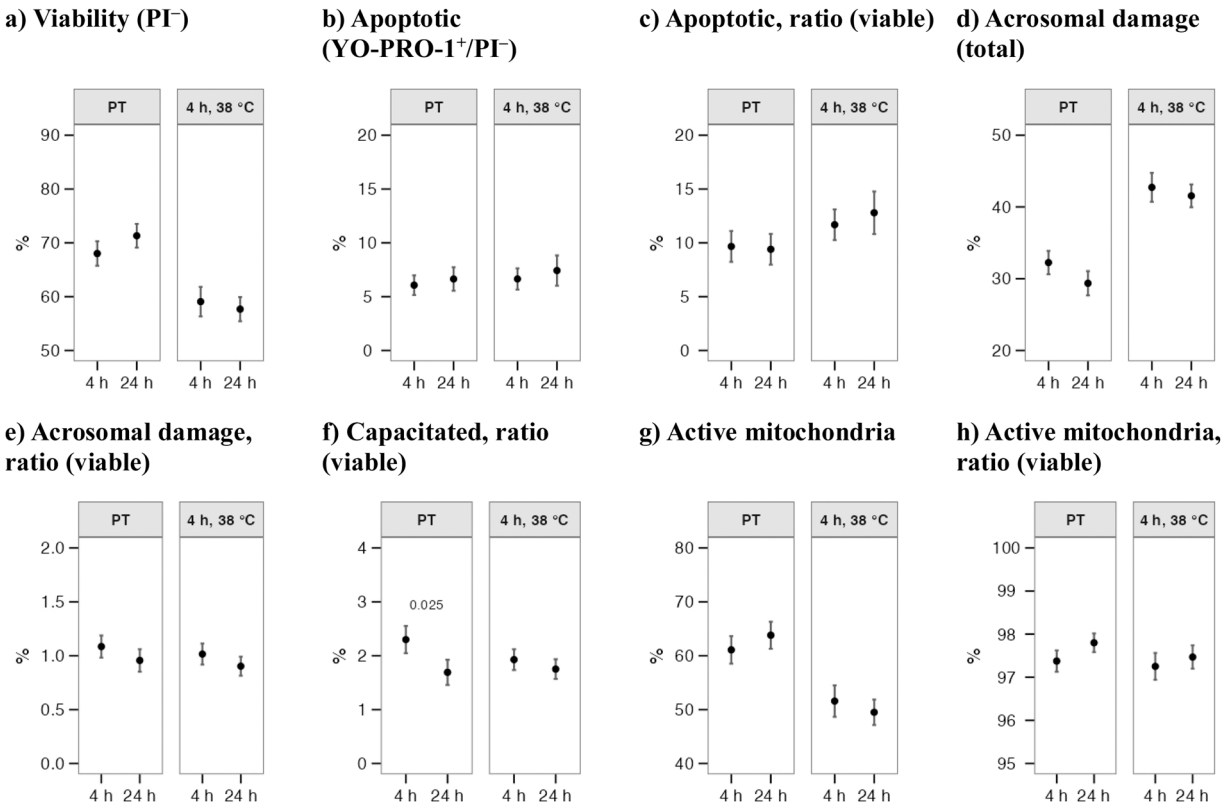


Fig. 4. Effect of the extension of the equilibration time from 4 h to 24 h using OPTIXcell on the sperm physiology assessed by flow cytometry (n = 12 bulls). The plots show mean ± SEM for sperm viability (PI^-), apoptotic spermatozoa ($YO\text{-}PRO\text{-}1^+/PI^-$), apoptotic ratio (%$YO\text{-}PRO\text{-}1^+$ within PI^-), acrosomal damage (total PNA^+ and %PNA^+ within $H258^-$), capacitated spermatozoa (%$M540^+$ within $YO\text{-}PRO\text{-}1^-$), and active mitochondria ($MitoTracker^+$ and %$MitoTracker^+$ within $YO\text{-}PRO\text{-}1^-$), directly post-thawing (PT) and after a 4-h incubation (4 h at 38 °C). Insets show *P* values for the effects of equilibration time. The overall effect of the incubation was *P*<0.001 for viability, acrosomal damage (total), and mitochondrial activity (total).

shown that equilibration time is essential for adapting the sperm plasma membrane to new conditions. However, there is no agreement on which equilibration time is more appropriate for bull sperm. We add valuable information from some previous reports (Anzar et al., 2011; Fleisch et al., 2017; Foote and Kaprotht, 2002; Shahverdi et al., 2014), which suggested that increasing the equilibration time of bull and buffalo semen did not affect or only slightly decreased the post-thawing quality.

Whereas previous results indicated that a 24-h equilibration time might have no detrimental effects on bull spermatozoa (Anzar et al., 2011; Fleisch et al., 2017; Foote and Kaprotht, 2002), we found that the progressive motility and some kinetic parameters

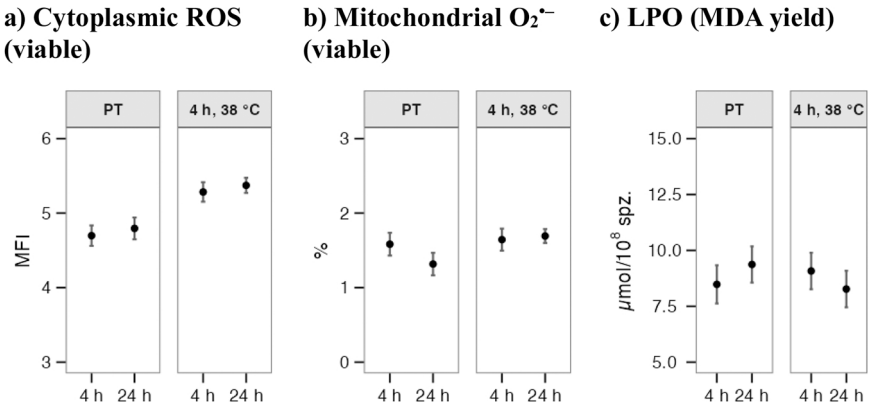


Fig. 5. Effect of the extension of the equilibration time from 4 h to 24 h using OPTIXcell on the oxidative stress variables (n = 12 bulls). The plots show mean ± SEM for cytoplasmic ROS (H_2DCFDA mean fluorescence intensity —MFI— within H285$^-$), and production of the superoxide anion $O_2^{\bullet-}$ in mitochondria (%MitoSOX$^+$ within H285$^-$), and lipoperoxidation (LPO) as malondialdehyde (MDA) production, directly post-thawing (PT) and after incubation for four hours (4 h at 38 °C). The equilibration time did not significantly affected these variables. The overall effect of the incubation was $P<0.001$ for cytoplasmic ROS and $P=0.037$ for mitochondrial $O_2^{\bullet-}$.

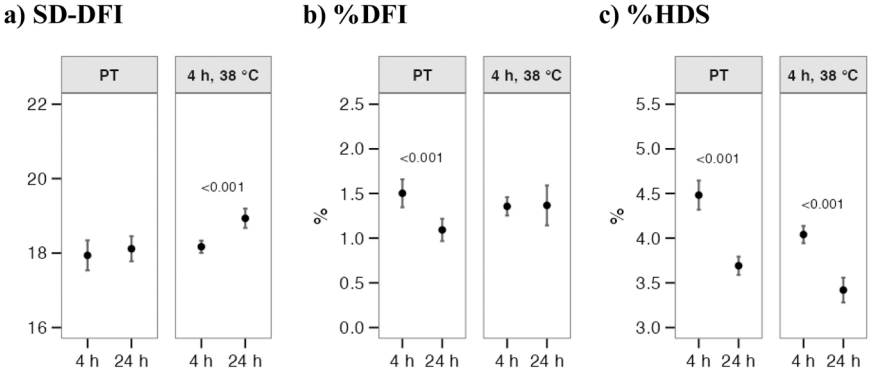


Fig. 6. Effect of the extension of the equilibration time from 4 h to 24 h using OPTIXcell (n = 12 bulls) on the SD of the DFI (sperm DNA fragmentation index), sperm DNA fragmentation (%DFI), and chromatin immaturity (%HDS), directly post-thawing (PT) and after incubation for four hours (4 h at 38 °C). Insets show *P* values for the effects of equilibration time. The overall effect of the incubation was $P<0.001$, only for %HDS.

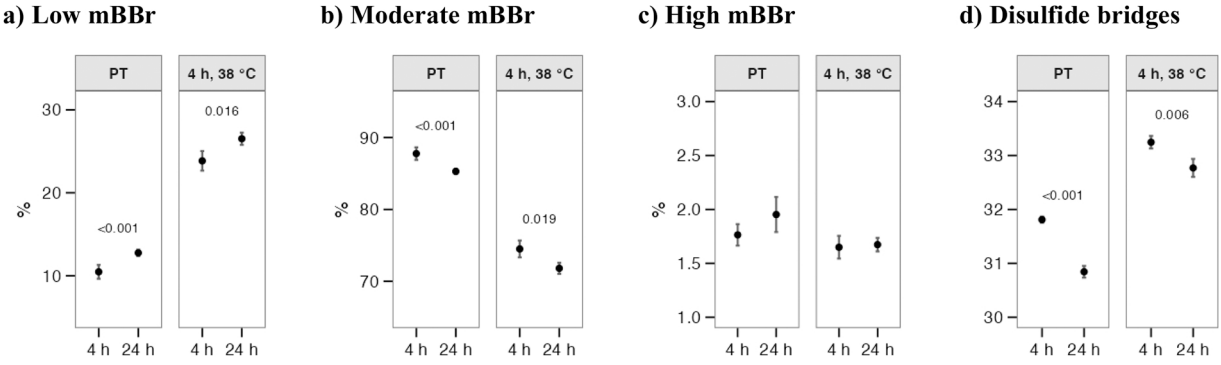


Fig. 7. Effect of the extension of the equilibration time from 4 h to 24 h using OPTIXcell (n = 12 bulls) on the evaluation of free thiols (as low, moderate, or high mBBr staining) and disulfide bridges (estimated from the mBBr assay), directly post-thawing (0 h) and after incubation for four hours (4 h at 38 °C). Insets show *P* values for the effects of equilibration time. The overall effects of the incubation were $P<0.001$, except for the high mBBr population ($P>0.05$).

decreased just after thawing. However, this decrease was relatively small and did not reflect on the sperm populations or the physiological parameters assessed by flow cytometry. Foote and Kaprotht (2002) found a positive effect of extending the equilibration time, however, with different conditions than the ones tested in our experiment: 18 h, using a whole milk-fructose extender and only considering total motility, which was overall lower (~45%) than in our experiment with OPTIXcell. The extender type affects the equilibration time's impact on the post-thawing semen quality. For instance, the Tris-egg yolk-based extender Biladyl resulted in higher post-thaw bull sperm quality, comparison to the soybean-derived extenders AndroMed and Biociphos Plus following the same 18 h equilibration time protocol (Muiño et al., 2007). However, these authors did not include a comparison with shorter equilibration times.

In a more extensive study, Fleisch et al. (2017) tested the commercial extenders AndroMed, Triladyl, and also OPTIXcell, and up to 96 h of extension, analyzing plasma membrane and acrosome integrity (PMAI), mitochondrial activity, and DNA fragmentation index (%DFI, not considering other parameters). While extending the equilibration time to 48 h and beyond seemed harmful, there were no significant effects for 24 h in motility, mitochondrial activity, %DFI, and even a positive impact for PMAI. Similar results were found in buffalo (Shahverdi et al., 2014), with the BioXcell extender yielding higher post-thawing progressive motility (but neither total motility, PMAI, nor %DFI) than Tris-citric egg yolk and no differences among 2, 4, 8, and 16 h of equilibration time. Our results follow these findings considering the membrane and acrosomal status, with no significant changes between 4 and 24 h equilibration time.

One of the purposes of this study was to expand the knowledge about the physiological and chromatin parameters by performing an exhaustive flow cytometry study to understand better the possible changes of the extension of the equilibration time. Thus, the present study found no differences in mitochondrial functionality between the equilibration times. This could be relevant to the lack of apoptotic-like membrane changes or evidence of oxidative stress since mitochondrial functionality is related to apoptosis pathways such as cytochrome c release from the mitochondria after functionality loss (Martin et al., 2007). Moreover, an increase in ROS yield is typical both in activation (such as capacitation) and in stressful events (Das and Roychoudhury, 2022). Our results explain previous studies, at least for the 24-h equilibration period. Moderate extension of the equilibration time might not be enough to induce cellular stress, explaining the lack of differences. It would be interesting to investigate if the detrimental changes observed in longer equilibration times (Fleisch et al., 2017) are related to oxidative stress or mitochondrial failure and, therefore, if extenders could be modified (e.g., with longer antioxidant capacity) to improve these results.

Interestingly, the proportion of live-capacitated spermatozoa decreased slightly after thawing in the 24-h equilibration time. We cannot confirm if this was due to the death of sensitive spermatozoa undergoing these changes or to better membrane stability, but in any case, the changes were small and possibly biologically non-important.

Effects on the sperm chromatin were minor but, in most cases, significant. Whereas some studies included analysis of DNA fragmentation (Fleisch et al., 2017), as far as our knowledge is concerned, there are no reports on the effects of extending the equilibration time on sperm chromatin structure. This information is relevant due to the influence not only of DNA fragmentation but also the overall chromatin structure on fertility (Dogan et al., 2015; Martínez-Pastor et al., 2009; Martins et al., 2021). We noticed a very low overall DNA fragmentation, and, interestingly, extending the equilibration time decreased the incidence post-thawing, whereas Fleisch et al. (2017) reported a sustained increase with equilibration time (but non-significant from 4 to 24 h). The lower DNA fragmentation at 24-h could be related to higher compaction of the sperm chromatin, as suggested by the %HDS variable of SCSA. However, the mBBr results indicate that the larger chromatin compaction when equilibrating for 24 h was not due to the formation of new disulfide bridges between protamines. Indeed, disulfide bridges decreased with a longer equilibration time, maybe due to the good antioxidant capacity of the OPTIXcell extender. Our results on oxidative stress markers and previous observations after adding potent antioxidants (Salman et al., 2021) support this hypothesis. In any case, whereas specific studies should investigate the mechanisms modulating the chromatin compaction, the results are encouraging since a high %HDS has been related to lower fertility in bull semen (Narud et al., 2021).

Using a stress test as the incubation at 37–38 °C is a simple and effective method to assess sublethal damage and resiliency after thawing, revealing potential cryoinjuries. Many authors have utilized this test at different times. For instance, 2 h (Anzar et al., 2011), 3 h (Fleisch et al., 2017), 4 h (Muiño et al., 2008b), or 5 h (Muiño et al., 2008a), and up to 9 h (Muiño et al., 2007). After the incubation, the results related to sperm physiology equalized, suggesting that the sample resiliency was similar. Using also OPTIXcell, Fleisch et al. (2017) found similar results after incubating the thawed samples, except for PMAI, which remained higher for the 24-h equilibration time. The freezing protocol could have influenced that result by affecting the membrane resiliency since the equivalent PMAI in our experiment (not shown and very similar to the overall viability) was higher for the 4-h equilibration. Nevertheless, the most interesting results were related to oxidative stress and chromatin status. The incubation increased ROS production very slightly, and the lack of response of an oxidative stress indicator as LPO indicated good protection by the extender (Fernández-Santos et al., 2009). This protection would have prevented noticeable changes in the chromatin integrity, as showed by the SCSA after the incubation. Nevertheless, the incubation at 38 °C could have enhanced the activity of the nuclear peroxidases (Conrad et al., 2005), explaining the higher amount of disulfide bridges.

Whereas the influence of the individual bulls was not a central goal of this experiment, it should be the focus of follow-up studies. We found that the individual variability affected not only the post-thawing sperm quality but also the resilience to the incubation and, to some extent, the results of the extended equilibration time. Interestingly, the interaction of both treatments with the male was significant, considering the sperm chromatin evaluation. Even if these parameters were satisfactory in all cases, the changes (positive and negative) in this crucial aspect of sperm quality could influence the vulnerability (Zubkova et al., 2005) and the fertility of the cryopreserved doses (Narud et al., 2021). Our results suggest that bull characterization should include tests such as post-thawing incubation. Moreover, assays of chromatin structure and not only DNA fragmentation could provide crucial information on the suitability of long equilibration times for the cryopreservation of semen from specific bulls.

Whereas we did not perform a fertility trial with samples equilibrated for 4 or 24 h, we explored if the sperm quality could be

associated with the AI records from the males used in this study (Christensen et al., 2011; Rodriguez-Martinez, 2003). Previous studies have shown no significant effects of increasing the equilibration time with field fertility, even if effects were noted on sperm quality (Fleisch et al., 2017; Foote and Kaprotht, 2002). It is relevant that the usual post-thawing analyses in the generally performed 4-h equilibration time were not associated with NRR. However, analyses after the post-thawing incubation or in the 24-h equilibration time presented many significant correlations, some with at least moderate strength. It is also noteworthy that the chromatin-associated variables predominated among the variables associated with NRR. Whereas the status of post-thawed spermatozoa might not be related to the AI results, a prolonged interaction of the spermatozoa with the extender might induce changes, reflecting a more accurate assessment of its potential fertility.

For instance, extenders contain lipidic components (Nasiri et al., 2012; Röpke et al., 2011; Towhidi and Parks, 2012), which could interact more efficiently with the membranes with extended equilibration times or the higher incubation temperature. Indeed, the consistency of the associations and their signs when using the change between the 4 and 24-h equilibration times support that modifications of the spermatozoa could be significant and that chromatin structure analyses (beyond DNA fragmentation) might add value to the usual sperm assessment panel. Research on the effect of different equilibration times in spermatozoon composition and intracellular signaling is warranted, together with extensive trials for determining the impact of those changes on field fertility.

5. Conclusions

The differences in the post-thawing semen variables between the two equilibration times in our experiments were slight. Thus, according to these findings and the previous studies, we can conclude that the application extension of the equilibration time to the day after semen collection is available, according to the AI center's requirement. Further studies are required to evaluate the fertilizing capacity of those samples to create a more comprehensive picture of these treatments.

CRediT authorship contribution statement

Amer Salman: Investigation, Data curation, Writing- Original draft preparation; Estela Fernández-Alegre: Investigation, Data curation; Rubén Francisco-Vázquez: Investigation, Resources; Rubén Gómez-Martín: Supervision, Conceptualization, Methodology, Investigation, Data Curation; Alejandro Fernández-Fernández: Investigation, Data Curation; Héctor Areán-Dablanca: Investigation, Resources, Funding acquisition; Juan Carlos Domínguez: Investigation, Resources, Funding acquisition; J. Ramiro González-Montaña: Investigation, Visualizaton; Néstor Caamaño: Supervision, Methodology, Investigation, Writing- Reviewing and Editing; Felipe Martínez-Pastor: Supervision, Methodology, Formal analysis, Visualization, Writing- Reviewing and Editing, Resources, Funding acquisition.

Conflict of interest

The authors state that there is no conflict of interest.

Acknowledgments

Xenética Fontao Artificial Insemination Center for providing the cryopreserved doses analyzed in this study; INDEGSAL researchers Andrea Núñez González and Itxaso Crespo Félez; and technicians Cristina Arija Domínguez, Lucía Tejerina García, and Beatriz de Arriba Ruiz for support during the analyses. Dr. Amer Salman was supported by a CSN (Sweden) studentship grant and the Excellence Network project AGL2016–81890-REDT (MINECO, Spain).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2023.107209.

References

Almeida, F.C., Silva, S.V., Souza, H.M., Gomes, W.A., Lima Filho, J. a C., Wicke, A.A., Batista, A.M., Guerra, M.M.P., 2017. Effects of glycerol, equilibration time and antioxidants on post-thaw functional integrity of bovine spermatozoa directly obtained from epididymis. Andrologia 49. https://doi.org/10.1111/and.12623.

Anzar, M., Kroetsch, T., Boswall, L., 2011. Cryopreservation of bull semen shipped overnight and its effect on post-thaw sperm motility, plasma membrane integrity, mitochondrial membrane potential and normal acrosomes. Anim. Reprod. Sci. 126, 23–31. https://doi.org/10.1016/j.anireprosci.2011.04.018.

Bailey, J.L., Bilodeau, J.F., Cormier, N., 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21, 1–7.

Christensen, P., Labouriau, R., Birck, A., Boe-Hansen, G.B., Pedersen, J., Borchersen, S., 2011. Relationship among seminal quality measures and field fertility of young dairy bulls using low-dose inseminations. J. Dairy Sci. 94, 1744–1754. https://doi.org/10.3168/jds.2010-3087.

Conrad, M., Moreno, S.G., Sinowatz, F., Ursini, F., Kölle, S., Roveri, A., Brielmeier, M., Wurst, W., Maiorino, M., Bornkamm, G.W., 2005. The nuclear form of phospholipid hydroperoxide glutathione peroxidase is a protein thiol peroxidase contributing to sperm chromatin stability. Mol. Cell. Biol. 25, 7637–7644. https://doi.org/10.1128/MCB.25.17.7637-7644.2005.

Das, A., Roychoudhury, S., 2022. Reactive oxygen species in the reproductive system: sources and physiological roles. Adv. Exp. Med. Biol. 1358, 9–40. https://doi.org/10.1007/978-3-030-89340-8_2.

DeJarnette, J.M., Marshall, C.E., Lenz, R.W., Monke, D.R., Ayars, W.H., Sattler, C.G., 2004. Sustaining the fertility of artificially inseminated dairy cattle: the role of the artificial insemination industry. J. Dairy Sci., Electron. Suppl. 87, E93–E104. https://doi.org/10.3168/jds.S0022-0302(04)70065-X.

Dogan, S., Vargovic, P., Oliveira, R., Belser, L.E., Kaya, A., Moura, A., Sutovsky, P., Parrish, J., Topper, E., Memili, E., 2015. Sperm protamine-status correlates to the fertility of breeding bulls. Biol. Reprod. 92, 92. https://doi.org/10.1095/biolreprod.114.124255.

Fernández-Alegre, E., Lacalle, E., Soriano-Úbeda, C., González-Montaña, J.R., Domínguez, J.C., Casao, A., Martínez-Pastor, F., 2022. *Bos taurus* and *Cervus elaphus* as non-seasonal/seasonal models for the role of melatonin receptors in the spermatozoon. Int J. Mol. Sci. 23, 6284. https://doi.org/10.3390/ijms23116284.

Fernández-Santos, M.R., Domínguez-Rebolledo, A.E., Esteso, M.C., Garde, J.J., Martínez-Pastor, F., 2009. Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity. Int. J. Androl. 32, 353–359. https://doi.org/10.1111/j.1365-2605.2008.00871.x.

Fleisch, A., Malama, E., Witschi, U., Leiding, C., Siuda, M., Janett, F., Bollwein, H., 2017. Effects of an extension of the equilibration period up to 96 h on the characteristics of cryopreserved bull semen. Theriogenology 89, 255–262. https://doi.org/10.1016/j.theriogenology.2016.10.018.

Foote, R.H., Kaprotht, M.T., 2002. Large batch freezing of bull semen: effect of time of freezing and fructose on fertility. J. Dairy Sci. 85, 453–456.

Herold, F.C., de Haas, K., Colenbrander, B., Gerber, D., 2006. Comparison of equilibration times when freezing epididymal sperm from African buffalo (Syncerus caffer) using Triladyl or AndroMed. Theriogenology 66, 1123–1130. https://doi.org/10.1016/j.theriogenology.2006.03.007.

Leite, T.G., do Vale Filho, V.R., de Arruda, R.P., de Andrade, A.F.C., Emerick, L.L., Zaffalon, F.G., Martins, J.A.M., de Andrade, V.J., 2010. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. Anim. Reprod. Sci. 120, 31–38. https://doi.org/10.1016/j.anireprosci.2010.04.005.

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., Levy, R., 2007. Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Hum. Reprod. 22, 380–388. https://doi.org/10.1093/humrep/del399.

Martínez-Pastor, F., Fernández-Santos, M.R., del Rocío Fernández-Santos, M., Domínguez-Rebolledo, A.E., Esteso, M.C., Garde, J.J., Biology of Reproduction Group, 2009. DNA status on thawed semen from fighting bull: a comparison between the SCD and the SCSA tests. Reprod. Domest. Anim. Zuchthyg. 44, 424–431. https://doi.org/10.1111/j.1439-0531.2008.01098.x.

Martins, M.C., Gonçalves, L.M., Nonato, A., Nassif Travençolo, B.A., Alves, B.G., Beletti, M.E., 2021. Sperm head morphometry and chromatin condensation are in constant change at seminiferous tubules, epididymis, and ductus deferens in bulls. Theriogenology 161, 200–209. https://doi.org/10.1016/j.theriogenology.2020.12.004.

Mata-Campuzano, M., Alvarez-Rodríguez, M., del Olmo, E., Fernández-Santos, M.R., Garde, J.J., Martínez-Pastor, F., 2012. Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37 °C in presence of several antioxidants. Theriogenology 78, 1005–1019. https://doi.org/10.1016/j.theriogenology.2011.12.018.

Muiño, R., Fernández, M., Peña, A.I., 2007. Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18h. Reprod. Domest. Anim. 42, 305–311. https://doi.org/10.1111/j.1439-0531.2006.00784.x.

Muiño, R., Rivera, M.M., Rigau, T., Rodriguez-Gil, J.E., Peña, A.I., 2008a. Effect of different thawing rates on post-thaw sperm viability, kinematic parameters and motile sperm subpopulations structure of bull semen. Anim. Reprod. Sci. 109, 50–64. https://doi.org/10.1016/j.anireprosci.2007.11.028.

Muiño, R., Tamargo, C., Hidalgo, C.O., Peña, A.I., 2008b. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. Anim. Reprod. Sci. 109, 27–39. https://doi.org/10.1016/j.anireprosci.2007.10.007.

Narud, B., Khezri, A., Zeremichael, T.T., Stenseth, E.-B., Heringstad, B., Johannisson, A., Morrell, J.M., Collas, P., Myromslien, F.D., Kommisrud, E., 2021. Sperm chromatin integrity and DNA methylation in Norwegian Red bulls of contrasting fertility. Mol. Reprod. Dev. 88, 187–200. https://doi.org/10.1002/mrd.23461.

Nasiri, A.H., Towhidi, A., Zeinoaldini, S., 2012. Combined effect of DHA and α-tocopherol supplementation during bull semen cryopreservation on sperm characteristics and fatty acid composition. Andrologia 44 (Suppl 1), 550–555. https://doi.org/10.1111/j.1439-0272.2011.01225.x.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, http://www.R-project.org/, Vienna, Austria.

Paul, R.K., Balaganur, K., Kumar, D., Singh, R., 2020. Pre-freezing equilibration for 22 h improves post-thaw sperm functions in cryopreserved ram semen by reducing cholesterol efflux. Cryobiology 96, 76–84. https://doi.org/10.1016/j.cryobiol.2020.07.013.

Rodriguez-Martinez, H., 2003. Laboratory semen assessment and prediction of fertility: still utopia? Reprod. Domest. Anim. 38, 312–318.

Röpke, T., Oldenhof, H., Leiding, C., Sieme, H., Bollwein, H., Wolkers, W.F., 2011. Liposomes for cryopreservation of bovine sperm. Theriogenology 76, 1465–1472. https://doi.org/10.1016/j.theriogenology.2011.06.015.

Salman, A., Caamaño, J.N., Fernández-Alegre, E., Hidalgo, C.O., Nadri, T., Tamargo, C., Fueyo, C., Fernández, Á., Merino, M.J., Martínez-Pastor, F., 2021. Supplementation of the BIOXcell extender with the antioxidants crocin, curcumin and GSH for freezing bull semen. Res. Vet. Sci. 136, 444–452. https://doi.org/10.1016/j.rvsc.2021.03.025.

Shah, S. a H., Andrabi, S.M.H., Qureshi, I.Z., 2016. Effect of equilibration times, freezing, and thawing rates on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. Andrology 4, 972–976. https://doi.org/10.1111/andr.12214.

Shahverdi, A., Rastegarnia, A., Rezaei Topraggaleh, T., 2014. Effect of extender and equilibration time on post thaw motility and chromatin structure of buffalo bull (Bubalus bubalis) spermatozoa. Cell J. 16, 279–288.

Sieme, H., Oldenhof, H., Wolkers, W.F., 2016. Mode of action of cryoprotectants for sperm preservation. Anim. Reprod. Sci., Spec. Issue 10th Bienn. Conf. Assoc. Appl. Anim. Androl. 169, 2–5. https://doi.org/10.1016/j.anireprosci.2016.02.004.

Towhidi, A., Parks, J.E., 2012. Effect of n-3 fatty acids and α-tocopherol on post-thaw parameters and fatty acid composition of bovine sperm. J. Assist. Reprod. Genet. 29, 1051–1056. https://doi.org/10.1007/s10815-012-9834-7.

Woods, E.J., Benson, J.D., Agca, Y., Critser, J.K., 2004. Fundamental cryobiology of reproductive cells and tissues. Cryobiology 48, 146–156.

Zubkova, E.V., Wade, M., Robaire, B., 2005. Changes in spermatozoal chromatin packaging and susceptibility to oxidative challenge during aging. Fertil. Steril. 84 (Suppl 2), 1191–1198. https://doi.org/10.1016/j.fertnstert.2005.04.044.