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Dry ice is a reliable substrate for the distribution of frozen mouse spermatozoa: A multi-centric study

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ABSTRACT

Disseminating mouse stocks as frozen materials offers both ethical and logistical advantages over live animal shipment, minimizing the welfare issues and avoiding some of the complex custom regulations that are associated with live animal transportation. Embryo freezing in liquid nitrogen (LN_2) at −196 °C has traditionally been the method of choice for archiving mouse lines. However, spermatozoa freezing is emerging as a more convenient alternative due to the application of innovative cryopreservation and recovery protocols. In addition, frozen spermatozoa are less sensitive to post-freezing temperature fluctuations.

Here we demonstrated that spermatozoa frozen using standard laboratory protocols can be safely stored in dry ice (−79 °C) for at least seven days. The protocol we report here is robust and has been validated in a multi-centric study involving mouse spermatozoa samples exchanged between five European Mouse Mutant Archive (EMMA) nodes. Furthermore, following shipment on dry ice the spermatozoa can be returned to LN_2 for long term storage without any noticeable detrimental effect. This protocol permits frozen spermatozoa to be shared and shipped in dry ice between biorepositories, networks and scientific institutions at low cost, using common courier companies, while avoiding the complexities, risks and hazards associated with using a traditional LN_2 dry-shipper.

1. Introduction

Following the sequencing and analysis of whole human and mouse genomes (2001 and 2002) large scale mouse gene knockout programs have accelerated the production of novel genetically altered (GA) lines [1,2]. To fully exploit these emerging resources large collections of mouse models must be cryopreserved and made readily available to the international biomedical research community [3]. Moreover, the establishment of distributed and collaborative research infrastructures for systematic phenotyping, e.g. the International Mouse Phenotyping Consortium (IMPC) [4,5] is increasing the demand for these novel strains of mice while new approaches for the generation (e.g. CRISPR/Cas9) [6] are accelerating the production of GA mice. Similarly, the distribution of GA mouse lines as embryos and spermatozoa are being refined in order to reduce the dependency on the transportation of live mice. Considering the above issues and the imperative to attain the highest welfare [7] (3Rs principle) and legal standards (Directive 2010/63/EU and related national legislations), every effort should be made to disseminate mouse strains worldwide, as frozen germplasm. Since cryopreservation of the laboratory mouse was established in 1972 [8–10] cryobiologists have developed a wide range of procedures that can be exploited to archive, as well as, recover mouse stocks from frozen embryos, gametes and ovarian tissue [11–15].

At −196 °C, well below the glass transition temperature of water (−134.5 °C), all biochemical and photodynamic reactions are effectively stopped [16] and it is accepted wisdom that keeping samples at LN_2 temperature is the best way to transport and store germplasm indefinitely. The current preference for exchanging stocks as live mice is of course influenced by the traditional animal handling procedures and/or, perhaps, the recipient's inability to handle frozen materials properly. Technological advancements, procedural standardization and continuing training programs are needed to ensure that laboratory

Abbreviations: IMPC, International Mouse Phenotyping Consortium; GA, Genetically Altered; IVF, In Vitro Fertilisation; IKMC, International Knockout Mouse Consortium; EMMA, European Mouse Mutant Archive

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[1] https://www.infrafrontier.eu/infrafrontier-research-infrastructure/international-collaborations-and-projects/european-mouse.

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0093-691/

personnel can fully exploit the benefits of exchanging frozen embryos and spermatozoa.

Historically, embryo freezing has been the preferred method for archiving and transporting mouse lines as frozen stocks across Europe and worldwide. This approach has been extensively validated as reliable and applicable to most mouse strains and it has the added advantage of permitting the cryopreservation of both diploid genomes and strains carrying multiple genetic mutations. However spermatozoa freezing has recently emerged as a more economical and practical alternative for archiving mouse strains, particularly since robust methods for spermatozoa freezing and *in vitro* fertilisation (IVF) were released [17–20] A major advantage of spermatozoa freezing is that it is much easier for beginners and does not require any expensive equipment. Moreover it enables repositories to rapidly and economically archive the huge number of mutants being generated by large national and international programs. Although spermatozoa freezing is now the preferred method for archiving mutant mouse lines from international large-scale mutagenesis programs it does have the drawback of only preserving the haploid genome and should not be used for multiple mutations.

Here we show that spermatozoa from wild type and GA mouse strains on a variety of genetic backgrounds (both inbred and outbred) can be stored for at least seven days in a −80 °C freezer or on dry ice. We tested the spermatozoa using three freezing methods commonly used in mouse repositories, namely: a) the classical protocol using 18% raffinose 3% skim milk as the cryoprotectant [21], b) the classical protocol as modified by Ostermeier et al. [14,17] and c) the classical protocol as modified by Takeo & Nakagata [15,18–20].

Upon thawing from −80 °C the spermatozoa maintained their potency with no loss of fertility compared with spermatozoa stored in LN_2. Using the protocol described by Kenyon et al. [22] we extended and validated the procedure by exchanging frozen spermatozoa on dry ice among five EMMA/Infrafrontier [23–25] nodes located in five countries (CNR-Monterotondo, IT; MRC-Harwell, UK; CNB-CSIC-Madrid, ES; Helmoltz Centre-Munich, DE; and Institut Clinique de la Souris-Strasbourg, FR). We also proved, that this protocol can be applied to wild type and GA strains of different inbred and outbred genetic backgrounds, frozen with different freezing methods. In addition, spermatozoa held on dry ice can be returned to LN_2 for long term storage without noticeable detrimental effects. Our results show that shipping frozen spermatozoa in dry ice is a robust, safe and economical way to disseminate mouse strains across members of the scientific community. This simple protocol only requires a polystyrene box filled with approximately 5 Kg of dry ice to allow for a transit time of up to three days. A larger box or scheduled replenishment of dry ice at intermediate stations may be needed for longer journey times, i.e. sending samples between continents.

2. Materials and methods

2.1. Animals

All animal experiments described in this paper were approved by the animal use and welfare committees at each institute involved in this study. Male mice used for spermatozoa freezing in this study were sexually mature 3–4 months of age, while the females used as oocyte donors for the IVF were between 3 and 4 weeks of age [26]. CD1 females were used as embryo transfer recipients. All embryo transfers were performed under general anaesthesia (gaseous or injectable) accompanied by pain relief in the form of injectable analgesia.

All genetically altered and wild type mice were sourced from the institute's own animal houses and were reared in SPF facilities under standard husbandry conditions as follows; temperature of 20–22 °C, relative humidity of 40–70% with 15–20 air changes per hour and under controlled (12:12 h) light–dark cycle (7am-7pm). The mice had ad libitum access to water and a standard rodent diet.

When required the mice were culled by trained personnel using gaseous anaesthesia followed by a rising concentration of CO_2 and cervical dislocation to confirm death or cervical dislocation alone.

2.2. Experiments

2.2.1. Experiment 1: short term storage (seven days) at −80 °C of spermatozoa harvested from six wild type strains of mice

2.2.1.1. Spermatozoa cryopreservation

To test the practicality of holding spermatozoa at temperatures warmer than −196 °C, a series of preliminary studies were set up in which at −80 °C freezer was used as a surrogate for dry ice. These first experiments were carried out at CNR-Monterotondo only. In these experiments spermatozoa were harvested from mature males from five different inbred strains, i.e. C57BL/6J, C57BL/6N, FVB/NJ, BALB/c, 129/SvJ, and one outbred stock CD1 males. Spermatozoa were then cryopreserved using three different methods commonly used by mouse repositories. These methods were:

1) the CPA-MTG protocol described by Ostermeier et al. [17] (CPM);
2) the CPA-LGlu protocol described by Takeo and Nakagata [20](gCPA);
3) the classical CPA protocol described by Nakagata and reviewed by Sztein [27–29] (CPA).

After dissection the spermatozoa were pooled from two males from each strain and frozen using the three techniques being tested. Each of these protocols used LN_2 as the refrigerant. All spermatozoa straws were initially stored in LN_2.

After freezing, the straws were subject to one of three treatment groups. Group A: straws were transferred to an ultra-low temperature freezer at −80 °C for seven days; Group B: straws were transferred to an ultra-low temperature freezer at −80 °C for seven days and then returned to a LN_2 tank for at least 10 min (refrozen samples), and Group C: straws were maintained in the LN_2 tank and were used as control samples.

2.2.1.2. In vitro fertilisation (IVF)

A series of IVF recoveries were set up in order to test the ability of the spermatozoa from the three treatment groups to fertilise mouse oocytes. The protocol used for the IVF recoveries was matched to the published protocol used to freeze the spermatozoa. In brief, the spermatozoa frozen using the CPM method used Cook media in the IVF recovery [17]. The spermatozoa frozen using gCPA and CPA methods used a high calcium HTF media in the IVF recoveries as described by Takeo and Nakagata [18–20].

The IVFs were performed using oocyte donor females matched to the genetic background of the spermatozoa donors: C57BL/6J, C57BL/6N, FVB/NJ, BALB/c, 129/SvJ and CD1. For each strain and each method three straws (Group A, Group B and Group C) were compared for every IVF session. These experiments were repeated three times and for each session two IVF dishes were analysed for each experimental group. IVF results are the average of the three repetitions and are expressed as percentage of 2-cell embryos produced per oocyte used.

The quality of the embryos generated by IVF was assessed by *in vitro* embryo culture, as well as, birth rates following embryo transfer. The *in vitro* assessment was performed by culturing IVF derived

2-cell embryos in potassium simplex optimized medium (KSOM) [30] for 72 h and annotating the development rate achieved. The *in vivo* assessment was performed by surgically transferring 30 embryos per experimental group into the oviducts of two pseudopregnant CD1 females, as described by Sztein et al. [21].

Results are expressed as percentage of pups born per embryos transferred.

These quality control checks were repeated for each of the five inbred strains and the outbred stock.

2.2.2. Experiment 2: short term storage (seven days) at −80 °C of spermatozoa harvested from genetically altered mice (GA)

The studies outlined in Experiment 1 were repeated in order to confirm that holding spermatozoa harvested from GA mice at −80 °C did not compromise the ability to recover the mutant using IVF techniques since it has been reported that mutants have different fertility compared to wild type mice [31]. Spermatozoa were obtained from 3 to 4 month-old GA mice on the same six backgrounds as in Experiment 1, namely five inbred strains C57BL/6J, C57BL/6N, FVB/NJ, BALB/c, 129/SvJ and one outbred stock CD1 previously tested as wild type. Two GA strains from each genetic background were used, (Table 1), all of which were obtained from the EMMA repository.

Following the paradigm set out in Experiment 1, spermatozoa were frozen using three different methods commonly used by mouse repositories. The experiment was carried out as described in experiment 1 and repeated three times for each strain listed in Table 1. This second experiment was also carried out at CNR-Monterotondo.

2.2.3. Experiment 3: shipment simulation: comparison between spermatozoa maintained for seven days at −80 °C in ultra freezer and in dry ice (−79 °C)

Spermatozoa were obtained from 3 to 4 month-old mutant mice from C57BL/6J and C57BL/6N inbred backgrounds. For each background one GA strain was used (Table 2) and sourced from the EMMA repository. Spermatozoa of each GA line had previously been frozen using the CPM protocol described by Ostermeier et al. [17]. We chose this method because it is the one routinely used at CNR-Monterotondo.

Table 1
GA strains used for spermatozoa freezing (Experiment 2).

Background	Strain
C57BL/6J	EM00327 (B6Crl; 129P2Gja1^{tm1Kwi}/Cnrm)
	EM01967 (B6.Cg-Tg(ITGAMcre)2781Gkl/Cnrm)
C57BL/6N	EM07097 (B6N.Cg-Piwil2$^{tm2.1Doca}$/Cnrm)
	EM09116 (C57BL/6NTac-Myh4$^{tm2(EGFP/Cre/ERT2)Wtsi}$/Cnrm)
FVB/NJ	EM00407 (FVB; 129P2-Cdkn2a^{tm2Brn}/Cnrm)
	EM01183 (FVB/N-Tg(Nes-rtTA)306Rvs/Cnrm)
129/SvJ	EM00158 (B6; 129S7-Prnp^{tm1Cwe}/CweCnrm)
	EM01139 [B6; D2-Tg(Cd4-cre)1Cwi/CwiCnrm]
Balb/c	EM01746 (C.129P2-Fcgr1^{tm1Sjv} Fcgr3^{tm1Sjv}/Cnrm)
	EM01745 (C.129P2-Fcgr3^{tm1Sjv}/Cnrm)
CD1	EM02449 (CD1.129-Sp6^{tm1Ibmm}/Cnrm)
	EM02389 (CD1.129P2-Afp^{tm1Ibmm}/Cnrm)

Table 2
GA strain used for spermatozoa freezing (Experiment 3).

Background	Strain
C57BL/6J	EM02598 [B6; 129S7-Del(16T10-Hira)4Bld/Cnrm]
C57BL/6N	EM04742 [C57BL/6N Scnn1b$^{tm1a(EUCOMM)Hmgu}$/Cnrm]

Each strain was divided into five groups:

1) Control: Frozen control straw kept in LN_2;
2) -80 °C: Frozen straw transferred to an ultra-low temperature freezer at −80 °C for seven days;
3) -80 °C refrozen: Frozen straw transferred to ultra-low freezer (−80 °C) for seven days then refrozen into LN_2 for at least 10 min;
4) Dry ice: Frozen straw transferred from LN_2 into a Styrofoam box (size W: 29 × D: 29 × H: 25 cm) packed with dry ice chips (about 5 kg). This box was maintained at Room Temperature for seven days (refilling the box with dry ice when necessary);
5) Dry ice refrozen: Frozen straw transferred to dry ice into a Styrofoam box (dimensions above) for seven days (refilling with dry ice when necessary) then refrozen into LN_2 for at least 10 min.

After seven days IVF recoveries were performed using the Ostermeier method as previously described [17]. This third experiment was also carried out at CNR-Monterotondo and repeated three times. *In vivo* assessment was performed as previously described.

2.2.4. Experiment 4: multi-centric study: exchanging frozen GA spermatozoa in dry ice among EMMA partners

To confirm that spermatozoa could be transported between laboratories on dry ice under operational conditions, a sample exchange programme was set up among five EMMA/Infrafrontier nodes located in Monterotondo-Rome (Italy), Madrid (Spain), Munich (Germany), Strasbourg (France) and Harwell (UK). Spermatozoa were harvested from three GA strains on two different genetic backgrounds (C57BL/6J, C57BL/6N), see Table 3. The spermatozoa had been frozen down using both standard methods previously described by Ostermeier et al. [17] or Takeo and Nakagata [18–20]. We chose these two methods because they are the most commonly used among the EMMA/Infrafrontier nodes.

Two straws from each strain and method were sent from Monterotondo-Rome to the other participating centres in a Styrofoam box packed with ∼5 kg dry ice, using a common courier company.

Upon arrival the straws were held on dry ice until the 3rd day after shipment, at which point one straw from each GA strain was thawed and used in an IVF recovery using wild type oocyte donors of the appropriate genetic background (Group A). The remaining straw was returned to LN_2 for at least 10 min before being used for IVF (Group B). Straws kept in LN_2 were used as controls in IVF recoveries in the laboratory in Monterotondo-Rome (Group C).

Fertilisation rates were calculated as the average of the results obtained from all five participating EMMA nodes for each strain and method. For each strain thirty IVF derived 2-cell embryos per experimental group were transferred into pseudopregnant recipient females at each node. The females were allowed to give birth normally and the number of live born pups was recorded. The live born results are presented as the average of all five participating EMMA nodes.

Table 3
GA strain used for spermatozoa freezing (Experiment 4).

Background	Strain
C57BL/6J	EM02571 (B6; 129P2-Prndtm1Aag/Cnrm)
	EM00404 [B6; Cg-Tg(Cebpb-rtTA2S*S2)1Bjd/Cnrm]
	EM02462 [C57BL/6-Tg(Cr2-cre)3Cgn/Cnrm]
	EM04735 (B6NTac; B6N-A^{tm1Brd} Phyhip$^{tm1a(EUCOMM)Hmgu}$/Cnrm)
C57BL/6N	EM03992 [C57BL/6NTac-Stx8$^{tm2a(EUCOMM)Wtsi}$/Cnrm]
	EM05656 (C57BL/6N-Aldh3a1$^{tm1a(EUCOMM)Wtsi}$/Cnrm)

2.3. Statistical analysis

Each of the four experiments is internally organized as a complete factorial design, this allows for a consistent general linear model (GLM) analysis (that in the case of balanced groups is formally identical to ANOVA) taking explicitly into account the interactions between 'treatment' and other intervening factors, allowing a more thorough and accurate analysis of data. SAS v.8.2 Cary (NC), USA software was used for statistical analysis and $P < 0.05$ was considered significant. Spermatozoa fertilisation rates, and embryo transfer pup birth rates are expressed as mean (M) ± standard deviation (SD).

2.4. Ethical review procedure

All animal studies and procedures undertaken at all five EMMA/Infrafrontier nodes were reviewed by established Ethics Committees and approved by the corresponding competent authorities, in compliance with their respective national legislation on animal welfare, transposing both the 86/609/CEE and 2010/63/EU Directives on protection of animals used in research. The references for the Ethics approvals at every location are: CNR-Monterontondo (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, ref.13/2010-B), CNB-Madrid (ref. PROEX 344/15 in compliance with Spanish Royal Decree 53/2013), MRC-Harwell (ref.PEL30/4303), HMGU-Munich (ref.55.2-1-54-2532-212-14), ICS-Strasbourg (ref.APAFIS#4411-201603071800289v1).

3. Results

3.1. Experiment 1: short term storage (seven days) at −80 °C of spermatozoa harvested from six wild type strains of mice

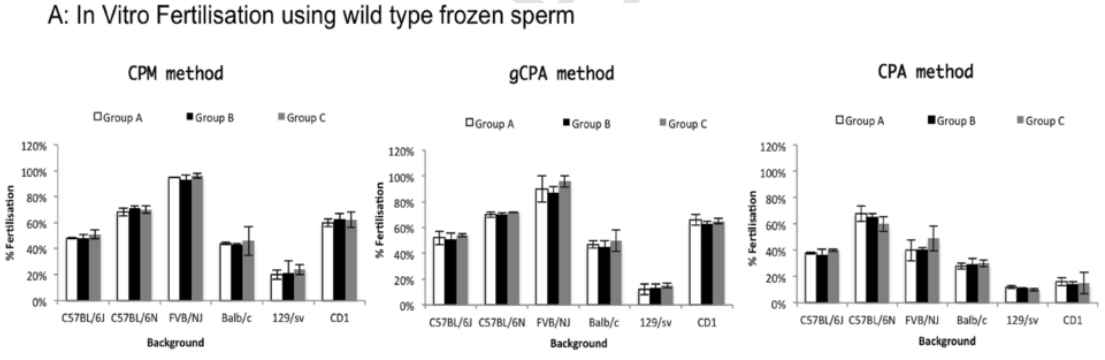
The IVF results of wild type spermatozoa derived from C57BL/6J, C57BL/6N, FVB/NJ, BALB/c, 129/SvJ and CD1 (expressed as percentage of 2 cell embryos produced/oocytes used) after seven days of storage at −80 °C are reported in Fig. 1A. The results show that regardless of the method used for freezing (CPM, gCPA or classical CPA), frozen spermatozoa can be stored at −80 °C for at least seven days (group A) without significantly lost of its fertilisation capability, as compared to samples maintained in the LN_2 tank that acted as control (group C).

In addition, results showed that spermatozoa held at −80 °C for at least seven days can be returned to LN_2 storage (group B) without a significant loss of fertilisation potential.

Statistical analysis show that only "freezing method" and "background" exerted a statistically significant effect on IVF, while treatment had no significant effect neither per se, nor with the other two modulating factors (see Supplemantary Data).

On the other side, it is already known that fertilisation rates are strongly dependent on the genetic backgrounds of the mouse strains used, as previously described by Ostermeier et al. [17]. For example, 129/SvJ spermatozoa gave the lowest fertilisation rates (10% ± 1% between group A and C) with the classical CPA method whereas C57BL/6N spermatozoa (group A, B and C) always resulted in fertilisation rates of ≥60%, regardless of the spermatozoa freezing method used. These results also confirmed that the classical CPA method, the oldest of the three methods testes in this study, is the least efficient spermatozoa freeze/recovery method of the ones tested, across all the strains, as previously reported [17–20].

Fig. 1B shows that there is no statistical difference in the number of live born pups obtained from implanted embryos fertilised with



B: Pup Birth Rate (%)

Embryos derived from CPM method

	PUPS BORN (% Mean ± SD)		
Background	Group A	Group B	Group C
C57BL/6J	58,85 ± 3,3	59,00 ± 2,9	60,60 ± 4,4
C57BL/6N	62,70 ± 0,9	60,90 ± 3,4	61,45 ± 2,6
FVB/NJ	57,70 ± 4,1	59,35 ± 3,0	59,80 ± 3,7
Balb/c	44,35 ± 3,9	46,00 ± 1,8	45,90 ± 4,2
129/sv	43,60 ± 2,8	42,85 ± 5,0	44,05 ± 3,2
CD1	61,50 ± 3,8	59,69 ± 4,8	60,25 ± 5,0

Embryos derived from gCPA method

	PUPS BORN (% Mean ± SD)		
Background	Group A	Group B	Group C
C57BL/6J	57,80 ± 3,1	59,10 ± 1,9	58,60 ± 3,4
C57BL/6N	54,35 ± 2,5	55,90 ± 1,8	55,40 ± 1,3
FVB/NJ	67,20 ± 3,3	65,85 ± 4,6	66,20 ± 2,4
Balb/c	44,25 ± 4,1	46,10 ± 3,4	45,55 ± 2,9
129/sv	33,60 ± 3,6	32,25 ± 2,7	33,30 ± 5,0
CD1	69,85 ± 5,7	70,98 ± 5,5	72,20 ± 9,1

Embryos derived from CPA method

	PUPS BORN (% Mean ± SD)		
Background	Group A	Group B	Group C
C57BL/6J	37,68 ± 3,9	35,98 ± 6,4	36,55 ± 4,7
C57BL/6N	35,99 ± 4,5	38,80 ± 5,4	39,40 ± 2,6
FVB/NJ	38,91 ± 4,6	39,15 ± 6,0	40,10 ± 4,2
Balb/c	41,33 ± 2,7	40,00 ± 4,3	40,00 ± 2,5
129/sv	26,96 ± 3,2	27,45 ± 2,8	27,70 ± 2,4
CD1	65,79 ± 3,4	66,55 ± 4,1	66,40 ± 4,7

Fig. 1. Short term storage (seven days) at −80 °C of spermatozoa of wild type strains frozen with three different methods. A. IVF rates of wild type spermatozoa, frozen with CPM, gCPA and CPA methods, after storage for seven days at −80 °C (Group A), −80 °C then LN_2 refrozen (Group B) and LN_2 (Group C). Males and females of different genetic background were used for IVF procedures. Each IVF procedure was repeated three times, illustrated as different bar-patterns in the graphic. Each bar is the average of the three IVF results (two IVF dishes for each session). There is no statistically significant difference between the three treatment groups $P > 0.05$. **B**. Pup birth rates of wild type embryos derived from IVF with spermatozoa of Group A, B and C. Results are expressed as percentage of pups born per embryos transferred. $P > 0.05$.

spermatozoa stored at −80 °C versus the control spermatozoa. The same applies to embryos obtained from spermatozoa stored at −80 °C and then refrozen in LN_2. This is true for all genetic backgrounds and for all methods used. For example the embryos obtained from C57BL/6N frozen spermatozoa in the gCPA method, resulted in similar figures, across all experimental groups: 55.40% ± 1.3% of pups for the Group C, 54.35% ± 2.5% for the group A and 55.90% ± 1.8% for the group B.

Birth rates, following embryo transfer, varied from a minimum of 26.96% for embryos on an inbred 129/SvJ genetic background produced by spermatozoa frozen using the classical CPA method to a maximum of 72.20% for embryos on and outbred CD1 background resulting from spermatozoa frozen using the gCPA method. These results are in accordance with the routine experience in the field.

3.2. Experiment 2: short term storage (seven days) at −80 °C of spermatozoa harvested from genetically altered mice (GA)

Experiment 2 was carried out as previously described for experiment 1 with the exception that the spermatozoa were harvested from two GA strains of the same genetic background as the wild type in experiment 1. The IVF results (expressed as percentage of 2-cell embryos produced/oocytes used) after seven days of storage at −80 °C are reported in Fig. 2A. The results show that regardless of the method used for freezing (CPM, gCPA or classical CPA) frozen spermatozoa harvested from GA males can be stored at −80 °C for at least seven days (group A) and refrozen (group B) without, significantly, losing its fertilisation potential compared to the group C control. In this experiment we confirmed that sample storage did not exert a treatment effect. Neither did we observe any specific interaction between of sample storage and the other factors as, method, strain and background. (see Supplemantary Data).

Spermatozoa storage at −80 °C did not affect the viability of embryos produced and their capacity to produce newborn pups. Results were background, strain and freezing method dependent, however for each embryo transfer session, there were no statistical significant differences in birth rates between the three treatment groups (Fig. 2B).

3.3. Experiment 3: shipment simulation: comparison between spermatozoa maintained for seven days at −80 °C in ultra freezer and in dry ice (−79 °C)

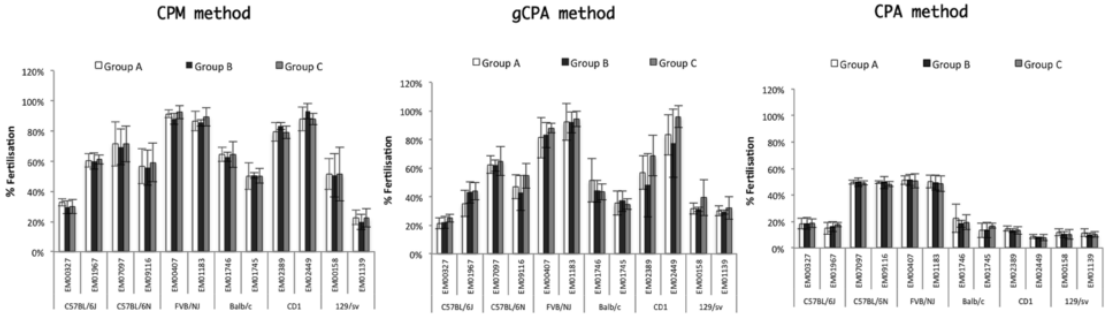
Spermatozoa from GA animals frozen with the CPM method, on either a C57BL/6N or a C57BL/6J background, were compared after seven days at −80 °C in ultra-low freezer and in dry ice (−79 °C). Control straws were maintained in LN_2 only. Results are shown in Fig. 3A.

For each genetic background, no statistical significant differences were seen between the different groups, whether the spermatozoa had been returned to LN_2 before the IVF recovery was set up, or not.

These results indicate that frozen spermatozoa can be maintained for up to seven days at −80 °C in an ultra-low freezer or in dry ice at −79 °C without significantly changing the capacity to fertilise eggs.

A 50% or greater live birth rate was obtained from all experimental groups for both genetic background (Fig. 3B). The factors taken into consideration for the analysis were treatment and background, again we confirmed the lack of any significant effect on both IVF and birth rate of Treatment both per se and as for its interaction with background (see Supplemantary Data).

A: In Vitro Fertilisation using GA frozen sperm



B: Pup Birth Rate (%)

Embryos derived from CPM method

Background	Strain	PUPS BORN (% Mean ± SD) Group A	Group B	Group C
C57BL/6J	EM00327	39,33 ± 8,6	43,67 ± 4,2	44,20 ± 2,5
	EM01967	42,33 ± 11,1	47,67 ± 1,1	48,23 ± 1,1
C57BL/6N	EM07097	50,70 ± 4,6	51,13 ± 5,5	50,80 ± 3,7
	EM09116	49,67 ± 6,0	48,97 ± 5,1	47,97 ± 3,6
FVB/NJ	EM00407	29,57 ± 4,2	32,20 ± 1,0	32,03 ± 1,6
	EM01183	30,33 ± 3,1	30,23 ± 2,7	32,40 ± 2,9
Balb/c	EM01746	36,00 ± 1,0	38,00 ± 1,0	36,23 ± 1,1
	EM01745	39,60 ± 0,5	41,93 ± 3,1	40,00 ± 1,0
CD1	EM02449	54,80 ± 2,3	53,33 ± 2,3	54,13 ± 1,2
	EM02389	49,93 ± 2,0	50,33 ± 2,0	50,73 ± 1,6
129/sv	EM00158	40,00 ± 2,0	38,50 ± 4,8	39,73 ± 6,0
	EM01139	32,13 ± 1,2	31,67 ± 2,1	32,03 ± 5,3

Embryos derived from gCPA method

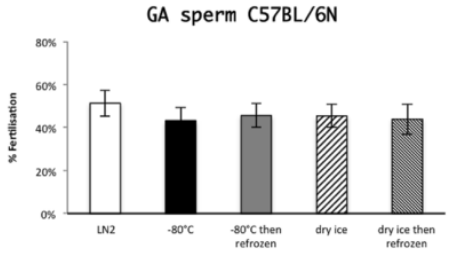
Background	Strain	PUPS BORN (% Mean ± SD) Group A	Group B	Group C
C57BL/6J	EM00327	49,40 ± 4,7	48,20 ± 2,3	50,00 ± 1,0
	EM01967	48,63 ± 0,7	48,33 ± 4,7	47,00 ± 1,0
C57BL/6N	EM07097	46,67 ± 2,3	48,83 ± 2,4	48,87 ± 4,3
	EM09116	47,90 ± 5,5	49,33 ± 4,0	49,07 ± 5,5
FVB/NJ	EM00407	35,50 ± 2,3	35,83 ± 2,7	36,27 ± 0,6
	EM01183	30,33 ± 2,5	30,67 ± 1,5	30,00 ± 0,9
Balb/c	EM01746	34,67 ± 2,5	32,13 ± 2,1	33,33 ± 3,8
	EM01745	37,33 ± 4,5	36,27 ± 3,0	35,13 ± 5,0
CD1	EM02449	55,13 ± 3,7	53,93 ± 8,0	54,00 ± 5,6
	EM02389	48,80 ± 3,5	49,13 ± 3,0	47,67 ± 3,2
129/sv	EM00158	34,00 ± 1,0	31,67 ± 3,5	34,17 ± 2,7
	EM01139	28,43 ± 1,8	29,40 ± 2,4	28,30 ± 1,1

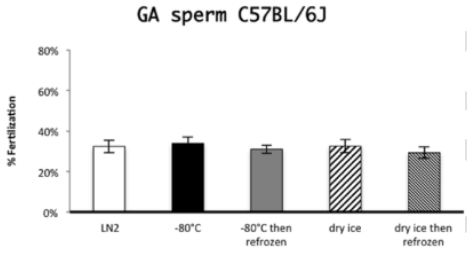
Embryos derived from CPA method

Background	Strain	PUPS BORN (% Mean ± SD) Group A	Group B	Group C
C57BL/6J	EM00327	33,00 ± 3,0	32,67 ± 4,1	33,20 ± 4,2
	EM01967	31,33 ± 4,8	31,20 ± 2,3	31,67 ± 4,7
C57BL/6N	EM07097	37,67 ± 2,5	37,20 ± 2,2	38,67 ± 2,3
	EM09116	36,33 ± 3,5	36,00 ± 3,5	35,03 ± 4,0
FVB/NJ	EM00407	29,00 ± 4,4	30,00 ± 1,0	28,67 ± 5,1
	EM01183	31,00 ± 4,4	30,33 ± 7,1	29,70 ± 6,4
Balb/c	EM01746	28,23 ± 2,4	30,33 ± 0,6	30,17 ± 3,8
	EM01745	35,17 ± 1,8	32,83 ± 2,6	34,47 ± 5,6
CD1	EM02449	29,17 ± 2,0	28,87 ± 1,8	30,67 ± 1,2
	EM02389	38,70 ± 2,0	34,97 ± 4,6	37,33 ± 3,8
129/sv	EM00158	31,00 ± 6,6	31,67 ± 5,3	30,67 ± 9,1
	EM01139	27,00 ± 5,3	28,67 ± 3,2	27,27 ± 2,0

Fig. 2. Short term storage (seven days) at - 80 °C of GA spermatozoa frozen with three different methods. A. IVF rates of GA spermatozoa, frozen with CPM, gCPA and CPA methods, after storage for seven days at −80 °C (Group A), −80 °C then LN_2 refrozen (Group B) and LN_2 (Group C). Two GA strains were used for each genetic background. Each IVF procedure was repeated three times, illustrated as different bar-patterns in the graphic. Each bar is the average of the three IVF results (two IVF dishes for each session). $P > 0.05$. **B.** Pup birth rates of GA embryos derived from IVF with spermatozoa of Group A, B and C. For each group 30 embryos were reimplanted into 2 CD1 pseudopregnant females. $P > 0.05$.

A. In Vitro Fertilisation





B. Pup Birth Rate (%)

	PUPS BORN (% Mean ± SD)	
Treatment for 7 days	GA C57BL/6N	GA C57BL/6J
CTRL LN_2	58,50 ± 3,5	54,35 ± 6,1
-80°C	54,25 ± 5,3	50,90 ± 2,0
-80°C then refrozen	55,20 ± 4,5	52,65 ± 4,3
Dry ice	49,95 ± 5,6	55,00 ± 2,0
Dry ice then refrozen	52,36 ± 6,1	49,96 ± 4,3

Fig. 3. Spermatozoa storage for seven days: comparison between an ultra-low freezer (at −80 °C) and dry ice (at −79 °C). **A**. IVF rates of GA frozen spermatozoa, from C57BL/6N and C57BL/6J background, after seven days storage. Control spermatozoa were maintained in LN_2, -80 °C and −80 °C then refrozen were maintained in an ultra-low freezer, dry ice and dry ice then refrozen were maintained into a Styrofoam box. One GA strain was used for each genetic background. Each IVF procedure was repeated three times. Each bar is the average of the three IVF results (two IVF dishes for each session). $P > 0.05$. **B.** Pup birth rates of GA embryos derived by IVF with spermatozoa stored for seven days as previously described. For each group 30 embryos were reimplanted into 2 pseudopregnant females. $P > 0.05$.

3.4. Experiment 4: multi-centric study: exchanging frozen GA spermatozoa in dry ice among EMMA partners

Spermatozoa samples from three GA strains on C57BL/6N background and from three GA strains on C57BL/6J background were frozen with both the CPM and the gCPA methods. Two straws for each strain/method were shipped in dry ice from Monterotondo-Rome to other four EMMA nodes. Results from this multicentric exchange study are shown in Fig. 4A. Fertilisation rates vary between the GA strains from 37,2% (for the strain EM03992 CPM) to 79.4% (for the strain EM02571 CPM), but in all cases exposure of the spermatozoa to LN_2 only (CTRL), dry ice only or dry ice then refrozen to LN_2 did not significantly affect fertilisation rates. Each EMMA partner succeeded in generating 2-cell embryos from the IVF recoveries they set up. These embryos were successfully implanted into recipient females which gave birth to live born pups with a high efficiency (from 32,4% ± 2.1%–74.80% ± 7.3%), as shown in Fig. 4B. The lack of any statistically significant difference across centres was actually demonstrated (see Supplemantary Data). The spermatozoa can be transported in dry ice for at least three days without losing fertilisation ability, regardless the freezing method used and genetic background.

4. Discussion

Facilitating the cryopreservation and distribution of important models to the research community is one of the principle functions of mouse repositories. However, shipping live animals raises welfare and ethical issues and is expensive. Consequently, shipment of frozen materials is promoted whenever possible but this tends to rely on LN_2 dry-shippers which have their own limitations, risks and hazards. Finding a cheaper alternative to the use of LN_2 dry-shippers would help reduce the reliance on the exchange of live animals and improve animal welfare.

Dry ice (−79 °C) has several advantages compared to LN_2 for shipping, it is not considered dangerous or hazardous by couriers, it doesn't need special dry-shippers and can be used in conjunction with a simple styrofoam box which does not need to be returned to the vendor/sender. Dry ice is also extremely cheap and most couriers are prepared to transport dry ice boxes whereas only a few transport LN_2 dry shippers reliably. Given all these advantages we explored the possibility of maintaining frozen spermatozoa in dry ice for sufficient time to permit worldwide transportation, without losing fertilisation capacity.

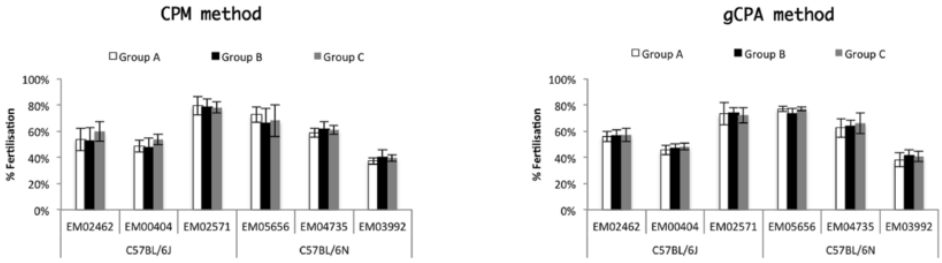
It has been known since the 1950s [32] that bovine semen could be stored on dry ice for several months could remain fertile even though some loss of viable spermatozoa was detected probably due to temperature fluctuations. After 1960, liquid nitrogen replaced dry ice as the medium of choice for long term storage [33]. More recent papers showed that bovine semen could be maintained for short time on dry ice [34] or at −80 °C [35] but not at −30 °C, without losing its ability to fertilise eggs. In contrast, human spermatozoa stored and transported in dry ice quickly loose their motility and vitality [36]. Short term exposure (more than 24 h) to dry ice compromised spermatozoa motility regardless of type of cryoprotectant used [37].

With regard to mouse spermatozoa, Okamoto et al. reported [38] that outbred or hybrid stocks' spermatozoa could be stored at −79 °C up to 4 months before losing fertility or could be successfully transported nationally in dry ice.

In order to demonstrate the feasibility of holding spermatozoa at dry ice temperatures, the fertilisation potential of wild type and GA spermatozoa maintained for up to seven days in an ultra-low freezer at −80 °C was evaluated. This evaluation included spermatozoa that had been frozen using three different spermatozoa freezing methods commonly used by the scientific community [17–20].

Our results show that short term exposure to −80 °C does not affect the potency of frozen spermatozoa, regardless of the spermatozoa freezing method used. As an extension to this observation we show that spermatozoa held at −80 °C can be returned to LN_2 for long term

A. In Vitro Fertilisation



B. Pup Birth Rate (%)

CPM method

	PUPS BORN (%Mean ± SD)		
GA strain	Group A	Group B	Group C
EM02462	47,16 ± 2,3	55,60 ± 12,0	49,38 ± 4,0
EM00404	34,80 ± 3,3	35,20 ± 3,3	32,80 ± 3,1
EM02571	68,56 ± 3,6	74,12 ± 6,6	70,78 ± 4,1
EM05656	47,80 ± 5,4	49,22 ± 4,7	56,56 ± 7,9
EM04735	64,40 ± 3,4	69,60 ± 7,3	65,96 ± 5,6
EM03992	34,44 ± 2,5	36,20 ± 4,5	32,40 ± 2,1

gCPA method

	PUPS BORN (%Mean ± SD)		
GA strain	Group A	Group B	Group C
EM02462	53,20 ± 4,1	63,00 ± 10,3	55,60 ± 6,9
EM00404	49,60 ± 2,1	49,60 ± 2,9	47,20 ± 3,0
EM02571	63,80 ± 3,5	70,38 ± 8,1	65,56 ± 5,4
EM05656	52,64 ± 7,2	55,10 ± 6,0	60,28 ± 8,0
EM04735	67,96 ± 3,7	74,80 ± 7,3	70,60 ± 5,1
EM03992	44,98 ± 4,9	48,30 ± 1,9	48,58 ± 1,7

Fig. 4. GA spermatozoa exchange among the five participating EMMA nodes (HMGU, MRC, CNB, ICS and CNR) in dry ice. A. IVF rates of GA frozen spermatozoa, from C57BL/6N and C57BL/6J background, after shipment in dry ice. Both freezing methods (CPM and gCPA) were used for each GA strain. The IVF was performed the 3rd day using: control straws maintained in LN_2 (group C), straws shipped in dry ice (group A) and straws shipped in dry ice then LN_2 refrozen (group B). Three GA strains were used for each genetic background. Each IVF procedure was repeated in all centres. For each method the graphic shows the average of results obtained from the five nodes. There is no significance between experimental groups $P > 0.05$. **B.** Pup birth rates of GA embryos derived from IVF using shipped spermatozoa, as previously described. For each group 30 embryos were reimplanted into 2 pseudopregnant females. Results are the average of the five centers. $P > 0.05$.

storage without detrimental effects. This offers the convenience that IVF recoveries do not need to be set up immediately upon arrival of the spermatozoa shipment. Additionally, this minimises the degree of coordination between provider and recipient and makes the technique attractive to both high throughput operations and smaller less well equipped facilities.

Next, we explored whether dry ice rather than a −80 °C freezer would be applicable for dry ice shipments between laboratories. To confirm that this was the case frozen spermatozoa were exchanged on dry ice between EMMA/Infrafrontier nodes across Europe. To standardise the value of this study each partner extended the holding period in dry ice to a total of three days from the dispatch date, when the actual transit time was shorter than this. Each partner was able to recover live born pups following IVF recoveries set up after three days of exposure to dry ice with or without subsequent temporary storage in LN_2. No adjustments to the in-house IVF recovery protocols existing in Monterotondo-Rome were made, hence demonstrating that the technique was both robust and transferable to multiple laboratory environments. From an operational perspective, and according to the deliverables agreed for the Infrafrontier-I3 project, the EMMA/Infrafrontier repository is expected to promote alternatives to live animal transportation and disseminate detailed protocols on its website that describe the shipment of spermatozoa on dry ice (https://www.infrafrontier.eu/knowledgebase/protocols/cryopreservation-protocols).

The ethical and economical advantages of this protocol persuaded the EMMA/Infrafrontier repository to immediately implement the procedure before publishing it. This approach also complies with the openness and transparency commitments in science associated with this consortium which are designed to promote advances in technologies among all potential EMMA/Infrafrontier customers at the earliest time possible, without delays, for their benefit.

In this study we have illustrated the efficacy of this protocol by exchanging GA spermatozoa from males on different genetic backgrounds which were frozen using the most common freezing methods. More over, we also show that is possible to return spermatozoa samples to liquid nitrogen for long term storage without loss of fertilisation capacity. The EMMA/Infrafrontier repository has already used the protocol described in this manuscript to send out 135 shipments of frozen spermatozoa on dry ice to clients in Europe (n = 88), Asia (n = 3) and North America (USA and Canada) (n = 55). The preferred transport vessel is a polystyrene box (Air Sea Containers: Thermal Control Unit) but any box suitable for transporting dry ice would be sufficient. However, it is important to enclose the straw in a pre-chilled sleeve so that it is easy to locate and does not get damaged during transit. In our experience 5 kg of dry ice will be sufficient to maintain the samples in a frozen state for three days (trans-European shipments). We used 8 Kg of dry ice for international (Asia and North America) journeys. So far we have not experienced any specific problems when we shipped abroad. A simple declaration stating the shipment is not dangerous shipment and does not contain biologically hazardous material is sufficient for European Union countries (Great Britain requires also a supplementary health certification and a license for Importation). Non-EU countries may have different requirements. There are several advantages of using dry ice boxes over conventional dry shippers: a) the polystyrene boxes are cheap to buy and are disposable b) the boxes do not need to be returned to the vendor/sender which reduces shipping costs and c) dry ice parcels can be handled by a wider range of couriers than is the case for dry shippers.

In conclusion, this study has demonstrated that holding spermatozoa at −79 °C (dry ice) or −80 °C (ultra-low freezer) for up to seven days does not compromise fertility or the developmental potential of IVF derived progeny. It is worth noting that spermatozoa can be transferred to a LN_2 freezer for long term storage after temporary

storage in dry ice without noticeable loss of fertilisation capacity. Finally, this procedure is also suitable for both wild type and GA strains from a range of genetic backgrounds and offers a simple and inexpensive way to exchange frozen mouse spermatozoa.

We believe that this procedure offers an inexpensive, simple and robust alternative to the use of LN_2 dry-shippers for exchanging mouse frozen spermatozoa between biorepositories, networks and scientific institutions. Success with this technique is independent of the IVF recovery method used or the courier employed to handle the dry ice package which makes the protocol accessible to a wide cross section of users. The ability to transport frozen mouse spermatozoa on dry ice, along with other more traditional methods of shipping embryos and germplasm, will help in minimizing the use of live animal shipments when exchanging mouse stocks, therefore leading to a significant improvement in animal welfare standards.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.04.003.

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