Theriogenology 245 (2025) 117523

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

Cryopreservation at −80 °C impacts sperm integrity and fertility in a mouse strain-dependent manner

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ARTICLE INFO

Keywords:
sperm cryodamage
Cryopreservation
In vitro fertilisation
Mouse spermatozoa
-80 °C freezer

ABSTRACT

We previously developed a simple method to cryopreserve and maintain B6N spermatozoa at −80 °C, instead of using liquid nitrogen (LN_2), for up to one year without detrimental effects on fertility. The goal of the present study was to test the efficacy of this method in six different mouse strains (129/SvImJCnrm; 129, C57BL/6JCnrm; B6J, C57BL/6NTacCnrm; B6N, BALB/cByJCnrm; BALB/c, Crl:CD1(ICR); CD-1, and FVBN/JCnrm; FVB). At different time points up to one year, we evaluated the integrity and the fertility of the spermatozoa cryopreserved and stored at −80 °C in comparison to the classical LN_2 method. After 1 year, no differences in the *in vitro* fertilisation (IVF) rate and in the number of dead spermatozoa were observed in 129, B6N, CD-1 and FVB strains. In contrast, spermatozoa from B6J and BALB/c cryopreserved at −80 °C showed a significant reduction in the IVF rate, an increased number of dead spermatozoa and increased morphological abnormalities after 3 months (BALB/c) and 6 months (B6J). In all strains, the total sperm motility decreased after 1 month at −80 °C and increased ultrastructural damage was found in the −80 °C group. The present results indicate that spermatozoa from 129, B6N, CD-1 and FVB can be cryopreserved and stored at −80 °C for at least one year without impacting their fertility. However, spermatozoa from B6J and BALB/c are the most sensitive to temperature. Therefore, cryopreservation and storage of 129, B6N, CD-1 and FVB strains at −80 °C for up to one year is feasible, thereby circumventing the use of LN_2.

1. Introduction

Cryopreservation of spermatozoa is currently the simplest and most economical method for long-term archiving of genetically engineered mice used for biomedical research [1,2]. Transportation of cryopreserved spermatozoa has advantages over the shipment of live mice as it bypasses some of the health and welfare issues associated with live animal transport. Over the years, various sperm cryopreservation methods were developed using different types of cryoprotectants [3–5]. However, these methods require liquid nitrogen (LN_2) and expensive cryogenic storage systems to cryopreserve and store sperm samples.

We previously reported that spermatozoa from 129/SvImJCnrm (129), C57BL/6JCnrm (B6J), C57BL/6NTacCnrm (B6N), BALB/cByJCnrm (BALB/c), Crl:CD1(ICR) (CD-1), and FVBN/JCnrm (FVB) strains, cryopreserved using standard laboratory protocols and stored in LN_2, can be shipped in dry ice (−80 °C) for at least seven days and then returned to LN_2 for indefinite storage without any noticeable detrimental effect [6]. Dry ice has a huge advantage due to its safety and low costs compared to a LN_2 dry shipper. This method of shipment is now routinely used by mouse repositories such as the European Mouse Mutant Archive (EMMA)-Infrafrontier network to distribute cryopreserved spermatozoa. We expanded our studies by analysing longer

Abbreviations: COCs, Cumulus-Oocyte-Complexes; FELASA, Federation of European Laboratory Animal Science Associations; IVF, *In Vitro* Fertilisation; LN_2, Liquid Nitrogen.

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https://doi.org/10.1016/j.theriogenology.2025.117523
Received 21 January 2025; Received in revised form 19 March 2025; Accepted 2 June 2025
Available online 4 June 2025

periods of times of storage at −80 °C, proving that spermatozoa from different genetic backgrounds (B6J, B6N, CD-1 and B6D2F1) can be cryopreserved in LN_2 and stored in a −80 °C ultra-deep freezer for up to 2 years without affecting their fertilising potential [7]. More recently, we reported that it is possible to store mouse spermatozoa (B6J, B6N and CD-1), which were previously cryopreserved in LN_2, for up to 5 years in a −80 °C freezer with no significant differences compared to storage in LN_2 in terms of fertilising ability, sperm viability, intracellular calcium concentration, membrane lipid disorder and mitochondrial activity [8].

We also demonstrated that B6N spermatozoa can be efficiently cryopreserved directly at −80 °C without the use of LN_2 and stored at −80 °C for 1 year. These samples showed similar viability and fertility compared to the samples cryopreserved and stored in LN_2. Furthermore, the embryos resulting from *in vitro* fertilisation (IVF) with these spermatozoa had similar vitality and were equally able to produce live animals [7].

Therefore, the goal of this study was to evaluate the implementation of this new, LN_2-free method, not only to B6N males, but also to 5 other mouse strains, namely, 129, B6J, BALB/c, CD-1, and FVB. Spermatozoa were cryopreserved directly at −80 °C and stored for 1, 3, 6, 9 or 12 months at −80 °C. For comparison, spermatozoa were also cryopreserved in LN_2. To investigate the integrity of the spermatozoa at −80 °C or in LN_2, after each timepoint, *in vitro* fertilisation (IVF), *in vitro* development as well as measurements of sperm viability, sperm kinetics, morphology and transmission electron microscopy were performed.

2. Materials and methods

2.1. Mice and husbandry

Mice belonging to the strains 129/SvImJCnrm (129), C57BL/6JCnrm (B6J), C57BL/6NTacCnrm (B6N), BALB/cByJCnrm (BALB/c), Crl:CD1(ICR) (CD-1) and FVBN/JCnrm (FVB) were bred in the Consiglio Nazionale delle Ricerche-European Mouse Mutant Archive (CNR-EMMA)-Infrafrontier specific pathogen-free (SPF) barrier unit (Monterotondo Scalo, Rome, Italy). They were housed in individually ventilated cages (Tecniplast, Gazzada, Italy) at a temperature of 20 ± 2 °C, relative humidity of 55 ± 15 % with 12–15 air changes per cage per hour and a 12/12-h light/dark cycle (7 a.m. - 7 p.m.). Certified dust-free wood bedding (Scobis one, Mucedola, Settimo Milanese, Milano, Italy) was provided in the cages. Mice were fed a standardised mouse diet (4RFN and Emma 23, Mucedola, Italy) and were provided chlorinated, filtered water *ad libitum*. They were tested for micro-organisms every 3 months using 6- to 8-week-old B6N sentinels. Serology was performed according to the FELASA recommendations [9].

2.2. Sperm cryopreservation

For each strain, spermatozoa from 3-month-old males were cryopreserved, as previously described [4]. The caudae epididymides and the *vasa deferentia* originating from three males per strain were placed in 3 mL of cryoprotective medium (CPM) consisting of 18 % w/v D-(+)-raffinose pentahydrate (Sigma-Aldrich, Darmstadt, Germany), 3 % w/v skim milk (BD Diagnostics, Le Pont de Claix, France) and 477 μM α-monothioglycerol (MTG, Sigma-Aldrich, Darmstadt, Germany). After 10 min at 37 °C, the pooled sperm was loaded into 0.25 mL french straws (IMV Technologies, France), which were then divided into two groups. The control group was cryopreserved by exposure to LN_2 vapour for 10 min before being plunged into LN_2. Then the straws were transferred into a LN_2 cryogenic tank. The −80 °C group was cryopreserved without the aid of LN_2 by transferring them directly into a −80 °C ultra-deep freezer and stored at −80 °C for 1–12 months.

2.3. In vitro fertilisation

IVF was performed, as previously reported [10], using the protocol described by Nakagata et al. [11] and modified by Ming-Wen Li et al. [12]. After 1, 3, 6, 9, and 12 months of storage, one straw each from LN_2 and from a −80 °C ultra-deep freezer were thawed in a water bath at 37 °C for 8 min and expelled into a 1.5 mL tube (Eppendorf, Hamburg, Germany). A volume of 30 μL spermatozoa was added to 90 μL capacitation medium (Krebs-Ringer bicarbonate solution (TYH) containing 1.0 mg/mL polyvinyl alcohol (Merck KGaA, Darmstadt, Germany) and 0.75 mM methyl-β-cyclodextrin (MBCD, Merck KGaA, Darmstadt, Germany) and incubated for 30 min. The oocytes were obtained from four-week-old 129, B6J, B6N, BALB/c, CD-1 or FVB after superovulation via an intraperitoneal injection of 5 IU pregnant mare's serum gonadotropin (PMSG, Intervet, Milan, Italy) followed by 5 IU hCG (Intervet, Milan, Italy) 48h later. At 12–14h post-hCG injection, the cumulus oocyte complexes (COCs) were released and incubated for 20 min in a fertilisation drop consisting of 250 μL modified human tubal fluid (HTF, Merck KGaA, Darmstadt, Germany) with 1 mM reduced L-glutathione (GSH, Merck KGaA, Darmstadt, Germany) and 5.14 mM Ca^{2+}. To reduce the female-to-female variability, the COCs from each female were distributed between the two experimental groups in the fertilisation drops. After capacitation, 20 μL of sperm were collected from the periphery of each capacitation drop and transferred to inseminate the COCs in each dish (final sperm concentration of 2–6 x 10^5 spermatozoa/mL). After 4h co-incubation, the oocytes were washed 3 times in 200 μL of HTF medium and cultured overnight in 100 μL M16 medium (Sigma-Aldrich, Taufkirchen, Germany). Twenty-four (24) hours post-insemination, the IVF rate, expressed as the percentage of 2-cell embryos developing from the number of oocytes inseminated, was determined. For each strain and time point, 3 replications were performed. Each replicate consisted of 2 fertilisation dishes per experimental group with COCs from 3 females each. For each time point, 18 females (9 for controls = LN_2 and 9 for the −80 °C group) were used.

2.4. In vitro development

The quality of the 2-cell embryos generated by IVF was assessed by *in vitro* embryo culture in potassium simplex optimized medium supplemented with amino acids ($KSOM^{AA}$) [13] for 72h until the blastocyst stage. For each group, 30 2-cell embryos were cultured and 3 replications were performed. Results are expressed as the percentage of blastocysts developing from the number of 2-cell embryos cultured.

2.5. Viability assessment by flow cytometry

Mouse sperm membrane integrity was assessed using flow cytometry (FACSCanto II, Becton Dickinson, USA) and the LIVE/DEAD® Sperm Viability Kit from Molecular Probe (ThermoFisher Scientific, Waltham, USA). The time points examined were the same as those for IVF. For each time point, straws containing the cryopreserved spermatozoa stored in LN_2 or at −80 °C were thawed as previously described for IVF. The sperm samples were then diluted in HEPES-buffered saline solution (10 mM HEPES, 150 mM NaCl, 2 % BSA, pH = 7.4) at a concentration of 1 x 10^6 cells/mL and incubated at 37 °C for 10 min with 1 μg/mL Hoechst 33342 (Molecular Probe, ThermoFisher Scientific, Waltham, USA). Staining with Hoechst 33342 was performed to identify only cells and to exclude debris, which may otherwise be counted as cells, as previously reported [10]. After centrifugation at 1000 rpm for 6 min, spermatozoa were re-suspended (1 x 10^6 cells/mL) and stained for 5 min with SYBR 14 at a concentration of 100 nM. The sperm samples were then stained by the addition of propidium iodide (PI) at a final concentration of 12 μM and incubated for 3 min before examination. A total of 10,000 events was analysed. The SYBR 14 and PI dyes were excited using a 488-nm blue laser. A 405-nm violet laser was used to excite Hoechst 33342. The selected sperm population was identified by SYBR 14 as live cells while PI-positive cells were considered as dead cells. After exclusion of the Hoechst 33342-negative cells, the sperm population was analysed by creating two-dimensional dot plot of PI versus SYBR 14. Only

spermatozoa that were SYBR 14-negative and PI-positive were considered to be dead. Fluorescence minus one control (FMO) was used to properly interpret the flow cytometry data. The data were analysed with FlowJo Software (BD, USA). For each strain and experimental group, 3 replicates were performed.

2.6. Measurement of sperm kinetics

Spermatozoa were thawed as described above and released into a pre-warmed (37 °C) sampling tube (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). They were allowed to disperse for 55 min at 37 °C in a moisture-saturated atmosphere of 5 % CO_2 and 95 % air in an incubator (Sanyo-Biomedical, Ewald Innovationstechnik GmbH, Bad Nenndorf, Germany). A homogeneous sperm suspension was obtained by gentle pipetting. Afterwards, an aliquot of 2 μL of the sperm suspension was diluted 1:50 with Cook's medium (Cook Medical, Brisbane, Australia). Sperm motility patterns were analysed with the Hamilton Thorne IVOS computerized semen analyzer (Hamilton Thorne, Beverly, MA, USA) operating at 30 video frames per sec (60 Hz). In total, 10 fields were recorded for each sample analysed. The result is given as a percentage of motile spermatozoa moving at a velocity strictly above 7.4 μm/s in any direction (mean of two measurements).

2.7. Evaluation of sperm morphology

An aliquot of the spermatozoa was smeared onto a glass slide (VWR, Radnor PA, USA), air-dried, and examined under bright-field microscopy (Leica Microsystems, Wetzlar, Germany) at a magnification of 40 X. The morphology of the head (normal or abnormal) and the tail (straight or bent) as well as the absence of a head were examined. The percentage of abnormal sperm was calculated from 200 spermatozoa examined.

2.8. Analysis of ultrastructural damages

Transmission electron microscopy was performed to visualize the potential ultrastructural damage caused by the cryopreservation method. Spermatozoa were thawed as described previously and incubated for 1h at 37 °C. An aliquot containing 800,000 spermatozoa was washed with 200 μL of 1X phosphate buffer (PB, 0.01M NaH_2PO_4*H2O and 0.04M Na2PO_4, pH 7.4), centrifuged at 1500 g (Hermle, Wehingen, Germany) for 2 min, and the pellet was re-suspended in 100 μL of PB 1X. The washing step was repeated 3 times. The final pellet was re-suspended in 60 μL of PB 1X and mixed with 60 μL of fixative (2 % formaldehyde, 2 % glutaraldehyde, 3 mM $CaCl_2$ in 0.1M sodium cacodylate buffer; Applichem, Darmstadt, Germany). After incubation at 4 °C for 48h, samples were washed with 0.1M sodium cacodylate buffer and centrifuged for 2 min at 1500g (Hermle, Wehingen, Germany). The supernatant was removed and the pellet was mixed at a ratio of 1:1 with 3 % low melting agarose, left for 10 min at 37 °C and hardened at 4 °C for 30 min. The pellets were cut in small pieces (125 mm^3) and washed four times for 15 min each with 0.1M sodium cacodylate buffer. Postfixation was applied using 1 % OsO_4 (Science Services, München, Germany), 1.25 % sucrose, 1 % potassiumferrocyanide in 0.1M sodium cacodylate buffer for 2h at 4 °C. Samples were washed four times with 0.1M sodium cacodylate buffer and dehydrated using an ascending ethanol series (50 %, 70 %, 90 %, 3 × 100 %) for 15 min each. Samples were incubated with a mixture of ethanol/propylenoxide (1:1) and two times with pure propylenoxide for 15 min each. They were then infiltrated with a mixture of epon/propylenoxide (1:1) followed by epon/propylenoxide (3:1) for 2h each at 4 °C as well as with pure epon overnight at 4 °C. The next day, the epon was refreshed and samples were incubated for 2h at room temperature. Samples were mounted onto epon blocks and cured for 72h at 60 °C. Ultrathin sections of 70 nm were cut using an ultramicrotome (Leica Microsystems, Wetzlar, Germany) and a diamond knife (Diatome, Biel, Switzerland) and stained with 1.5 % uranyl acetate for 15 min at 37 °C and 3 % lead citrate solution for 4 min. Images were acquired using a JEM-2100 Plus Transmission Electron Microscope (JEOL, Freising, Germany) operating at 80 kV equipped with a OneView 4K camera (Gatan, Pleasanton, USA). Images of the head and the midpiece of the spermatozoa were collected to obtain a minimum of 50 spermatozoa per sample. The spermatozoa were classified depending on their ultrastructural damages as Class I: intact plasma membrane (slightly wavy membrane accepted) and mitochondria without any specific abnormality, Class II: plasma membrane strongly rippled without interruption and mitochondria with minor abnormalities (diminution of cristae or roundness deviation) or Class III: spermatozoa with significant disturbances, damages and breaks in the plasma membrane or major abnormalities in the mitochondria.

2.9. Statistical analysis

The fertilisation rate, described as the number of 2-cell embryos developing from the number of oocytes incubated (Fig. 1), was treated as a binomially distributed variable. Therefore, 2x2-table Chi-Squared tests (Fisher-tests) were employed for the assessment of treatment effects, that is, differences in IVF rates (%) for LN_2 vs. −80 °C stratified by strain (129, B6J, B6N, BALB/c, CD-1 and FVB) as well as by storage duration in months (1, 3, 6, 9 or 12 months). An analysis of variance (ANOVA) was performed to analyse the blastocyst development (Fig. 2) depending on the two cryopreservation temperature at the different time points. The strains (129, B6J, B6N, BALB/c, CD-1, and FVB), treatment (storage in LN_2 or storage at −80 °C for 1, 3, 6, 9 or 12 months), and specific distributions of dead spermatozoa (Fig. 3) for all 6 pairwise status contrasts was compared using two-sample t-tests allowing for potential heterogeneity of the variances (method Satterthwaite). For the association between treatment (LN_2 vs. −80 °C) and morphological abnormalities (Fig. 5) as well as the association between treatment and the ultrastructural damages (Fig. 6), Chi-Squared tests were conducted by mouse strain and duration of storage. Statistical analysis was not possible with the total motility data (Fig. 4) since duplicate measurements were performed with only one sample each. Otherwise, data are presented as mean ± standard error of the mean (SEM). P-values <0.05 were considered statistically significant and asterisks correspond to: *** p-value <0.001; 0.001 ≤ ** p-value <0.01; 0.01 ≤ * p-value <0.05. Statistical analyses were performed with the software package SPSS Statistics 23 (IBM Corp., Amonk) and with SAS/STAT software 9.4 (SAS Institute Inc: SAS/STAT User's Guide, Cary NC: SAS Institute Inc, 2014). Graphical representations were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, USA).

3. Ethical review procedure

All animal studies were approved by the Institutional Animal Care and Use Committee of the CNR. Experiments were performed in accordance with general guidelines regarding animal experiments and approved by the Italian Ministry of Health in compliance with the Legislative Decree 26/2014 and 116/1992. The animal study protocol was approved by the Institutional Review Board (Organismo Preposto al Benessere degli Animali, OPBA) of the Institute of Biochemistry and Cell Biology-EMMA/Infrafrontier (Protocol number 0000079 of January 18, 2016) for studies involving animals.

4. Results

4.1. The in vitro fertilisation rate was maintained over time in spermatozoa cryopreserved and stored at −80 °C compared to liquid nitrogen except in B6J and BALB/c strains

The IVF rate of 129, B6N, CD-1 and FVB did not show significant differences after cryopreservation and storage at −80 °C compared to the liquid nitrogen control even after 12 months (Fig. 1). After 6 months,

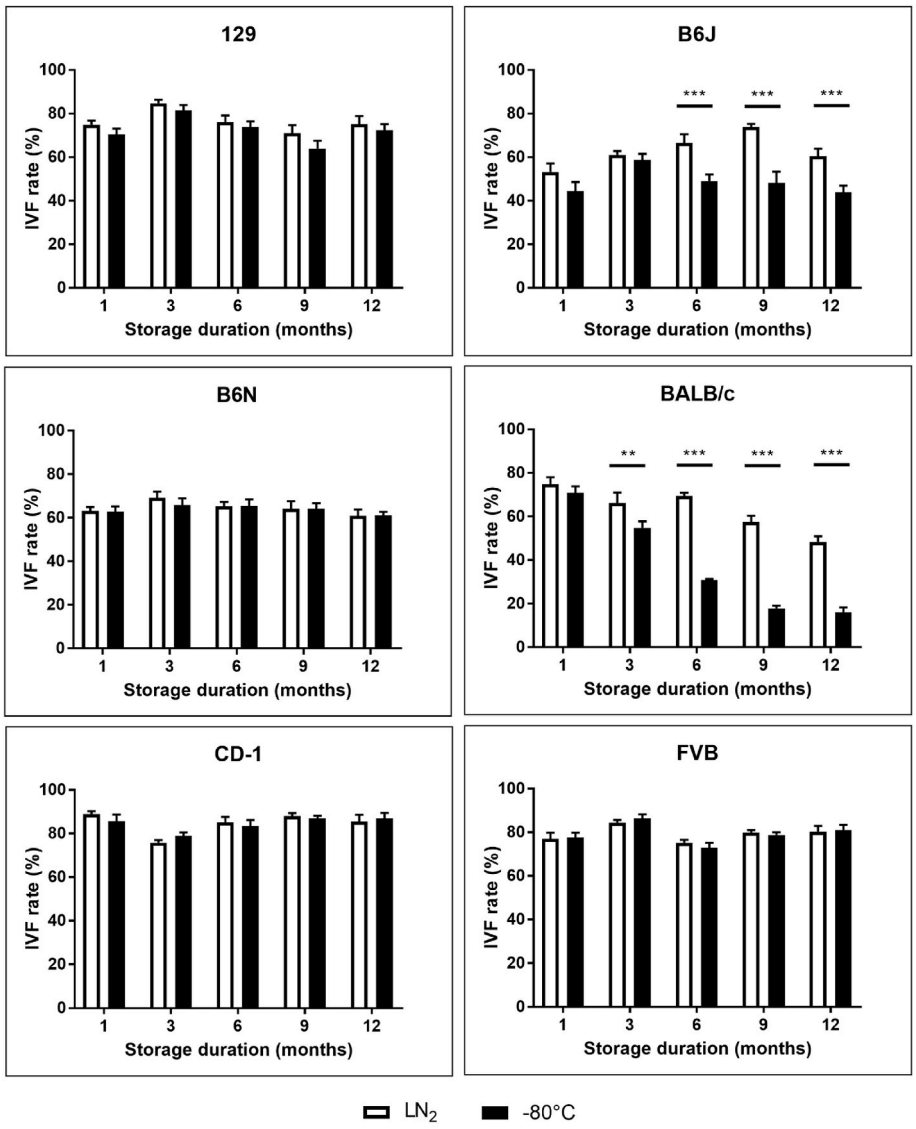

Fig. 1. Effect of cryopreservation and storage temperature on sperm fertility. The graphic represents the *in vitro* fertilisation rate of spermatozoa from 129, B6J, B6N, BALB/c, CD-1 and FVB males cryopreserved in LN_2 or at −80 °C and then stored for 1–12 months. It corresponds to the resulting 2-cell embryos (considered as fertilised oocytes) in 6 assays using a pool of spermatozoa from 3 animals, ** p-value <0.01 and *** p-value <0.001).

the percentage of 2-cells obtained from B6J sperm cryopreserved directly at −80 °C was significantly reduced compared to the control (49.0 ± 3.0 % vs 66.4 ± 4.1 %, $p < 0.001$). This reduction remained constant (about 70 % of the value for the control) up to 12 months. After 3 months, the IVF rate of BALB/c sperm cryopreserved directly at −80 °C was significantly reduced compared to the control (54.8 ± 13.0 % vs 66.2 ± 4.9 %, $p < 0.01$). This reduction increased over time up to 12 months when the percentage of 2 cells was only 16.1 ± 2.2 % compared to 48.4 ± 2.6 % in liquid nitrogen.

4.2. Embryo development was not affected by the sperm cryopreservation temperature and the storage duration

The sperm cryopreservation temperature did not affect the quality of embryos obtained from *in vitro* fertilisation. For all strains examined, there were no differences between the percentage of blastocysts obtained from culture of the 2-cells that originated from spermatozoa cryopreserved in LN_2 and directly at −80 °C (Fig. 2).

4.3. In B6J and BALB/c, the percentage of dead spermatozoa increased significantly over time when they were cryopreserved and stored at −80 °C

Cryopreservation and storage at −80 °C did not affect the spermatozoa from 129 mice except at 6 and 12 months of storage at −80 °C when the percentage of dead spermatozoa increased from 37 % in LN_2 to 43 % and 48 %, respectively ($p < 0.05$). In the B6N strain, there was an increase in the percentage of dead spermatozoa from 1 (37 %) to 9 months (49 %, $p < 0.05$) at −80 °C compared to the LN_2 control (30 %, Fig. 3). No differences were observed for the dead populations in CD-1 mice, where the percentage of dead spermatozoa ranged from 22 % in LN_2 samples to 35 % after 12 months at −80 °C ($p > 0.05$). Also, in FVB mice, we observed a similar result in the percentage of dead cells at −80 °C compared to samples in liquid nitrogen over time (40 %, 41 %, 42 %, 43 % and 37 % vs. 51 % respectively, $p > 0.05$). In B6J males, cryopreservation at −80 °C significantly reduced sperm viability after 6 months of storage reaching the peak after 12 months (76 %, $p < 0.001$) compared to 53 % in the LN_2 control. This observation was also pronounced in BALB/c males, where the percentage of dead spermatozoa was significant already after 3 months, increasing from 32 % in the LN_2 control to 57 % after 12 months ($p < 0.001$).

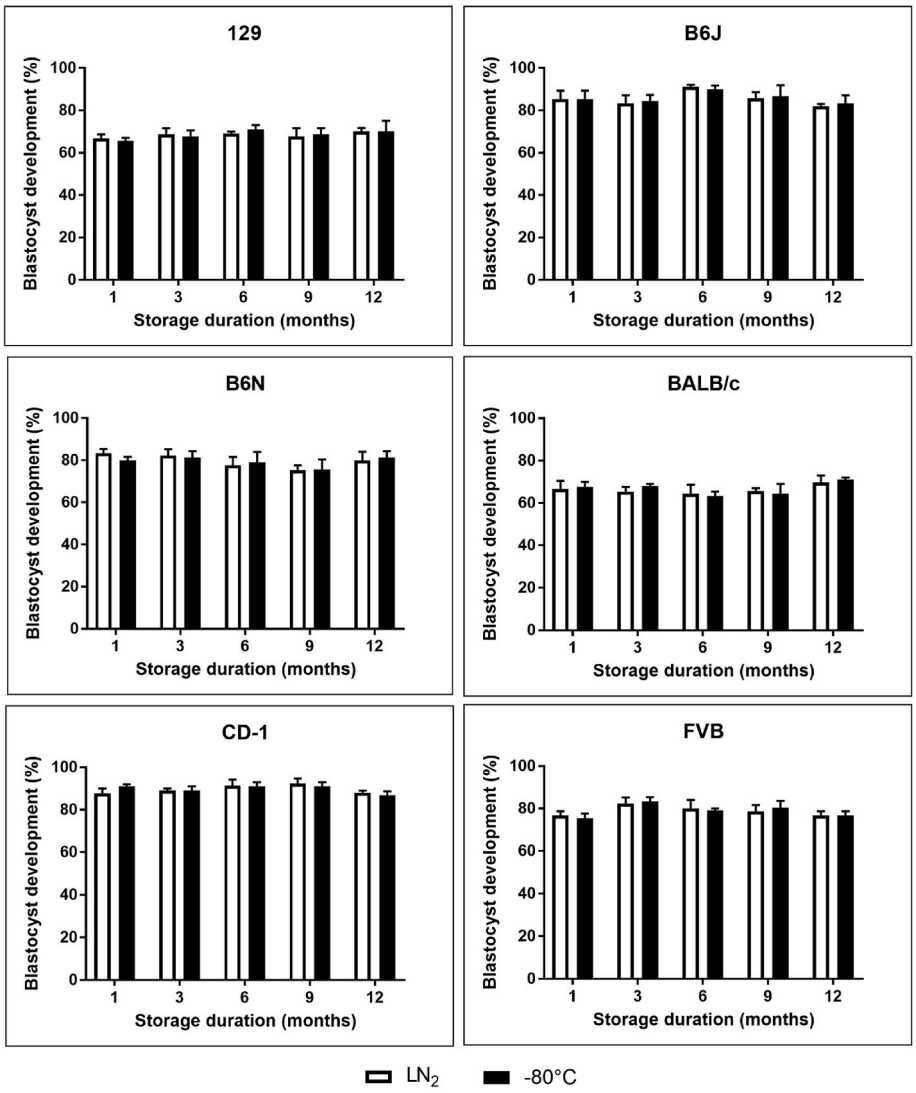

Fig. 2. Effect of cryopreservation and storage temperature on embryo development. The graphic represents the blastocyst development after *in vitro* culture of IVF 2-cell embryos produced from 129, B6J, B6N, BALB/c, CD-1 and FVB spermatozoa, which were cryopreserved and stored for 1–12 months in LN_2 or at −80 °C.

The Supplementary Fig. S1 shows the negative correlation between the percentage of dead sperm and the percentage of 2-cells obtained; an increase in the percentage of dead spermatozoa was associated with a reduction of the IVF rate ($r < 0$). In B6J, the percentage of dead spermatozoa showed a good association with the IVF outcome ($R^2 = 0.57$). However, the regression parameters were close to being significant ($p = 0.08$). In BALB/c, the R^2 reached 0.74 and was statistically significant ($p < 0.05$).

4.4. In all mouse strains, cryopreservation and storage at −80 °C decreased sperm motility

In all mouse strains, the total motility was always lower in the −80 °C groups (storage for 1, 6 and 12 months) compared to LN_2 (storage for 12 months). As shown in Fig. 4, the total motility of spermatozoa cryopreserved and stored in LN_2 for 1 year was highest in B6N and 129 compared to the other mouse strains, where more than 20 % of the spermatozoa had a velocity greater than 7.4 μm/s. In contrast, BALB/c spermatozoa had the lowest total motility (less than 10 % of spermatozoa had a velocity greater than 7.4 μm/s). In particular, when spermatozoa were cryopreserved and stored at −80 °C for 1 month compared to LN_2, we obtained a 2-fold decrease in B6N sperm motility (from 24 % to 12 %). Similarly, we obtained a 1.25-fold decrease in B6J (from 10 % to 8 %), a 1.17-fold decrease in BALB/c (from 7 % to 6 %), a 1.63-fold decrease in CD-1 (from 13 % to 8 %), a 1.25-fold decrease in FVB (from 15 % to 12 %), and a 1.77-fold decrease in 129 (from 23 % to 13 %). Generally, the total motility was maintained from 1 to 12 months at −80 °C in all strains except in B6J (decrease from 8 % at 1 month to 4 % at 12 months).

4.5. In all mouse strains, cryopreservation and storage at −80 °C increased sperm abnormalities

Spermatozoa were classified according to the type of abnormality observed and the results are shown in Fig. 5. Regarding the LN_2 group, the percentage of total abnormalities was approximately 20 % in all mouse strains except B6J and BALB/c, where 29.2 % and 41.6 % of spermatozoa, respectively, had at least one morphological abnormality. In all strains except BALB/c, the total number of abnormalities increases, but not significantly, between LN_2 and 1 month at −80 °C (from 18.8 % to 25.6 % in B6N, from 29.2 % to 35.7 % in B6J, from 22.5 % to 27.7 % in CD-1, from 24.9 % to 28.5 % in FVB and from 20.8 % to 28.4 % in 129). Spermatozoa from BALB/c showed a significant increase in total abnormalities already after 1 month at −80 °C compared to LN_2 (from 41.6 % to 53.3 %, $p < 0.05$). However, prolonged storage at −80 °C for 12 months significantly increased the percentage of total abnormal

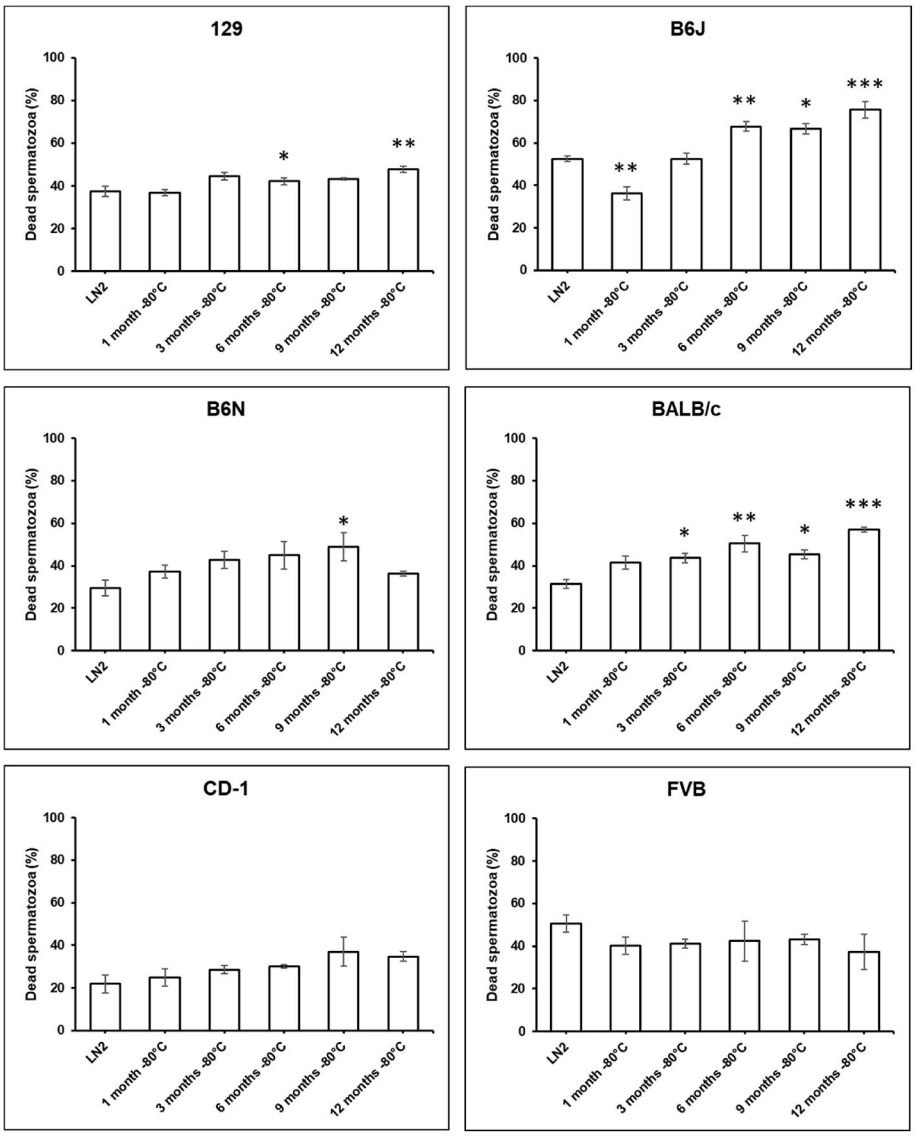

Fig. 3. Effect of cryopreservation and storage temperature on sperm viability. Sperm suspension from 129, B6J, B6N, BALB/c, CD-1 and FVB mice cryopreserved in LN_2 or at −80 °C and then stored for 1–12 months were analysed by flow cytometry to detect dead spermatozoa (10,000 events per assay, 3 assays using a pool of spermatozoa from 3 animals, * p-value <0.05, ** p-value <0.01 and *** p-value <0.001).

spermatozoa in all strains. Also, specifically in BALB/c, most (more than 20 %) abnormal spermatozoa were due to abnormalities in the head of the spermatozoa compared to the tail (less than 15 %). In all other strains, most abnormalities were found in the tail of the spermatozoa.

4.6. Cryopreservation and storage at −80 °C altered the sperm ultrastructure in all mouse strains

Ultrastructural damage was analysed using transmission electron microscopy images. Spermatozoa were classified into class I (intact), class II (slightly damaged) and class III (severely damaged) as shown in Fig. 6A. Two different organelles were examined, the nucleus and the mitochondrion as well as the plasma membrane. In the representative images, the nucleus of the spermatozoa is black. The plasma membrane around the nucleus was considered intact (class I), waved (class II), or broken (class III). Mitochondria were also visible in the transverse section of the central part of the spermatozoa. They were considered intact (class I), slightly damaged with loss of cristae and roundness (class II) or severely damaged with significant loss of cristae and roundness (class III).

Fig. 6B shows the classification of spermatozoa from each strain as a function of cryopreservation method and storage time. The percentage of severely damaged spermatozoa (class III) significantly increased when spermatozoa were cryopreserved at −80 °C and stored at this temperature for 1 month compared to LN_2 in all mouse strains. We also observed a gradual increase in class III spermatozoa stored at −80 °C with time for most strains. The opposite effect was observed for intact (class I) spermatozoa. Finally, the highest rate of sperm ultrastructural damage in LN_2 and at −80 °C was found in BALB/c. Specifically, 95.8 % of BALB/c spermatozoa were damaged (class II + class III) after 1 year at −80 °C compared to only 54.5 % in B6N.

5. Discussion

Sperm cryopreservation has traditionally been performed using LN_2. However, the use of LN_2 can be challenging for reasons of safety, cost and availability. Available alternatives to LN_2 could be the use of ultra-low temperature freezers, having a temperature of −80 °C. Several studies on B6N sperm cryopreservation and storage at −80 °C have already shown promising results [8,10]. Therefore, to expand our

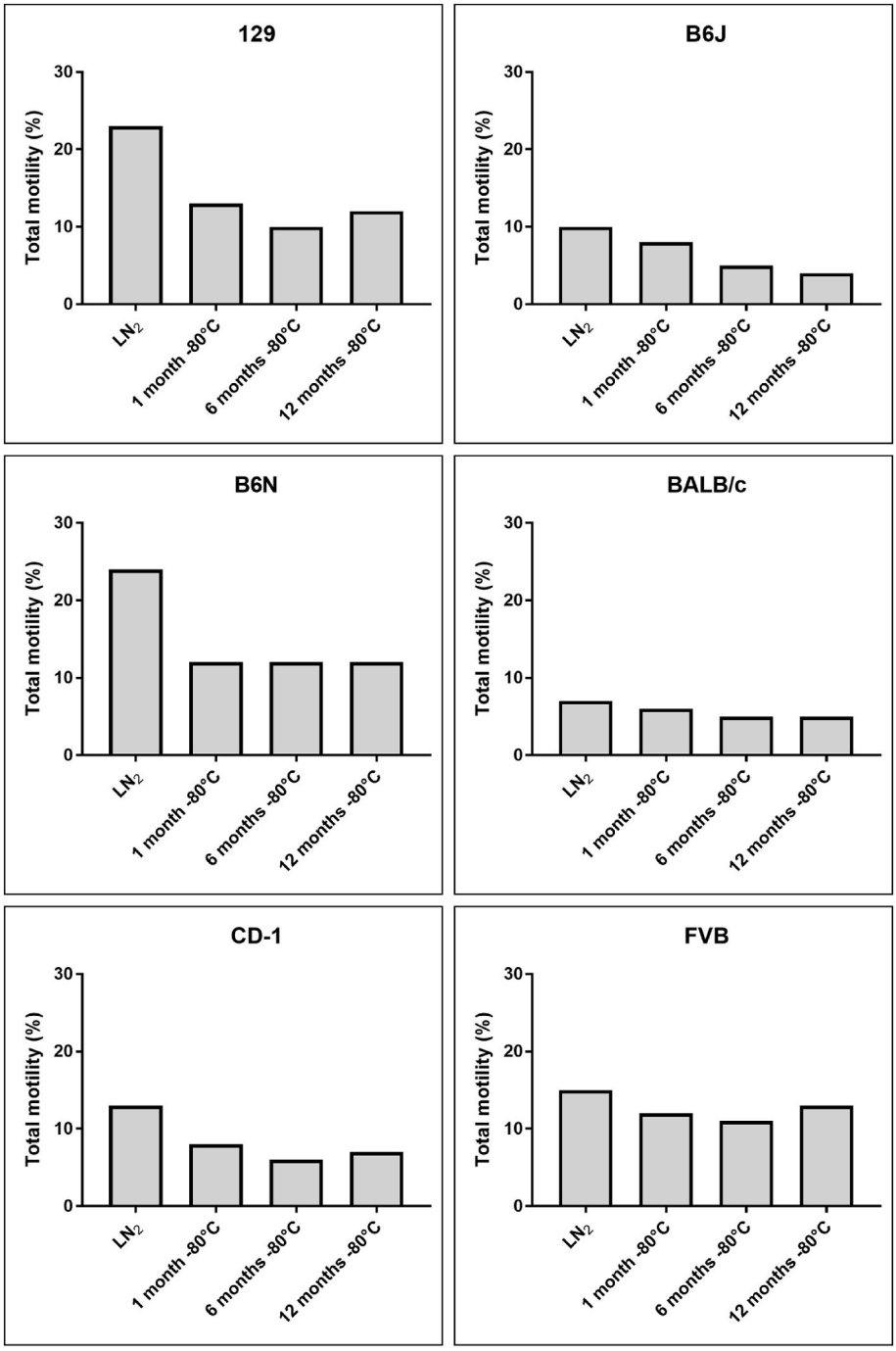

Fig. 4. Effect of cryopreservation and storage temperature on sperm motility. The percentage of motile spermatozoa (velocity >7.4 μm/s in any direction) from 129, B6J, B6N, BALB/c, CD-1 and FVB males after cryopreservation and storage in LN_2 or at −80 °C for 1–12 months was measured using the semen analyzer Hamilton Thorne IVOS (mean of 2 measurements from a pool of 3 animals).

knowledge on the applicability of sperm cryopreservation and storage at −80 °C, the current study was extended to include diverse mouse genetic backgrounds that are commonly used in the biomedical field, with a focus on sperm integrity and fertility. For routine conditions, usually spermatozoa from 2 males are cryopreserved. In the present study, spermatozoa from 3 males per strain were pooled and straws from this pool were used for all corresponding experiments. Based on our experience and previous scientific data in this field, we think that the data show rigor. The present results indicate that the temperature of −80 °C impact the integrity and fertility of cryopreserved spermatozoa in a mouse strain-dependent manner.

The current study showed that cryopreservation and storage at −80 °C for up to one year can be considered for spermatozoa from 129, B6N, CD-1 and FVB mouse strains as no decline in the IVF success was observed. Also, embryo development and viability of spermatozoa from these strains were maintained for up to one year at −80 °C. However, changes in sperm motility, morphology and ultrastructure were detected in all these mouse strains.

An initial decrease of approximately 40 % in total sperm motility was found when spermatozoa were cryopreserved at −80 °C compared to LN_2. However, the total motility was generally maintained from 1 to 12 months. This suggests that defects in motility are due to the initial

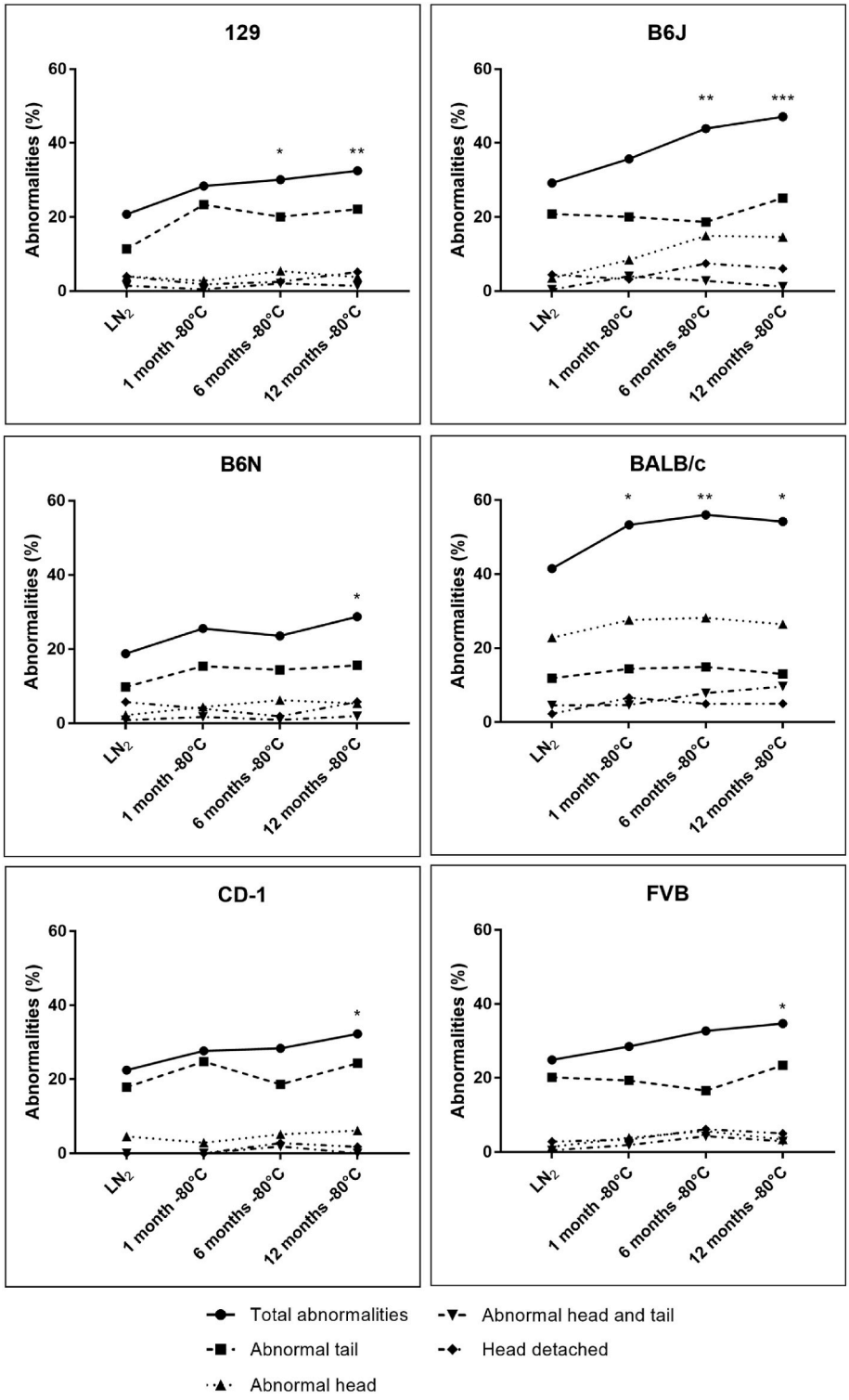

Fig. 5. Effect of cryopreservation and storage temperature on sperm morphology. Level of abnormalities in spermatozoa from 129, B6J, B6N, BALB/c, CD-1 and FVB mice cryopreserved in LN_2 or at −80 °C and then stored for 1–12 months were analysed. Spermatozoa were classified depending on their morphology: abnormal head, abnormal tail, abnormal head and tail, head detached and total abnormalities. Results are shown as percentage of abnormal spermatozoa found in each condition and strain, 200 spermatozoa examined from a pool of 3 animals, * p-value <0.05, ** p-value <0.01 and *** p-value <0.001.

cryopreservation procedure but not to storage at −80 °C. Changes in the ultrastructure of the sperm flagella may occur at an early stage of cryopreservation, especially during the cooling phase since mouse sperm motility is known to depend on the cooling rate [14].

A prolonged storage period of 12 months at −80 °C significantly increased the percentage of abnormalities. In contrast to motility, our results suggest that the duration of storage but not the initial cryopreservation at −80 °C damages sperm morphology in these strains. Studies of sperm cryopreservation and storage at −80 °C in other species have not reported an increase in sperm abnormalities. However, the duration of storage was limited to 5 days in mandarinfish [15], 1 month or 3 months in humans [16,17], and 4 months in dogs and cats [18,19].

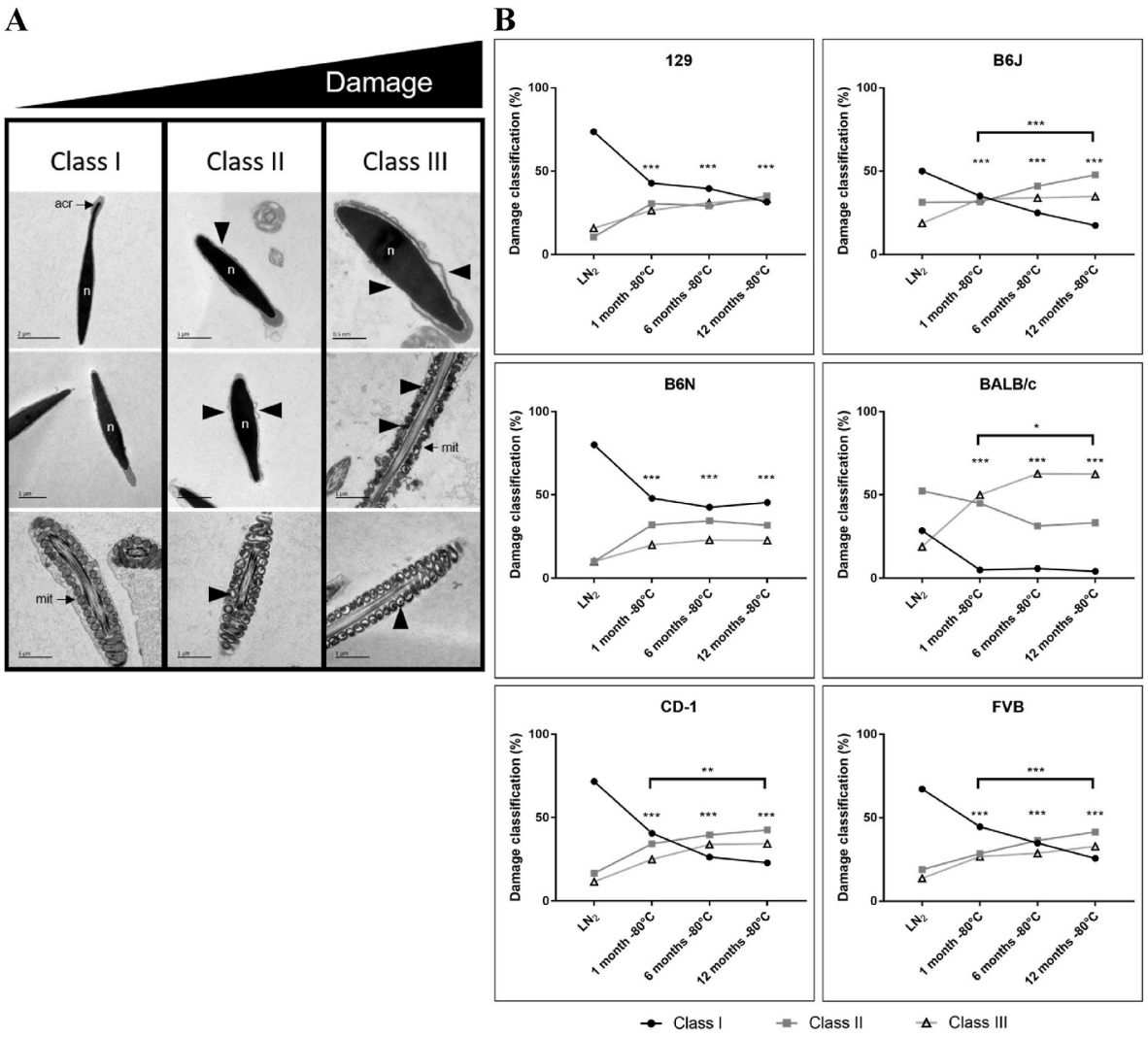

Fig. 6. Effect of cryopreservation and storage temperature on sperm ultrastructure. (A) Transmission electron microscopy images of longitudinal sections of spermatozoa from 129, B6J, B6N, BALB/c, CD-1 and FVB males cryopreserved in LN_2 or at −80 °C and then stored for 1–12 months were obtained, focusing on the head of the spermatozoa and the midpiece that contains mitochondria. Spermatozoa were classified depending on the ultrastructural damages found: class I have an intact or slightly waved plasma membrane and intact mitochondria, class II have heavily waved plasma membrane (arrowhead) without breaks and/or mitochondria with minor abnormalities (arrowhead showing loss of cristae) and class III have significant disturbances, damages and breaks of the plasma membrane (arrowhead) and/or major abnormalities on the mitochondria (arrowhead for abnormal shape and loss of cristae); n: nucleus, acr: acrosome, mit: mitochondria. (B) Percentage of class I, class II and class III spermatozoa depending on the cryopreservation method in each strain, 50 to 70 spermatozoa were examined from a pool of 3 animals, * p-value <0.05, ** p-value <0.01 and *** p-value <0.001.

Therefore, it is possible that a prolonged storage period at −80 °C affects sperm morphology in these species. In addition to morphological abnormalities, increased ultrastructural damage was found already after 1 month of storage at −80 °C and continued over time, except for spermatozoa from B6N and 129, where it remained stable from 1 to 12 months. Thus, the ultrastructural damage of spermatozoa seems to depend not only on the cryopreservation temperature, but also on storage duration. Similar cryoinjuries have been reported in spermatozoa cryopreserved in LN_2 from mice [20], chickens [21] and rams [22]. Thus, it would be expected that a higher cryopreservation temperature of −80 °C would lead to at least the same type of ultrastructural cryoinjury.

The use of a −80 °C freezer for sperm cryopreservation and storage affects the integrity and fertility of mouse spermatozoa in a strain-dependent manner. Indeed, B6J and BALB/c spermatozoa behaved differently from the 129, B6N, CD-1 and FVB strains for most of the parameters studied. First, a decrease in IVF was observed in relation to the duration of storage at −80 °C, starting at 6 months for B6J and 3 months for BALB/c. The storage duration at −80 °C also affected sperm viability in B6J and BALB/c and the timing corresponded to the decline in the IVF rate. In addition, a correlation was found between the IVF success rate and sperm viability for these two strains (Supplementary Fig. S1), indicating that viability is a critical parameter for IVF success in these genetic backgrounds. The reasons for the increased death of spermatozoa may be related to the significant damage to the plasma membrane, especially in the acrosome region, resulting in abnormal sperm morphology [23–25].

In the present study, BALB/c spermatozoa appeared to be the most sensitive to cryopreservation in LN_2 and at −80 °C. The lowest motility rate and the highest abnormality rate were obtained in cryopreserved spermatozoa from BALB/c even when LN_2 was used. Importantly, the type of abnormalities was different in cryopreserved spermatozoa from BALB/c compared to the other strains. The percentage of abnormal head was much higher than the percentage of abnormal tail in BALB/c, while we observed the opposite relationship in the other strains. Furthermore, the percentage of severely damaged spermatozoa was the highest in

BALB/c under −80 °C cryopreservation conditions.

The decline in the motility and integrity of 129, B6N, CD-1 and FVB spermatozoa was not associated with a decrease of the IVF rate. Considering sperm motility, conflicting results about its association with the IVF success rate are found in the literature. Some studies indicate a correlation between sperm motility and IVF success in humans and mice [12,26,27]. In contrast, there are also studies in mice where sperm motility did not correlate with the fertilisation rate [28]. Sperm morphology is also considered an important parameter to assess the fertilising potential [29]. However, fertilisation is still possible even in the case of abnormal sperm morphology [30].

One possible explanation for the maintenance of the IVF success rate despite sperm abnormalities would be that the remaining intact (or less damaged) gametes are sufficient to support a normal fertilisation rate. After all, the most motile spermatozoa are collected for IVF, only one spermatozoon is needed to fertilise an oocyte, and the number of oocytes per dish are usually in the range of 100–200. Therefore, the concentration of spermatozoa used for IVF would play an important role in compensating for the loss of sperm function due to cryoinjuries.

In the current study, a temperature of −80 °C was used to simulate conditions, which are often found even in smaller laboratories where a −80 °C freezer is often available or affordable and since dry ice is often used for transport between institutions. Since a temperature of −80 °C was not suitable for cryopreservation and storage of B6J and BALB/c spermatozoa a temperature of −152 °C, which is provided by some ultra-low temperature freezers, could be examined if one wants to also implement a LN_2-free cryopreservation method for cryopreservation and long-term storage of B6J and BALB/c spermatozoa. The glass transition temperature of water is −134.5 °C [31] so that it should not be expected that cryoinjuries at −152 °C will be higher than those observed using LN_2 but this would require further research. Notably, there are reports of cryopreservation and storage at −152 °C of caprine semen for up to 1 year without affecting the kidding rate [32]. Also, canine semen was cryopreserved and stored at −152 °C for 120 days without showing a decrease in motility or viability nor an increase in abnormal spermatozoa and abnormal acrosomes [33] and for 360 days without altering abnormality and motility [34]. Also, the feasibility of the use of other cryoprotectants for cryopreservation and storage at −80 °C should be investigated.

CRediT authorship contribution statement

Manon Peltier: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Marcello Raspa:** Writing – review & editing, Writing – original draft, Project administration, Conceptualization. **Sabrina Putti:** Methodology, Investigation, Data curation. **Renata Paoletti:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Ferdinando Scavizzi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Esther Mahabir:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Funding

This work was supported by the Infrafrontier-I3 project under EU contract Grant Agreement Number 312325 of the EC FP7 Capacities Specific Program at CNR, Institute of Biochemistry and Cellular Biology and intramural funding to EM.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marcello Raspa, Ferdinando Scavizzi reports financial support was provided by Infrafrontier-I3 project under EU contract Grant Agreement Number 312325 of the EC FP7 Capacities Specific Program at CNR, Institute of Biochemistry and Cellular Biology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to thank the CECAD imaging facility for their support in sample processing and transmission electron microscopy images, Hagen Scherb for help with the statistical analyses as well as Arsenio Armagno, Tiziana La Penna, Alessio Braconcini and Fabrizio Bonaventura for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.117523.

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