ORIGINAL ARTICLE

ANDROLOGY WILEY

The impact of five years storage/biobanking at −80°C on mouse spermatozoa fertility, physiology, and function

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Abstract

Background: We previously demonstrated how mouse spermatozoa can be efficiently stored for two years in a −80°C freezer, maintaining their ability to fertilize mouse eggs.

Objectives: The main objective here was to evaluate the effects of five years at −80°C compared to liquid nitrogen storage (LN_2, control condition) on mouse sperm viability, physiological parameters, and fertilization capacity.

Materials and methods: Three different strains were used: C57BL/6N, C57BL/6J and CD1. Flow cytometry experiments were performed to analyze sperm viability (SYBR-14 + Propidium Iodide +Hoechst33342), the intracellular calcium concentration (Fluo 3-AM), the membrane lipid disorder (Merocyanine 540), and the mitochondrial activity (MitoTracker Red) in live spermatozoa. The *in vitro fertilization* (IVF) was used to evaluate the sperm fertilizing ability.

Results: Flow cytometry analysis showed that the percentage of live cells are reduced in B6N and B6J, but not in CD1 mice. However, in the live population no differences in terms of intracellular calcium concentration, membrane lipid disorder, and mitochondrial activity were reported when comparing both biobanking methods. Spermatozoa stored at −80°C for 5 years successfully fertilized the eggs and developed mouse embryo normally both in culture and in vivo, generating live pups with no differences compared to control samples stored in LN_2.

Discussion: Long-term mouse sperm storage at −80°C (five years) could be considered an ideal alternative to the most common LN_2 approach, giving economical and logistic advantages. Moreover, the precise information originated from the flow cytometry analysis stands up this technique as an optimal strategy to evaluate the sperm quality and ranking.

Conclusion: It is demonstrated here the possibility to store mouse spermatozoa for up to five years in a −80°C freezer with no significant differences compared to the storage in LN_2 in terms of fertilizing ability, sperm viability, intracellular calcium concentration, membrane lipid disorder, and mitochondrial activity.

Raspa and Putti authors contributed equally to this work.

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1 | INTRODUCTION

Preservation of laboratory mouse genomes is a key task in biomedical research and translational science. Virtually, all the branches of medicine and physiology had enormous benefits from the investigations of the molecular and genetic mechanisms of healthy and pathological functions carried out using animal models and, in particular, the mouse. Indeed, mice and humans share nearly the same set of genes, protein-coding regions, and a common ancestor approximately 80 million years ago. Almost every gene found in one species so far has been found in a closely related form in the other. Both the mouse and human genomes contain about 3.1 billion base pairs and on average, the protein-coding regions of the mouse and human genomes are 85% identical. Therefore, the genomes of all mammals are comparably similar, while the mouse and human genomes are the most studied and the first produced and analyzed (HGP:2000; MGP:2002). In this context, the development of a platform for genome conservation plays a key role to make available and share precious resources for the scientific community worldwide. Cryopreservation of mouse spermatozoa has become the most achievable and less expensive method for long-term storage, manipulation, and transit of Genetic Altered (GA) resources in biomedical research.[1,2] Over the years, various freezing methods have been developed using different types of cryoprotectants.[3-6] However, all these methods use liquid nitrogen (LN$_2$) as storage and transport systems, which has several problems in terms of cost, safety, and hazard. Liquid nitrogen and cold nitrogen vapors can cause extensive tissue damage. Although nitrogen is non-toxic and inert, it can act as a simple asphyxiant by displacing oxygen in air to levels below the required to support life. Inhalation of nitrogen in excessive amounts can cause dizziness, nausea, vomiting, and loss of consciousness. Oxygen level monitoring should be provided for areas where cryogenic containers are stored and each container should be equipped with pressure relief devices designed to control the internal pressure. The Pressure Systems Safety Regulations 2000 (Health and Safety Executive 2014) apply to all systems containing liquefied gas operating at a pressure greater than 0.5 bar above atmospheric. These regulations require users to ensure the proper maintaining of the systems, which should be periodically examined and operated within the established safe operating limits. Shipping of samples in LN$_2$ requires the dry shippers, which makes the system expensive. Furthermore, improperly prepared dry shippers present a risk of liquid nitrogen leakage and are subject to regulation by the United States Department of Transportation (USDOT) should spillage occur. Violations of USDOT shipping regulations may result in civil and/or criminal penalties.

Our group previously reported that frozen mouse spermatozoa from different backgrounds can be stored at −80°C for 7 days, shipped in dry ice (−79°C) and, if necessary, transferred back to LN$_2$, maintaining their fertilization capacity.[7] Dry ice provides a huge advantage for safety and cost compared to LN$_2$ dry shipper. This preferential method is now routinely used to archive and distribute genome resources within the EMMA Repository–Infrafrontier, as many others worldwide (www.infrafrontier.eu/knowledgebase/protocols/cryopreservation-protocols).

The main goal of the present study was to extend the timing of −80°C storage up to 5 years and analyze the fertilizing ability and function of spermatozoa from C57BL/6N, C57BL/6J, and CD1 mouse strains.

To evaluate the effects of an extended storage on mouse spermatozoa preserved at −80°C, morphological and functional analysis were performed. In particular, the loss of acrosome integrity was studied and the percentage of living and dead cells were established. Then, three parameters related to the sperm function were analyzed by flow cytometry: the intracellular calcium concentration, the membrane lipid disorder, and the mitochondrial activity. Finally, the last experiment compared the performance of the two methods of storage in terms of IVF outcomes, embryo quality and birth rates in terms of viability and fertilization ability.

The importance of this work is prospectively relevant for the whole Mouse Resources Biorepository Network and strategic research infrastructures, but also for peripheral research laboratories with no constant access to LN$_2$. More in detail, the use of a more accessible −80°C freezer that maintains the back-up storage could be advantageous for laboratories with a massive generation of GA lines (i.e., by CRISPR/Cas9) and small research groups and Institutes that do not desire to saturate the limited space of a LN$_2$ tank.

2 | MATERIALS AND METHODS

2.1 | Mice and husbandry

C57BL/6N (B6N), C57BL/6J (B6J), and CD1 mice were bred at the Consiglio Nazionale delle Ricerche-European Mouse Mutant Archive (CNR-EMMA)-Infrafrontier specific pathogen-free (SPF) barrier unit (Monterotondo Scalo). Mice were housed in individually ventilated cages (Tecniplast) at a temperature of 20 ± 2°C, relative humidity of 55 ± 15% with 12–15 air changes per hour and a 12/12-hour light/dark cycle. Mice were fed following a standardized mouse diet (4RFN and Emma 23, Mucedola) and were provided chlorinated, filtered water ad libitum. Mice were

tested for micro-organisms every 3 months using 6- to 8-week old B6N sentinels. Serology was performed according to the FELASA recommendations.[8]

2.2 | Reagents

D-(+)-raffinose pentahydrate, α-monothioglycerol (MTG), reduced L-glutathione (GSH), bovine serum albumin (BSA, embryo-tested), polyvinyl alcohol (PVA), methyl-β-cyclodextrin (MBCD), Hoechst33342 (trihydrochloride trihydrate: H342), and embryo-tested water were purchased from Sigma-Aldrich (Merck KGaA). Skimmed milk was sourced from BD Diagnostics and human tubal fluid (HTF) medium from Millipore (Merck, KGaA). Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Intervet. For sperm capacitation, prior to IVF, a modified Krebs-Ringer bicarbonate solution (TYH) containing PVA 1.0 mg/ml and MBCD 0.75 mM of medium was prepared in-house.

The human tubal fluid (HTF) medium was modified with the addition of $CaCl_2$ to increase the Ca^{2+} concentration from 2.04 mM (regular concentration) to 5.14 mM (high concentration).

LIVE/DEAD® Sperm Viability Kit and Hoechst 33342 were purchased from Molecular Probe (Thermo Fisher Scientific). Fluo 3-AM was purchased from Thermo Fisher Scientific, Merocyanine M540 and Pisum sativum agglutinin (PSA) staining were acquired from Sigma-Aldrich (Merck KGaA), Near Infrared dye, MitoTracker Red, and 7-Aminoactinomycin D (7AAD) were purchased from BD Biosciences.

2.3 | Sperm cryopreservation

Spermatozoa from 3-month-old males were cryopreserved as previously described.[5] For each strain, the *caudae epididymides* and the *vasa deferentia* of two males were collected and pooled in a sperm collection dish (Falcon) containing 2 ml of the cryoprotective medium (CPM). The CPM contained 18% w/v raffinose, 3% w/v skim milk, and 477 µM MTG. Spermatozoa was released from the caudae epididymides and the vasa deferentia and allowed to disperse from the tissues for 10 min at 37°C. Then, the pooled spermatozoa was loaded into 0.25 ml French straws (IMV Technologies). Four aliquots of 12 ml each of spermatozoa and CPM were loaded into each French straw (sperm concentration 15–30 × 10^6 spermatozoa/ml).

Straws were then frozen by exposure to liquid nitrogen vapor for 10 min before being plunged into LN_2. After freezing, the straws were transferred either to a LN_2 cryogenic tank or to a −80°C freezer and kept for 5 years.

2.4 | Viability assessment by flow cytometry

Mouse sperm membrane integrity and viability was assessed by using the flow cytometry FACSCanto II (Becton Dickinson). The robust sperm population was identified and gated based on FSC and SSC parameter. On the selected population, non-sperm events were gated out of analyses by selecting only those cells H342 positive in order to exclude particles without nuclei and with the same scatter properties as spermatozoa. The use of a DNA stain (such as Hoechst342) for excluding cell debris that is the same size as spermatozoa has been recommended by several researchers.[9-11]

Then, the selected sperm population was identified by Sybr14 as live cells while propidium iodide (PI)-positive cells were considered as dead cells (LIVE/DEAD Sperm Viability Kit). Briefly, frozen straws stored in LN_2 or at −80°C for 5 years were transferred in liquid nitrogen and thawed as previously described. 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 × 10^6 cells/mL and were incubated at 37°C for 10 min with 1 µg/ml Hoechst342. After centrifugation at 1000 rpm for 6 min, cells were resuspended (1 × 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 addition of PI (final concentration 12 µM) and incubated for three minutes before examination.

A total of 10.000 events were analyzed. The SYBR-14 and PI dyes were excited using a 488 nm Blue Laser. A 405 nm Violet laser was used to excite H342.

Fluorescence minus one control (FMO) was used to properly interpret flow cytometry data. These data were analyzed with FlowJo Software (BD, USA). For each strain and experimental group, three replicates were analyzed.

2.5 | Flow cytometry analysis of intracellular calcium, membrane lipid disorder, and mitochondrial activity

Flow cytometry analysis was as well performed to evaluate the differences between the spermatozoa frozen with LN_2 and those preserved in a −80°C freezer in terms of (a) the concentration of intracellular calcium; (b) the sperm membrane lipid disorder; and (c) the sperm mitochondrial activity. For each experiment and each mouse line, three different straws containing spermatozoa from two different animals were used.

To overcome the limitation due to the subject-specific response to cryoprocedures, that could be a confusing factor in interpreting the comparison between the two methods, a pool of 500,000 spermatozoa obtained from six different males and from each mouse line were placed in a flow cytometry tube and incubated at room temperature and gently shaking with (a) Fluo 3-AM at 1 µM for 15 min to study the intracellular calcium concentration; (b) M540 at 2.7 µM for 15 min to represent the membrane lipid disorder; and (c) MitoTracker Red at 0.2 nM for 45 min to check the activation of the mitochondria. In order to distinguish between the dead and live spermatozoa, three different stainings were used, depending on the fluorescence emission spectra of the different probes: PI at 5 µM for 5 min; 10 µl 7AAD (BD Via-Probe™, Catalog Number: 555815, BD Biosciences)

for 10 min; and 1 µl LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Catalog number: L10119, Thermo Fisher Scientific) for 10 min, respectively. After the incubation time, 100.000 events/sample were acquired by flow cytometry (FACSCanto, BD Biosciences—three laser, eight color configuration). Each reagent was titrated (8-point titration) under assay conditions; dilutions were established based on achieving the highest signal (mean fluorescence intensity, MFI) for the positive population and the lowest signal for the negative population, representing the optimal signal to noise ratio, and stain indexes were calculated. Instrument performances, data reproducibility, and fluorescence calibrations were sustained and checked by the Cytometer Setup & Tracking Beads (BD Biosciences). In order to evaluate non-specific fluorescence, Fluorescence Minus One (FMO) controls were used. Compensation was assessed using CompBeads and FACSuite FC Beads (BD Biosciences) and single stained fluorescent samples. Data were analyzed first using FACSuite v 1.0.5 (BD Biosciences) software, and then, FcsWizard Software was used to convert.fcs data to.csv format in order to perform an exhaustive analysis of the fluorescence emitted from each single spermatozoon for the various fluorescence probes. To that, the columns of data "FCS," "SSC," "Fluo-3AM," "PI," "M540," "7AAD," "Mito," and "NIR" with the data from the 100,000 events acquired were selected and filtered following these criteria: forward scatter (FCS) between 30,000 and 135,000 arbitrary units (a.u); side scatter (SSC) between 20,000 and 145,000 a.u; Fluo 3-AM, M540 and MitoTracker Red >0 a.u; PI between 0 and 30,000 a.u; near infrared between 0 and 20,000 a.u; and 7 AAD between 0 and 40,000 a.u.

2.6 | Viability and acrosome integrity evaluation by fluorescence microscopy

To assess sperm viability and corroborate flow cytometry analysis, spermatozoa were thawed, stained with Hoechst 33342 (to stain all spermatozoa) and with LIVE/DEAD Sperm Viability Kit as described above and examined at fluorescence microscope.

To evaluate the status of the sperm acrosome, cells were first fixed in ethanol 70% for 1 h and then stained with Hoechst 33342 and PSA for 10 min.

For both experiments, a suspension of spermatozoa (40 µl) of each experimental group was analyzed. After staining, 10 µl of the suspension was spotted onto thoroughly cleaned and pre-warmed slides (R. Langenbrinck, Germany). One hundred spermatozoa were randomly observed and counted. The experiments were independently repeated three times (total number of examined spermatozoa: 300 spermatozoa/group).

2.7 | In vitro fertilization

As previously reported,[12] IVF was performed using the protocol described by Nakagata et al.[13] and modified by Li et al.[14] Before thawing, the straws stored in liquid nitrogen (control) or a −80°C

freezer were transferred into a LN_2 Dewar flask for at least 10 min. That was performed to prove that spermatozoa can be transferred back to LN_2 for permanent storage.

The straws were thawed in a water bath at 37°C for 8 min. To allow sperm capacitation, 30 µl of thawed spermatozoa was added into 90 µl of capacitation medium (consisting of TYH[15] with 0.75 mM MBCD) and incubated for 30 min in a 5% CO_2 incubator at 37°C. The oocytes were obtained from four-week-old B6N, B6J, or CD1 superovulated by an intraperitoneal injection of 5IU PMSG followed by 5 IU hCG 48 h later. At 12–14 h post-hCG injection, the cumulus oocyte complex (COCs) were released into a fertilization drop (consisting of 250 µl HTF with 1 mM GSH) and incubated for 20 minutes. To reduce the female to female variability, the COCs from each female were divided between the two experimental groups.

After spermatozoa of capacitation, 20 µl of spermatozoa was collected from the peripheral part of each capacitation drop and transferred to inseminate the COCs (final sperm concentration 2 to 6×10^5 spermatozoa/ml). After 4 h, the oocytes inseminated were washed three times in 200 µL of HTF medium and cultured overnight. 24 hours after insemination, the IVF rate, expressed as the percentage of 2-cell embryos from the number of inseminated oocytes, was determined. For each strain, in vitro fertilization was repeated three times.

2.8 | In vitro embryo quality control

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[AA16] or 72 h until blastocyst stage, annotating the development rate achieved. For each group, 25 embryos were cultured and the experiment was repeated three times. Results are expressed as percentage of blastocysts per 2-cell embryos cultured.

2.9 | Embryo transfer

The quality of the 2-cell embryos was also assessed by birth rates following embryo transfer. 15 embryos per experimental group were surgically transferred into the oviducts of 4 Day 0.5 pseudopregnant Crl:CD1(ICR) female mice. Recipients were anaesthetized intraperitoneally with medetomidine (Domitor, Orion Pharma, Finland) at a dose of 0.7 mg/kg body weight and then with 2.5% inhalant isoflurane solution (Isothesia@ IsoFlo Abbott Laboratories Ltd). After surgery, recipients were allowed to recover on a warming plate at 37°C and monitored until they woke up and regained their locomotor abilities. The birth rates are expressed as the percentage of pups born in relation to the number of embryos transferred. Embryo transfer was repeated four times. 60 embryos were transferred for each group and strain, reaching a total of 360 embryos used.

2.10 | Statistical analysis

Data were analyzed using Statistical Analysis System software 9.4 (SAS Institute Inc: SAS/STAT User's Guide, Cary NC: SAS Institute Inc, 2014). Data are presented as means ± standard error mean (SEM). All data obtained were analyzed using one-way ANOVA. Differences were considered to be significant when their probability of occurring by chance was less than 5% ($p \leq 0.05$).

Flow cytometry data were analyzed with GraphPad Prism 6 Software (La Jolla, CA, USA). The data referred to the fluorescence emission were recorded for each spermatozoon, and a population study was carried out by dividing each sperm samples in a discrete number of subpopulations as follows:

$$N_{pop} \sim \sqrt{N_{sptz}}$$

N_{pop} = number of subpopulations.
N_{sptz} = number of spermatozoa analyzed in each sample.

Then, the data were assessed for normalcy with D'Agostino-Pearson normality test and further analysis were carried out with parametrical or non-parametrical tests, following the needs.

2.11 | Ethical review procedure

The study—within a 10 years research project—data collection and analysis were approved by the Institutional Animal Care and Welfare Body of the CNR-IBBC/EMMA/Infrafrontier. Laboratory experiments were performed in accordance with general guidelines regarding animal breeding, zoothecnics, and biotechnology, in compliance with the Legislative Decree 26/2014 and 116/1992. Frozen germplasm stocked for this long-term research plan is used to reconstitute animal colonies.
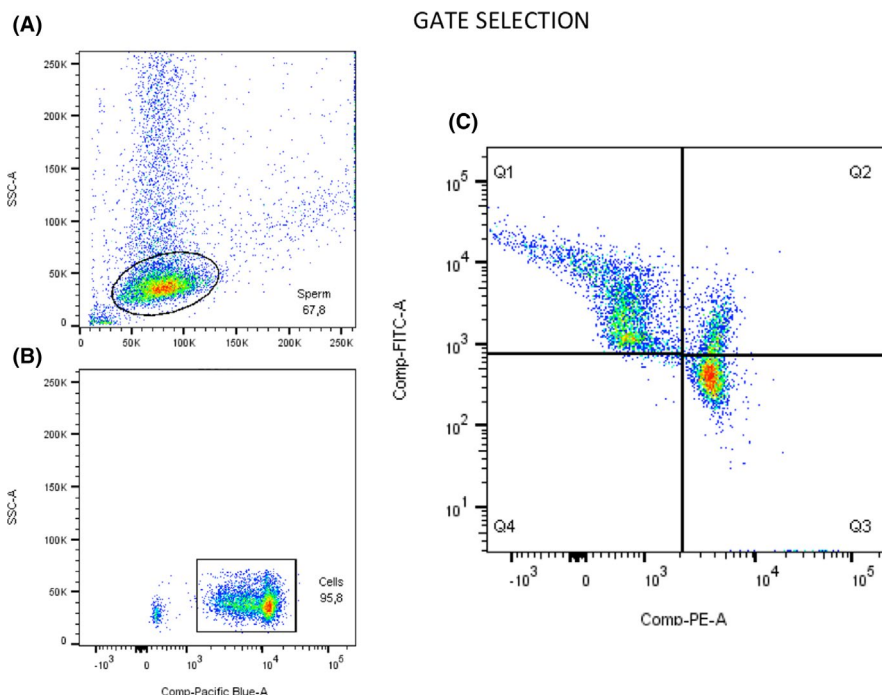
3 | RESULTS

3.1 | Storage at −80°C slightly modified the percentage of live cells in B6J and B6N, but not in CD1 mice

After exclusion of the H342 negative cells, the sperm population (Figure 1A and B) was analyzed by creating two-dimensional dot plot of PI versus SYBR-14 (Figure 1C). Only spermatozoa SYBR 14 positive and PI negative (population Q1) are considered to be alive.[17]

B6J samples kept at −80°C for 5 years displayed a light decrease of live, PI negative, spermatozoa compared to control (27% vs. 41%, $p < 0.05$). Also, in the case of B6N mice, we observed a similar reduction in the percentage of living cells in samples stored at −80°C compared to the liquid nitrogen group (25% vs. 42%, respectively, $p < 0.05$). No differences were reported for the live populations in CD1 mice (see Figure 2A and B). Moreover, no significant differences were found regarding the dead population of B6J, B6N, and CD1 spermatozoa when comparing the LN_2 and −80°C groups.

To validate the results obtained by flow cytometric analysis, mouse spermatozoa stained with Hoechst33342/PI/SYBR14 were observed using a fluorescence microscope. The results obtained showed that this triple staining gives an extremely clean and clear signal and the count of spermatozoa confirmed the data obtained by flow cytometry (Figure 3 and Table 1).

FIGURE 1 Flow cytometry analysis to assess sperm membrane integrity. (A) Selection of sperm population. Population P1 represents mouse sperm cells. (B) Dot plot of Hoechst33342 vs. SSCA. H342-negative events represent debris and are eliminated from data acquisition. (C) Cytometric two-dimensional plot. There are four populations of spermatozoa: SYR14 positive spermatozoa (Q1) considered live, double positive spermatozoa (Q2), PI positive (Q3) considered dead spermatozoa and unstained spermatozoa (Q4).
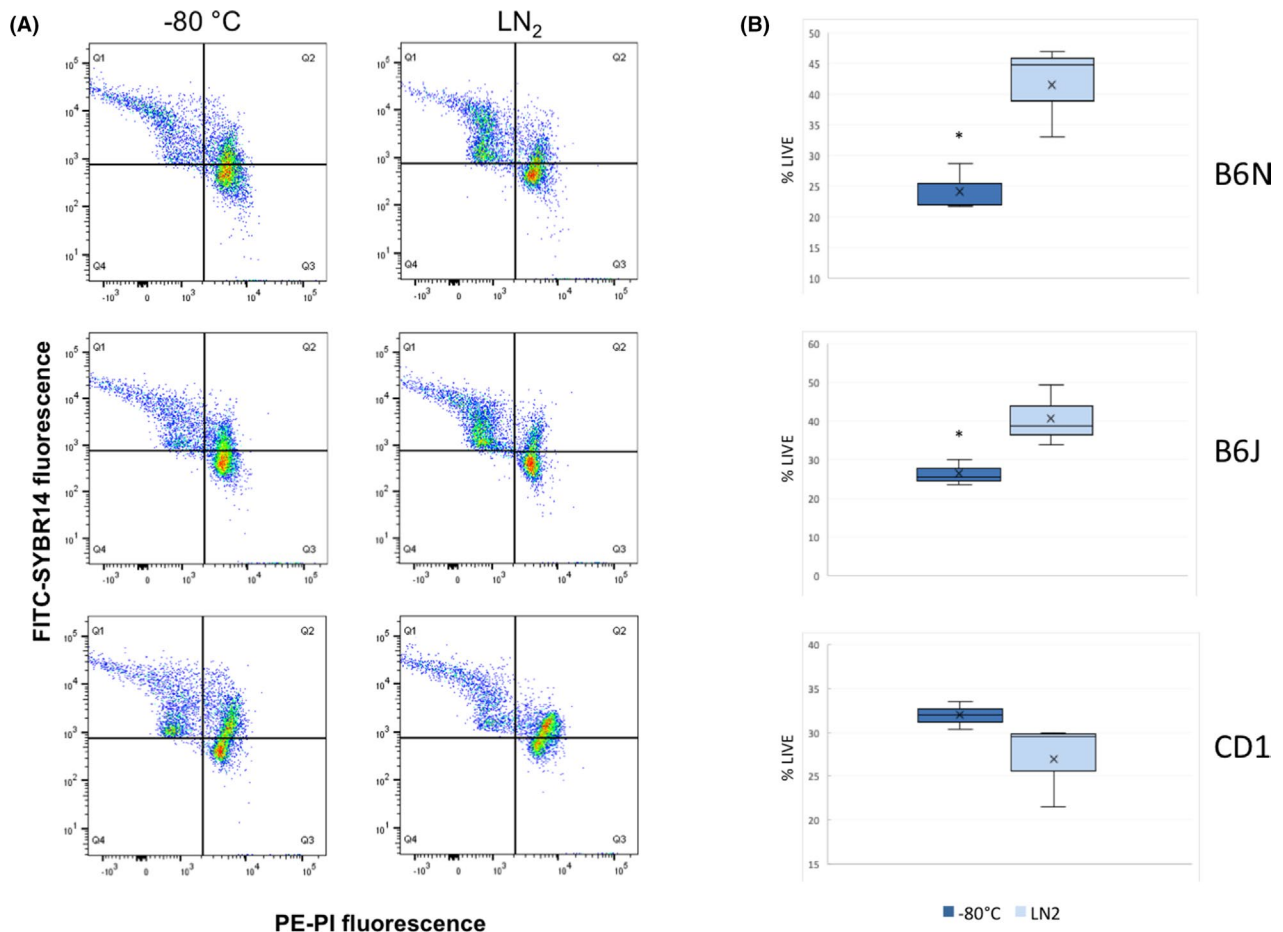
FIGURE 2 Plasma membrane integrity (A) Cytometric two-dimensional plot of B6N, B6J, and CD1 spermatozoa after 5 years of storage in LN$_2$ or in a −80°C freezer. (B) Graphical representation of live, SYBR14 positive PI negative, spermatozoa. Results are the mean of three replicates (*$p < 0.05$).

3.2 | Sperm storage at −80°C does not modify the intracellular calcium concentration, membrane lipid disorder, and mitochondrial activity

Once ensured the absence of differences in terms of acrosome damage comparing both storage methods for each strain (at least 100 cells were counted for each experimental group, $n = 3$), spermatozoa were subjected to flow cytometry analysis. After filtering and selecting the spermatozoa depending on the FCS, the SSC and the corresponding fluorescent dyes, the total number of spermatozoa collected from each group and used for the subsequent analysis and graphical representation is the following: 64081 for B6J (−80); 57411 for B6J (LN$_2$); 61771 for B6N (−80); 58045 for B6N (LN$_2$); 59087 for CD1 (−80); and 63402 for CD1 (LN$_2$).

We found no differences between spermatozoa stored at −80°C and those stored in LN$_2$ for the intracellular calcium concentration (Figure 4A) the membrane lipid disorder (Figure 4B) and the mitochondrial membrane potential (Figure 4C). The graphs represent the frequencies of the corresponding fluorescence for each strain, comparing the two cryopreservation methods.

3.3 | Sperm storage at −80°C does not alter the IVF rates

After 5 years of mouse sperm storage, the IVF rates do not show significant differences when comparing storage in LN$_2$ with the storage in a −80°C freezer for B6N, B6J, and CD1 mice (50% vs. 53%, 62% vs. 63%, and 93.3% vs. 94.9%, respectively, $p > 0.05$). The results of the IVF are shown in Table 2 and Figure 5.

3.4 | Sperm storage at −80°C does not alter the in vitro embryo quality

To assess the possible effect of the sperm storage at −80°C on the early embryo development, the quality of the embryos obtained by IVF was examined in vitro. As illustrated in Table 3, B6N, B6J, and CD1 spermatozoa stored at −80°C for 5 years are able to produce embryos that develop until the blastocyst stage the same as the controls ($p > 0.05$).

FIGURE 3 Fluorescence microscopy analysis. Spermatozoa were stained with Hoechst 33342, SYBR14, and PI. Fluorescence microscopy images of the same field showing: (A) Hoechst3342, (B) PI, (C) SYBR-14, and (D) the merged images: living (1) and dead spermatozoa (3) showed a green and red fluorescence, respectively. The spermatozoa (2), stained with both, appeared yellow (bar =10 µm).
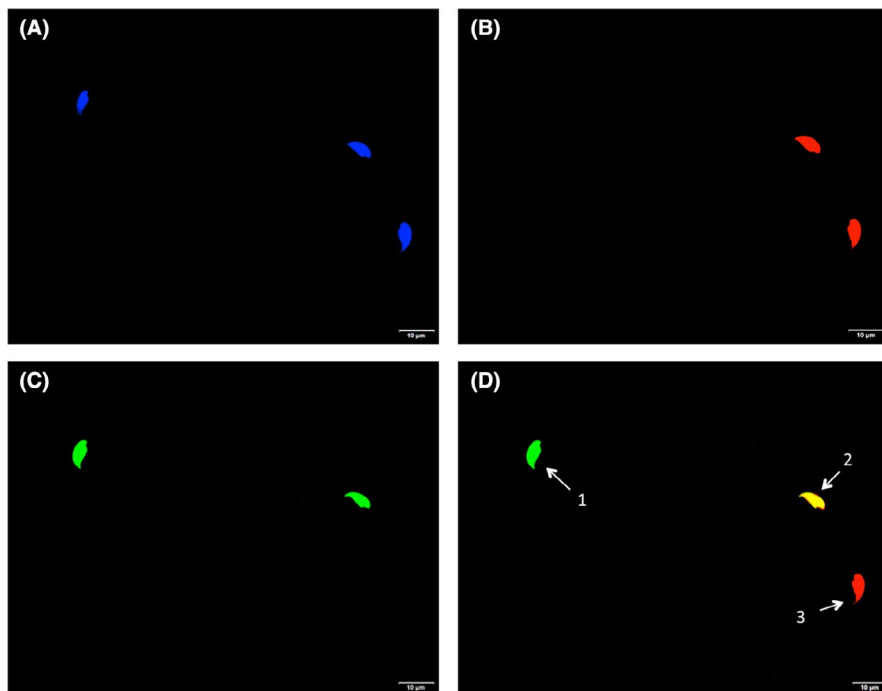
TABLE 1 Membrane integrity of mouse spermatozoa preserved for 5 years in LN$_2$ and at −80°C for the three different strains used (B6 N, B6 J, CD1) (*p < 0.05).

Strains	LN$_2$ Live (%)	−80°C Live (%)
B6N	43.3 ± 2.9	23.7 ± 2.6*
B6J	47.0 ± 5.1	27.7 ± 3.0*
CD1	31.3 ± 2.0	33.0 ± 1.5

3.5 | Spermatozoa stored at −80°C successfully developed live born pups

In vivo embryo transfer was performed to test the effect of long-term sperm storage at −80°C on embryo implantation and development. The birth rate of control and −80°C groups was compared for all strains (see Table 4). In the control groups, the birth rate ranged from 50% in B6J strain to 65% in CD1 strain, while in the −80°C groups the percentage of newborn pups obtained varied from a minimum of 51.7% for B6J strain to a maximum of 66.7% for CD1 strain. For all strains, spermatozoa at −80°C were not significantly affected after 5 years of storage and were able to produce live born pups, compared to controls (p > 0.05).

4 | DISCUSSION

Here, we investigated the reliability and safety of sperm preservation at −80°C for a medium-long-term outline (5 years). Three different widely used strains were used to this aim: C57BL/6N, C57BL/6J, and CD1.

Flow cytometry and microscopy results showed that after 5 years of storage the spermatozoa preserved under LN$_2$ maintain a percentage of living cells of about 40%, while the samples stored at −80°C display a reduced percentage of about 25% of living sperm cells for the two C57BL/6 inbred substrains. No difference was observed for the more robust CD1 outbred strain (Figure 2 and Table 4).

A sperm sample is the mix of several sperm subpopulations, each of them characterized by very different features, among which just a very small percentage of sperm cells are becoming able to fertilize being capacitated. As a consequence, only when capacitating spermatozoa are seriously damaged the whole fertilizing ability is affected. For this reason, we carried out further morphological and functional assays, such as the evaluation of acrosomes integrity, finding that the percentage of spermatozoa losing their acrosomes integrity after 5 years of storage under LN$_2$ or at −80°C did not differ significantly. This is a fundamental result, because the molecular mechanism involving the interaction between acrosome integrity and fertilizing ability has been extensively studied in the laboratory mouse and some dogmas have been *de-novo* discussed.[18] Until a few years ago, it was believed that only acrosome-intact spermatozoa could pass through the *cumulus oophorus*, and therefore, the acrosome exocytosis content could trigger by binding to the ZP. Recently, experimental evidence questioned this sequence of events.[19] Based on these considerations, it is very important to investigate the effect of −80°C storage on sperm capacitation. Unfortunately, to date it is impossible to unequivocally identify capacitated spermatozoa. The capacitation process is a complex ensemble of several molecular events developed in parallel and/or in series. Each event is necessary to acquire the fertilizing ability but not sufficient itself to identify the single sperm cell as a capacitated one. Consequently, a set of partial markers has been developed to study specific molecular mechanisms (i.e., increase in membrane fluidity, increase in intracellular calcium concentration, changes in energetic metabolism). Here, we studied the effect of −80°C freezing on some markers
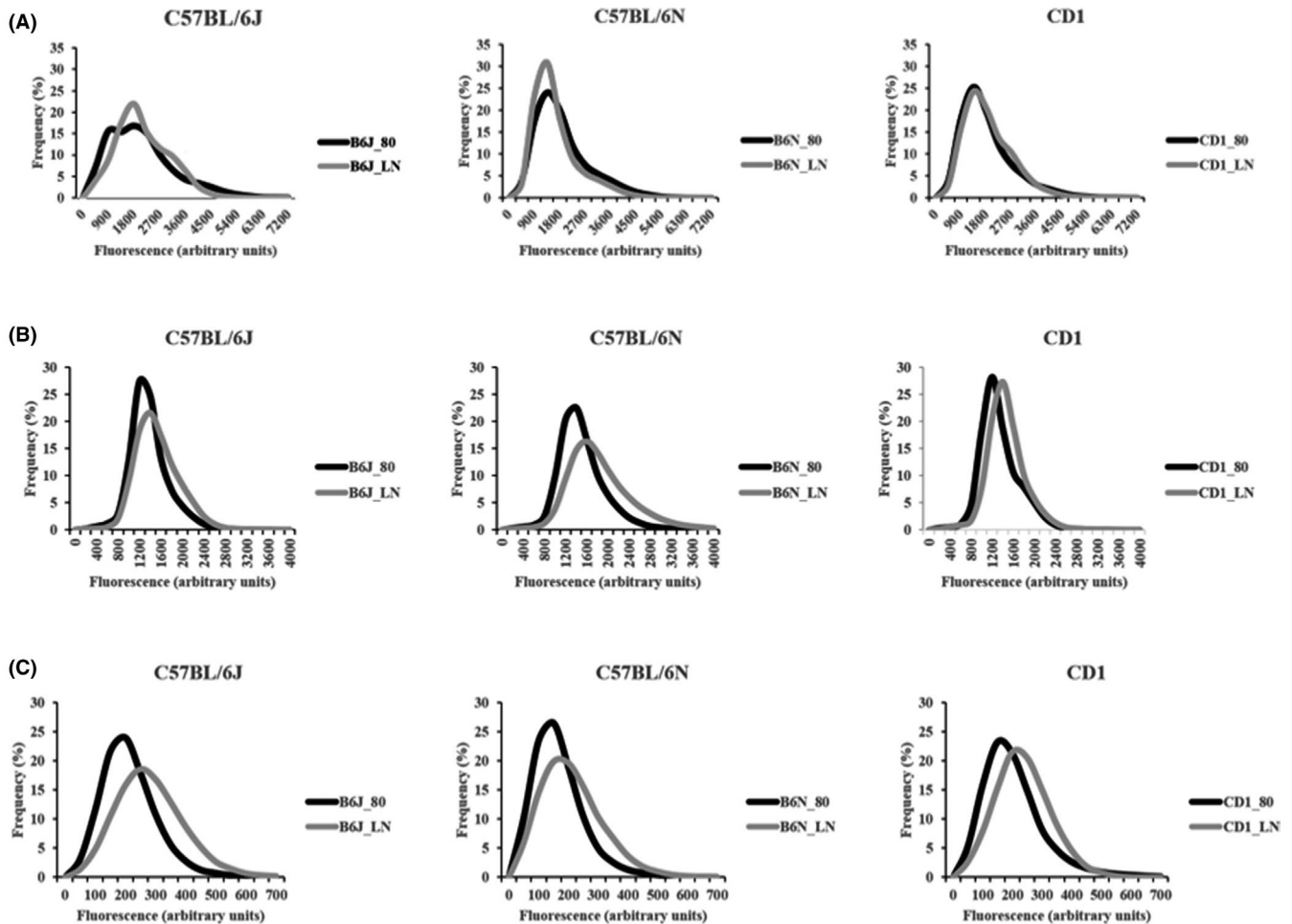
FIGURE 4 Flow cytometry analysis of intracellular calcium concentration, membrane lipid disorder and mitochondrial activity. The graphs represent the frequencies of the corresponding fluorescence of the live cells after staining with (A) Fluo 3-AM to assess intracellular calcium concentration; (B) Merocyanine-540 to assess membrane lipid disorder; (C) MitoTracker Red to evaluate the mitochondrial activity. No differences were found between spermatozoa stored at −80°C and those stored in LN_2 for the three different strains.

TABLE 2 Number of oocytes used for in vitro fertilization and number of 2-cell embryos produced after preservation in LN_2 or at −80°C for the three strains (B6N, B6J, CD1). No significant differences were observed ($p > 0.05$)

	LN_2		−80°C	
Strains	**No. of oocytes/ fertilization dish (mean ± SEM)**	**No. of 2 cells/ fertilization dish (mean ± SEM)**	**No. of oocytes/ fertilization dish (mean ± SEM)**	**No. of 2 cells/ fertilization dish (mean ± SEM)**
B6N	68.3 ± 2.9	34.3 ± 2.6	66.7 ± 3.3	35.3 ± 2.6
B6J	45.3 ± 2.9	28.7 ± 2.9	46.0 ± 4.7	28.7 ± 3.5
CD1	50.0 ± 4.6	47.7 ± 5.5	58.7 ± 2.9	54.3 ± 3.0

able to give information on key events in sperm signaling: the intracellular calcium concentration, the membrane disorder and the mitochondrial function. Interestingly, this is, to our knowledge, the first study analyzing the fluorescence of each single spermatozoon to evaluate the sperm quality and elucidate the potential differences between the two cryopreservation methods.

As a result, we found that PI negative cells (i.e., the cells with an intact membrane) immediately after thawing showed a lower fluorescence intensity of the intracellular calcium, without differences in the distribution of subpopulations when comparing LN_2 and −80°C

cryopreservation methods. These results are in accordance with the bibliography, since it is well described that spermatozoa need an external stimulus (as the follicular fluid)[20] in vivo or progesterone in vitro[21] able to produce the increase of the intracellular calcium and thus the initiation of the related processes.

This result is of great functional relevance, because the calcium fluxes are considered very important for the process of fertilization, since the ion Ca^{2+} can activate different intracellular pathways, initializing various signaling processes that lead the spermatozoa to the achievement of their fertilizing ability.[22]
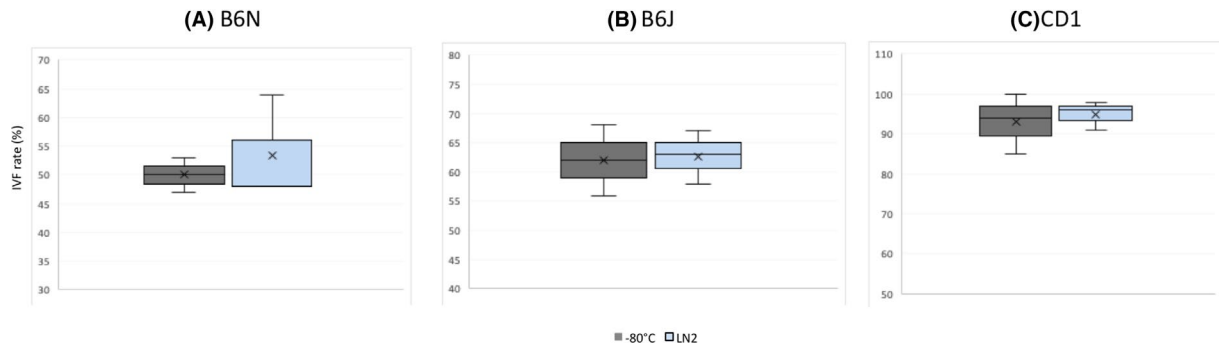
FIGURE 5 In vitro fertilization rates. The in vitro fertilization rate of (A) B6N, (B) B6J, and (C) CD1 spermatozoa stored for 5 years in LN$_2$ (control; light blue bar) or in a −80°C freezer (gray bar). Each result is the means of three IVF procedures. No differences were found between the conditions ($p > 0.05$).

TABLE 3 Blastocyst development of embryos derived from frozen spermatozoa kept in LN$_2$ or at −80°C for 5 years. No significant differences were observed ($p > 0.05$)

Strains	LN$_2$ Blastocyst development (%) (mean ± SEM)	−80°C Blastocyst development (%) (mean ± SEM)
B6N	88.0 ± 2.3	89.3 ± 1.3
B6J	80.0 ± 2.3	81.3 ± 1.3
CD1	90.7 ± 3.5	89.3 ± 2.7

TABLE 4 Birth rate of embryos derived from frozen spermatozoa kept in LN$_2$ or at −80°C for 5 years. No significant differences were observed ($p > 0.05$)

Strains	LN$_2$ Birth rate (%) (mean ± SEM)	−80°C Birth rate (%) (mean ± SEM)
B6N	56.7 ± 4.3	55.0 ± 3.2
B6J	50.0 ± 3.3	51.7 ± 3.2
CD1	65.0 ± 3.2	66.7 ± 2.7

Then, we have evaluated the membrane disorder by using a fluorescent probe, M540, combined with a live probe to discard the dead cells (Near Infrared staining, in this case). M540 is able to detect changes in the lipids reorganization and distribution within the sperm plasma membrane[23] functioning as an indicator of the membrane destabilization: The more fluid is the membrane, the more M540 intercalates into the membrane, as observed in many species.[24-29] The results obtained showed no differences for the membrane lipid disorder when comparing the two storage methods in all the three mice strains, confirming the stability of the mouse sperm membrane immediately after thawing.

The third event studied with this kind of analysis is the MMP, that is, the mitochondrial activity inside the spermatozoa. Mitochondria are known to be required for fertilization, and

defects in their ultrastructure, changes in their mtDNA, protein expression, lacking of cytochrome c, oxygen consumption, and mitochondrial respiratory efficiency, among others, have been correlated with a poor sperm motility, quality, and infertility (for further information, see the complete review from[30]). Here, the results obtained showed, also in this case, no differences between the two methods used to store the spermatozoa, standing up one more time the possibility to freeze the sperm cell at −80°C for up to 5 years preserving their quality.

Overall, these results seem to suggest that the live spermatozoa stored at −80°C did not differ significantly from those stored under LN$_2$.

Thus, we made a final experiment comparing the performance of the two treatments in term of IVF outcome, embryo quality and birth rate, finding no differences between the methods of storage. IVF results showed that, even if spermatozoa stored at −80°C have a slightly reduced number of the living cells, these are sufficient and perfectly capable of fertilizing eggs. It is not clear the function of SYBR14/ PI double positive spermatozoa but we have estimated them functionally dead as previously reported.[17,31] Embryos obtained from spermatozoa maintained at −80°C showed the same capacity to produce live and healthy animals than those produced by spermatozoa stored in liquid nitrogen.

In our opinion, this result is extremely relevant because it leads to take two important inferences:

- It is possible to successfully store mouse spermatozoa at −80°C for at least 5 years, without significant loss of their performance, in term of fertilizing ability. Possibly, this approach could be monitored further and implemented to give rise to an innovative approach on mouse genome biobanking.
- The storage at −80°C seems unable to cause health problems in new-borns. This last point is very important, because in humans there are a number of congenital disorders, termed imprinting disorders (IDs) which are caused by the disruption of imprinted genes, including Beckwith-Wiedemann syndrome (BWS), Silver-Russell syndrome (SRS), and Angelman syndrome (AS).[32] BWS and SRS appear to be associated with assisted reproduction, as well as the large offspring syndrome in bovine. Also in mice, artificial

reproductive technology (ART) may induce epigenetic instability and consequently compromising offspring health.[33]

In conclusion, storage in a −80°C ultra-freezer has several advantages compared to LN$_2$. Dedicated space/room is not required; it is cheaper than a LN$_2$ tank with constant refills, and it just needs electrical power. Furthermore, a −80°C freezer space is usually available also in small laboratories. Sperm cryopreservation at −80°C will lead to significant advantages for laboratories that, without the complex and expensive liquid nitrogen storage system, will be able to maintain a great number of mouse mutant lines without keeping the animals alive more than necessary, according to the highest welfare standards, the 3Rs principle. Future studies will help to understand possible temporal limits of this cryopreservation method. In any case, we believe that 5 years are a reasonable time to facilitate storage and exchange of frozen spermatozoa for the duration of a research project.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

MR: Conceptualization, Methodology, Investigation, Funding acquisition. SP: Conceptualization, Methodology, Investigation, Formal analysis. RP and MRS: Methodology, Investigation, Formal analysis. BB: Conceptualization. PL, MM, MD, CO, and SP: Methodology, Investigation. LV and NB: Conceptualization, Methodology, Investigation, Formal analysis, Manuscript drafting. FS: Conceptualization, Methodology, Investigation, Funding acquisition, Manuscript drafting, Supervision. All authors contributed to the manuscript and approved the submitted version.

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