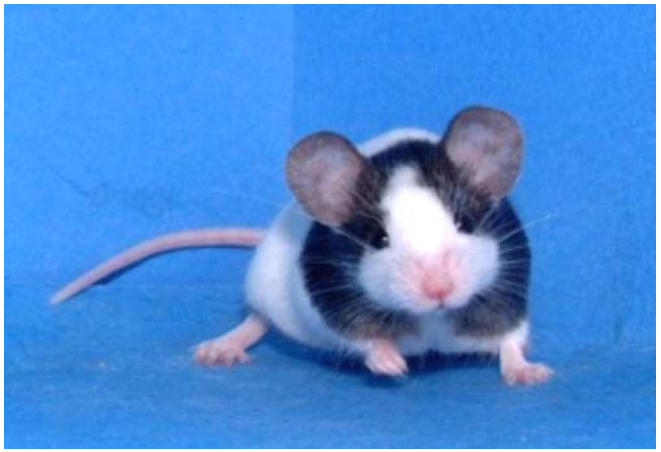
’2012 EMMA cryopreservation workshop

Transportation of frozen and unfrozen materials



RIKEN BioResource Center

Bioresource Engineering Division

Keiji Mochida

RIKEN Institutes

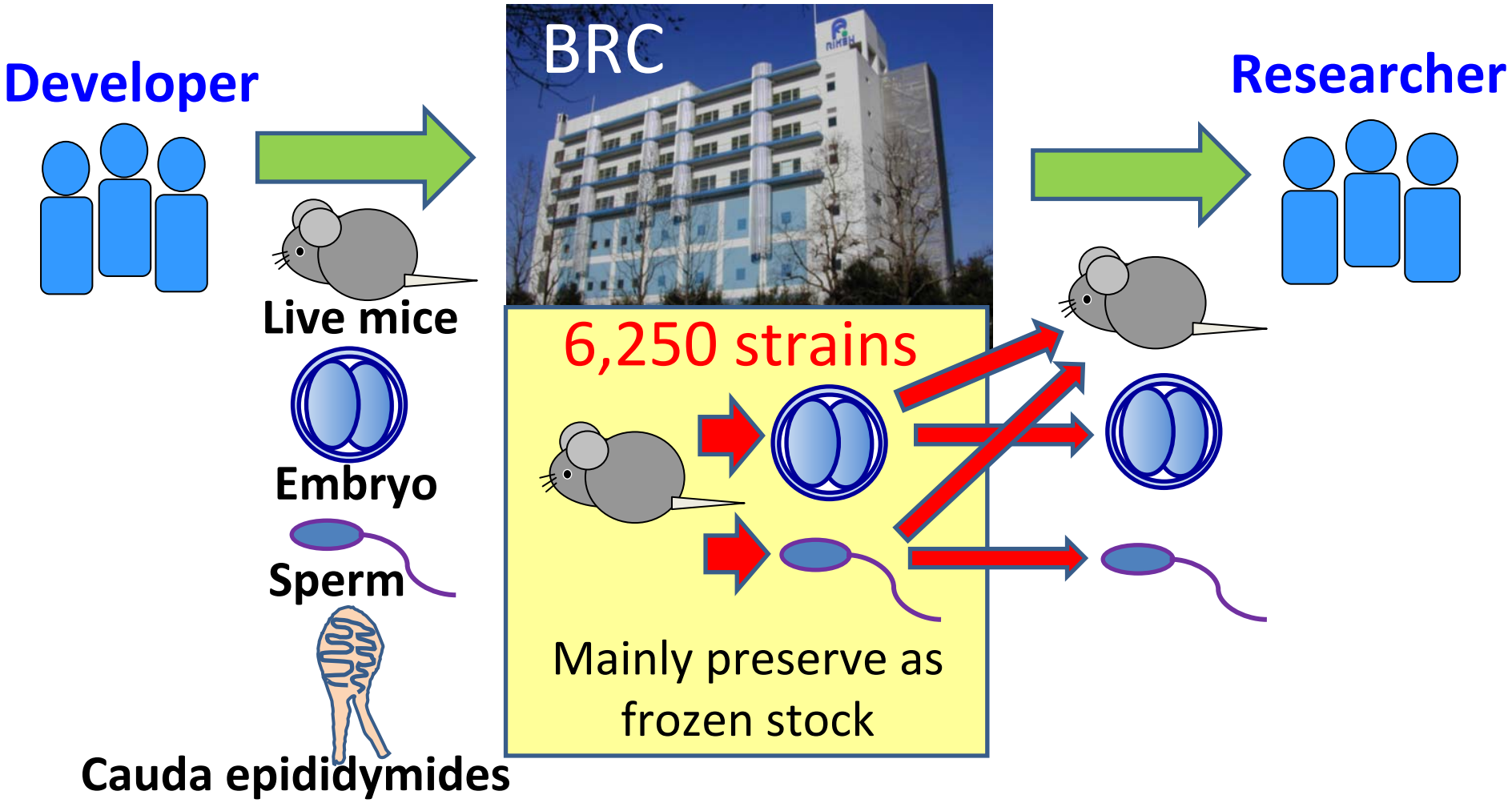
RIKEN
BRC

60km far
from Tokyo

RIKEN BRC (BioResource Center) as a mouse bank

Duties

Collection → Cryopreservation → Distribution



Archiving Mouse Resources at BRC

- 86% of strains have some genetically artificial modifications.
- 900 inbred strains containing 60 wild-derived strains

NBRP

IGTC

Cumulative no. since FY2001

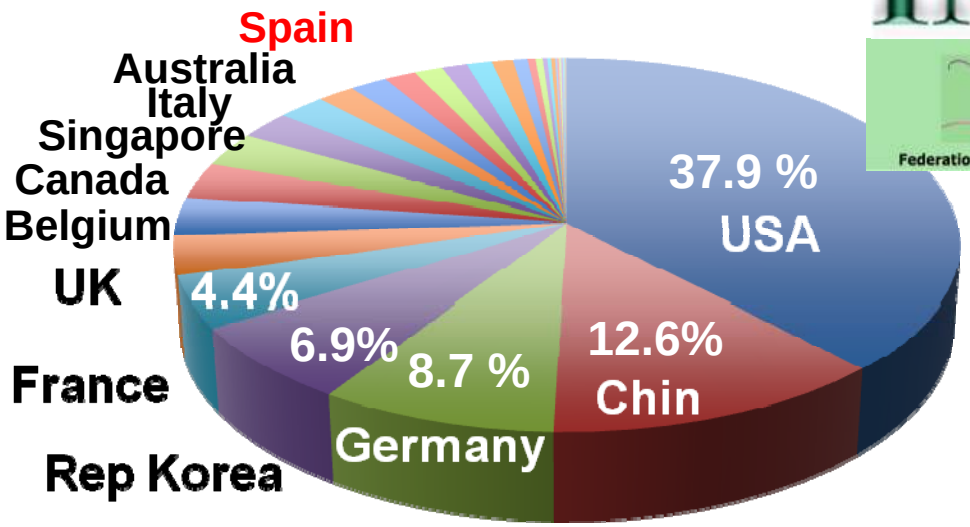
Depositor	No. Strains
Domestic academic	5,702
Domestic profit	285
Overseas academic	263
Total	6,250

BRC has collected unique strains mainly developed in Japan

International Distribution

We distribute mice to
510 organizations in
32 countries

in Europe
17 countries



List of formats for distribution

Format	No. items distributed
Live mice	18,217
Frozen embryos →	560
Recovered litters from frozen embryos	446
Frozen sperm →	32
Recovered litters from frozen sperm	54
Recoverd litters from FIMRe frozen embryos	6
Recoverd litters from FIMRe frozen sperm	2
Recoverd chimeras from FIMRe ES cells	3
Only MTA, indirect transfer	121
frozen or fixed tissues and organs	100
Genomic DNA	14
FOR 10 Years	19,555

About 25% of orders were distributed from frozen materials.

(Cumulative no. since FY2001)

Transportation methods until now

Live mice

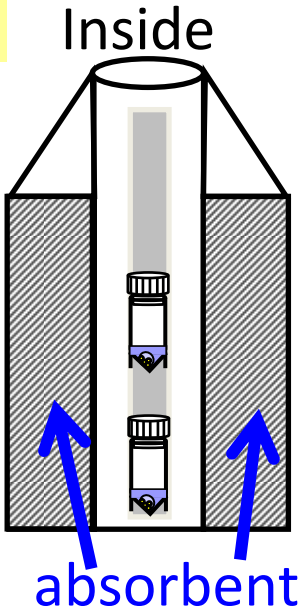
Advantage

1. Possible to use immediately
2. No need reproductive techniques

Disadvantages

1. Should keep temperature, fresh air...
2. Possibilities to die, escape, and spread murine diseases.
3. Cost of Transportation is expensive

Dry shipper



Advantage

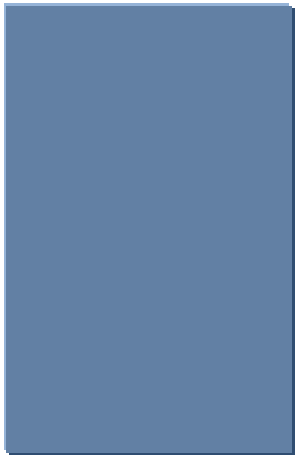
1. Stably keep at under -150℃

Disadvantages

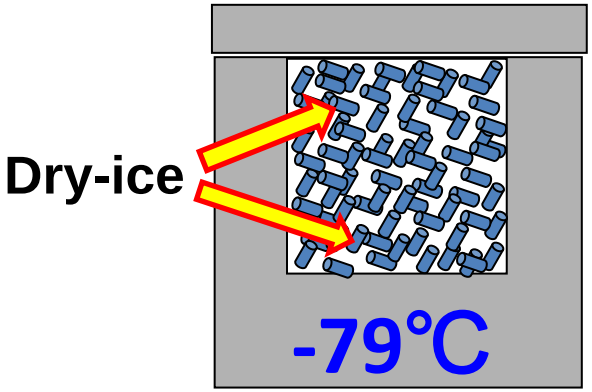
1. Large and heavy
2. Expensive
3. Reproductive techniques are needed
4. Incurs full fare for round trip

Experiments categorized by temperature

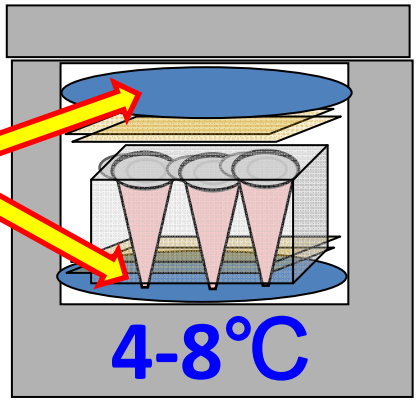
Transportation with



Dry shipper



Dry-ice package



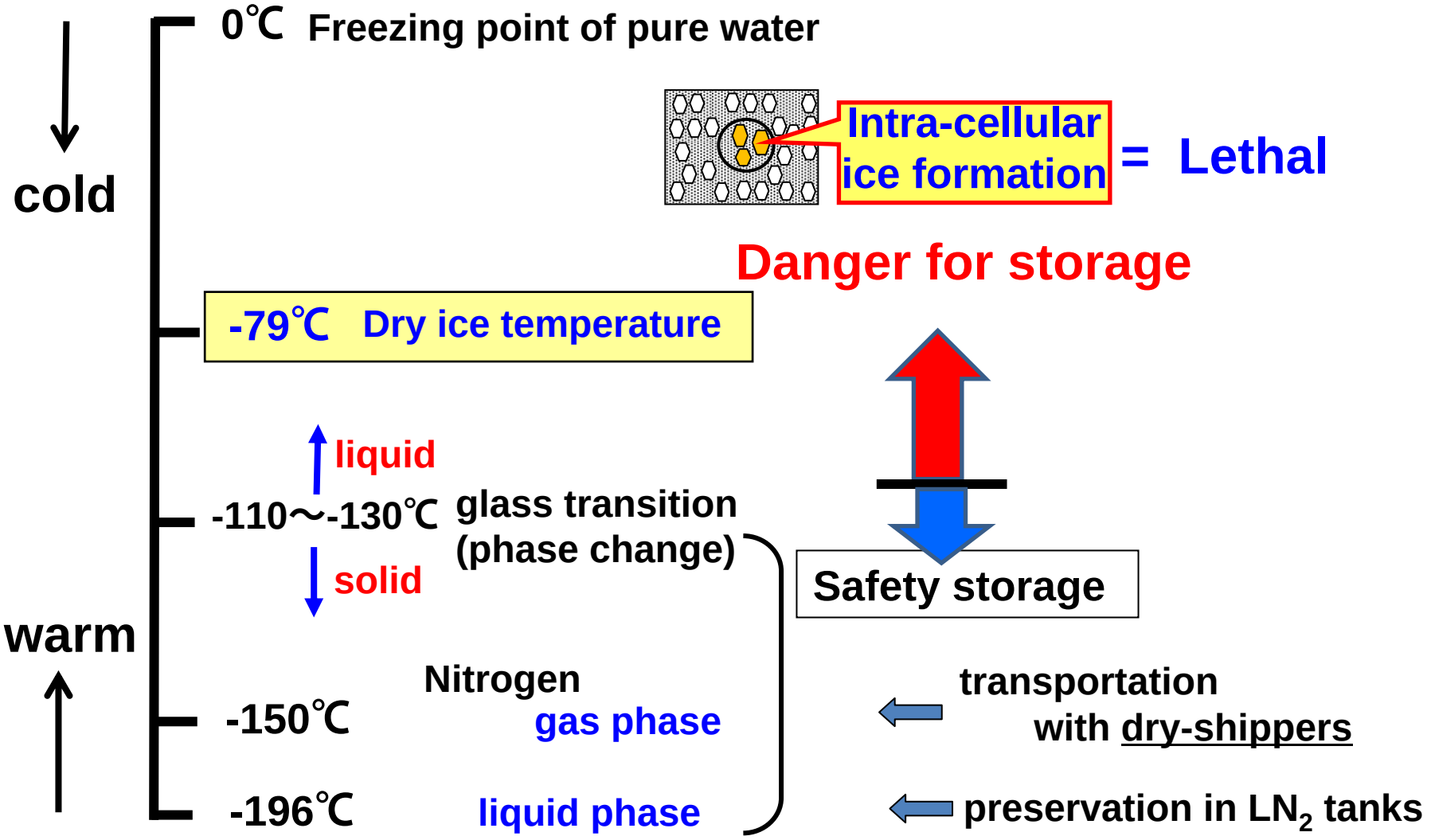
Refrigeration package

Embryo → Standard

Sperm → Standard

Experiment 1	Experiment 2
Experiment 3	Experiment 4

Summary of temperature and preservation condition



Exp.1 Transportation of embryos at -80℃

Strategy of novel development

1: Cryopreserve embryos without ice formation.
2: Using the high concentrated freezing solution.

↓

HOV method

(High Osmolality Vitrification)

even at -80℃

- **No ice formation.**
- **High survivability.**
- **Procedures are simple and quick.**

Exp.1 Transportation of embryos at -80℃

Embryos: 2-cell stage embryos of C57BL/6J strain

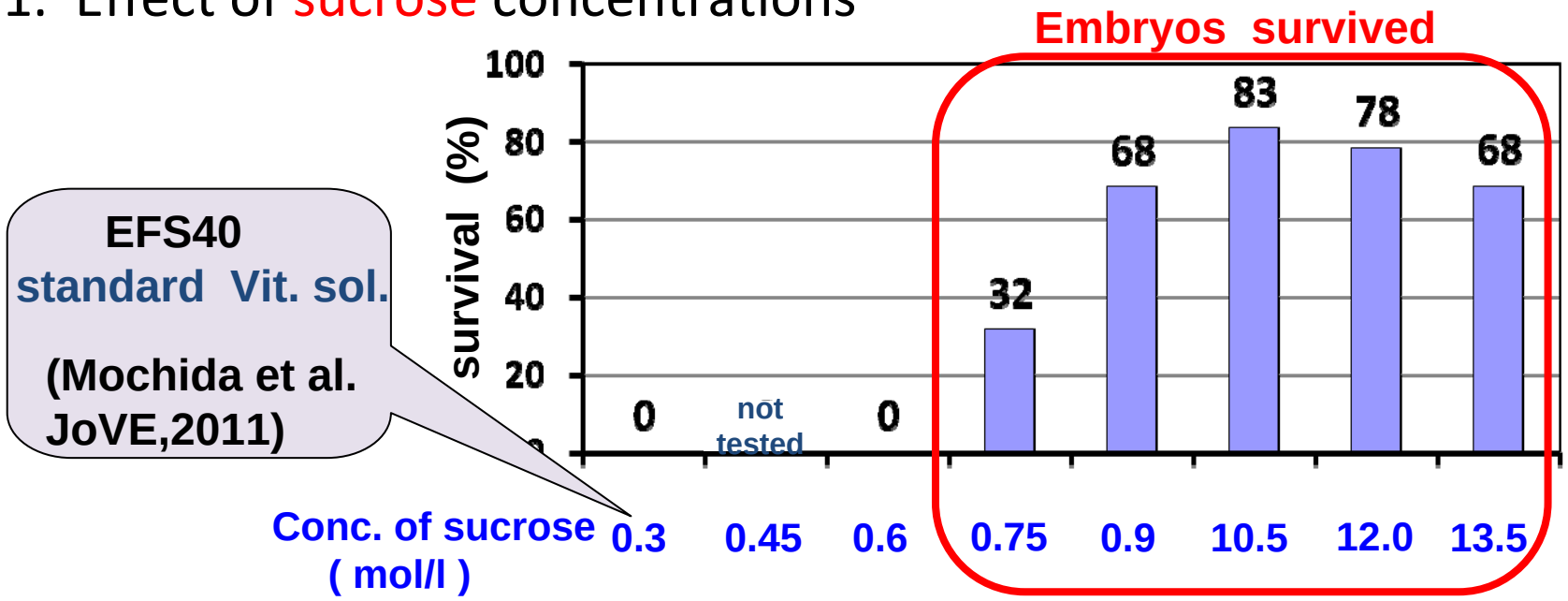
Storage: at -79ºC with dry ice pellets for 2 Days

Based vitrification sol.: EFS40 (40%EG, ficoll and sucrose)

Container: 1.2ml cryotube

developed by Kasai (1990)

1. Effect of sucrose concentrations



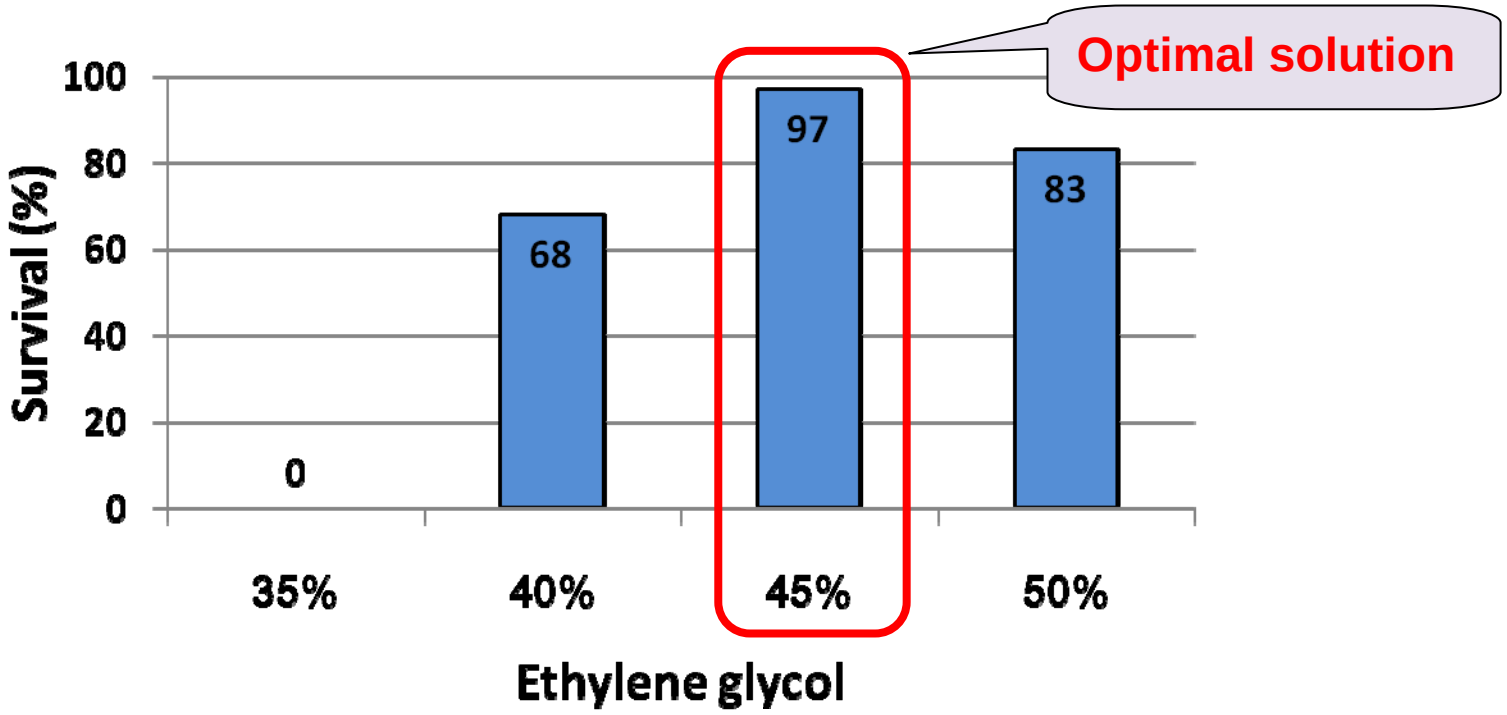
Results: After storage at –79ºC for 2days, embryos were survived.
But the survival rates were not enough. → defective method!

Exp.1 Transportation of embryos at -80℃

Vitrification solutions:

35, 40, 45, 50%EG were added in 18% ficoll and 1.5M sucrose

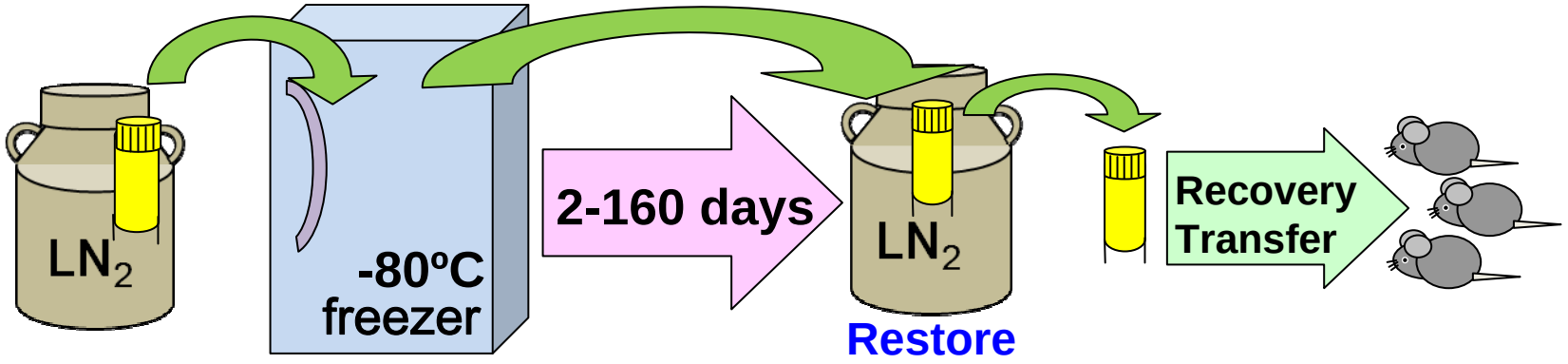
2. Effect of EG (ethylene glycol) concentrations



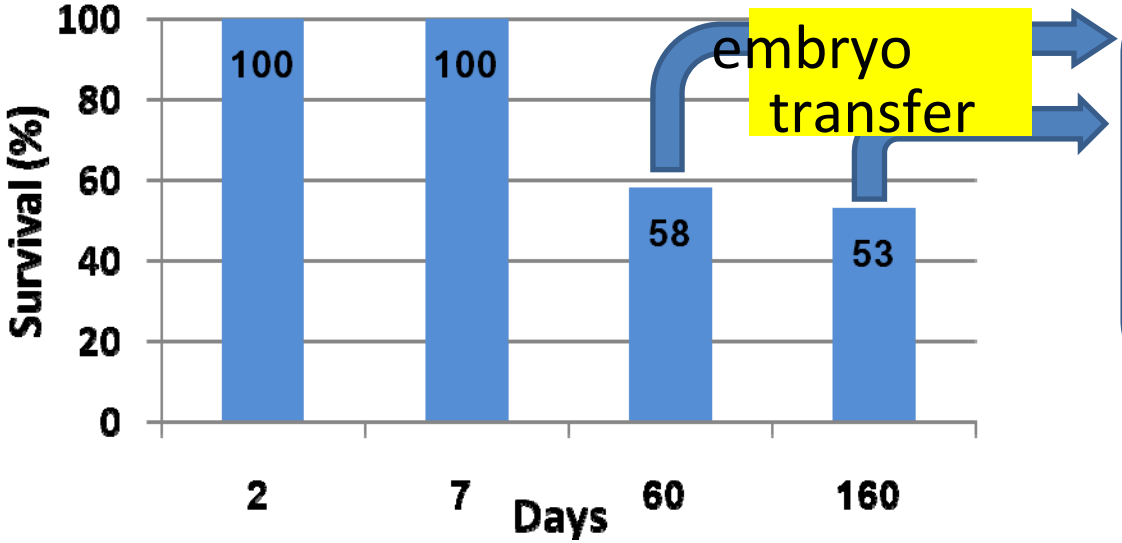
Results: **Survivability increased over 95%, when embryos were vitrified in solution contains both of sucrose and EG in high concentrations.**

→ **Optimal solution was found!**

Exp.1 Transportation of embryos at -80℃



3. Effect of duration at -80ºC



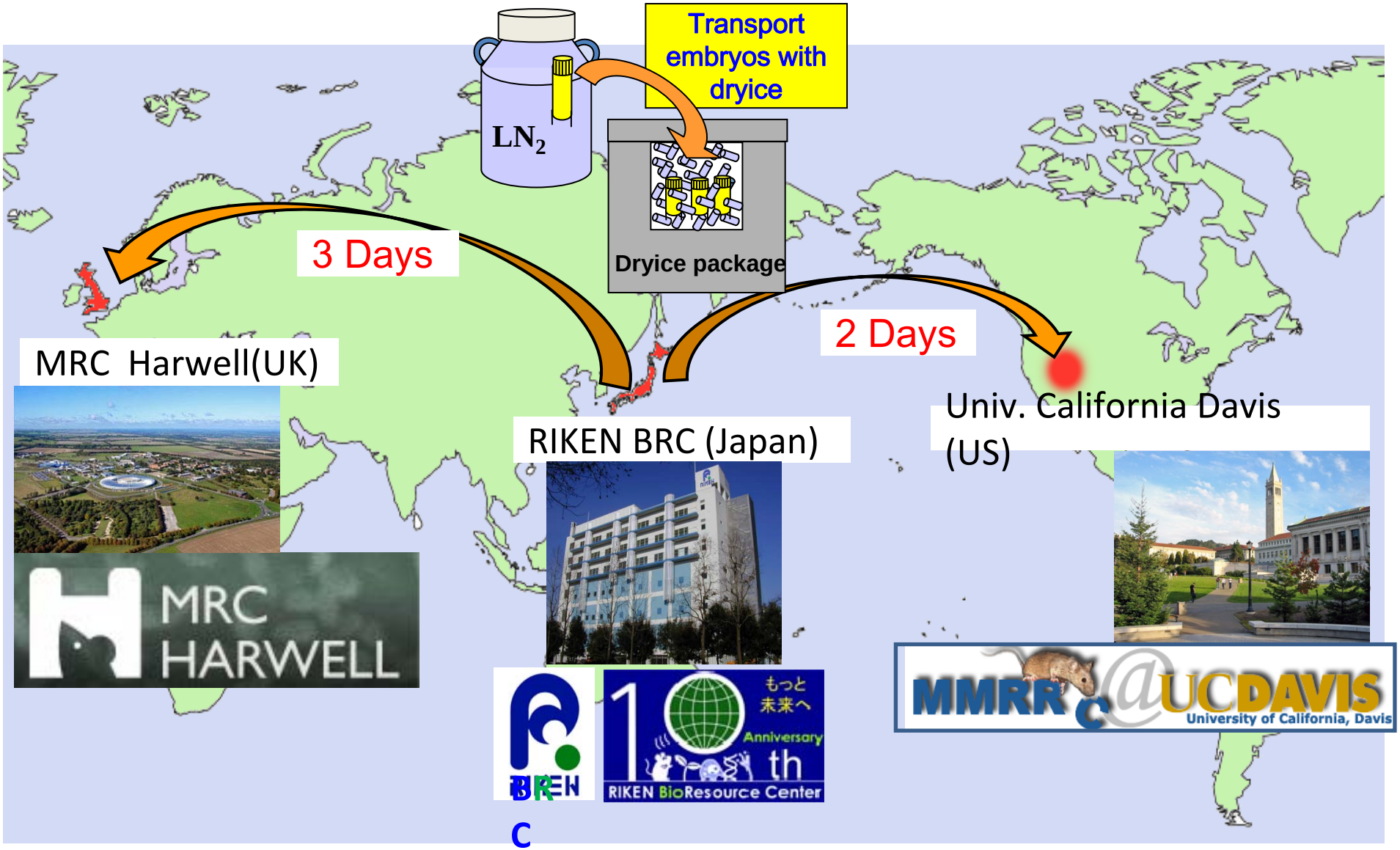
embryo transfer

32% and 12% of embryos developed into offspring

Results: Even after storage for 60 and 160 days in deep freezer, over 50% of embryos were survived and developed into offspring.

Exp.1 Transportation of embryos at -80℃

4. International transportation to FIMRe institutes



Exp.1 Transportation of embryos at -80℃

5. Results of international transportation to FIMRe institutes

Transportation	Recovery method	Transported embryos	Recovery (%)	Normal (%)	Pregnancy (%)	Embryos	Implant. (%)	Offspring (%)
-	Rapid	60	59 (98)	59 (100)	5 / 5 (100)	59	54 (92)	46 (78)
From Japan to MRC Harwell (UK)	Rapid	75	67 (89)	61 (91)	2 / 2 (100)	43	Not determined	17 (40)
From Japan to Univ. California Davis (US)	Slow	100	100 (100)	99 (99)	5 / 5 (100)	97	70 (72)	47 (48)

Results :

In both of institutes, over 90% of transported embryos were morphologically normal, then 40 and 48% of transferred embryos developed into offspring.

Transported embryos in dry-ice package were successfully recovered and developed into healthy mice.

Exp.1 Transportation of embryos at -80℃

6. Survivability of cryopreserved embryos by HOV method in major mouse strains

Strain	Total No. (%) of embryos			No of recipients pregnant/used (%)	No. of embryos		
	Vitrified	Retrieved (%)	Alive (%)		Transferred	Implanted (%)	Developed to offspring (%)
C57BL/6J	265	263 (97)	256 (97)	3/3 (100)	39	36 (92)	32 (82)
C57BL/6N	175	173 (99)	168 (97)	3/3 (100)	40	36 (90)	21 (53)
BALB/cA	210	210 (100)	206 (98)	3/3 (100)	40	31 (78)	18 (45)
129/SvJ	100	100 (100)	93 (93)	3/3 (100)	41	33 (80)	27 (66)
DBA/2N	200	200 (100)	193 (97)	6/6 (100)	77	44 (57)	25 (32)
C3H/HeN	100	99 (99)	96 (97)	3/3 (100)	41	27 (66)	19 (46)

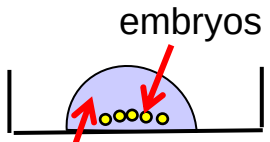
Results: **High survival rates (93-100%) and good ability to develop into offspring (32-82%) in six major inbred mouse strains were confirmed.**

Exp.1 Transportation of embryos at -80℃

7. Procedures of HOV method (optimized for major mouse strains)

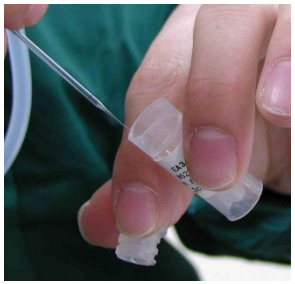
(1) Vitrification

Embryos were immersed in equilibrium solution for 3min.



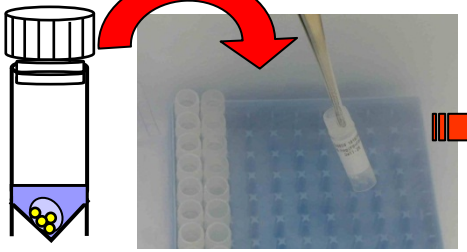
3 min

Transfer embryos into a tube containing vitrification solution with a glass capillary



1 min

Directly immerse in LN_2



Equilibrium solution
5%DMSO+5%EG-PB1, 50μl

Vitrification solution
42.5%EG+17%Ficoll +
1M Sucrose-PB1 , 50μl

preservation in LN_2

4-5 min

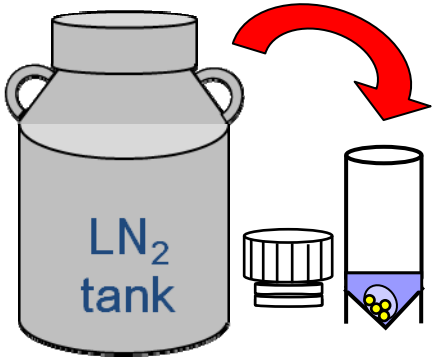
7-8 min

All procedures were performed at room temperature

(2) Liquefy (Slow thawing method)

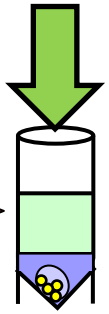
Don't need to hurry up !
Stable & high results.

Retrieve a tube and let it stand for 3min.



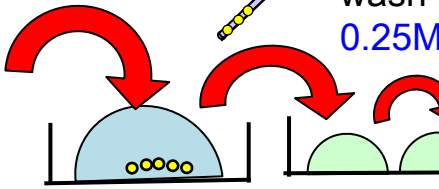
3 min

Add 850 μl of 0.75M sucrose-PB1



3 min

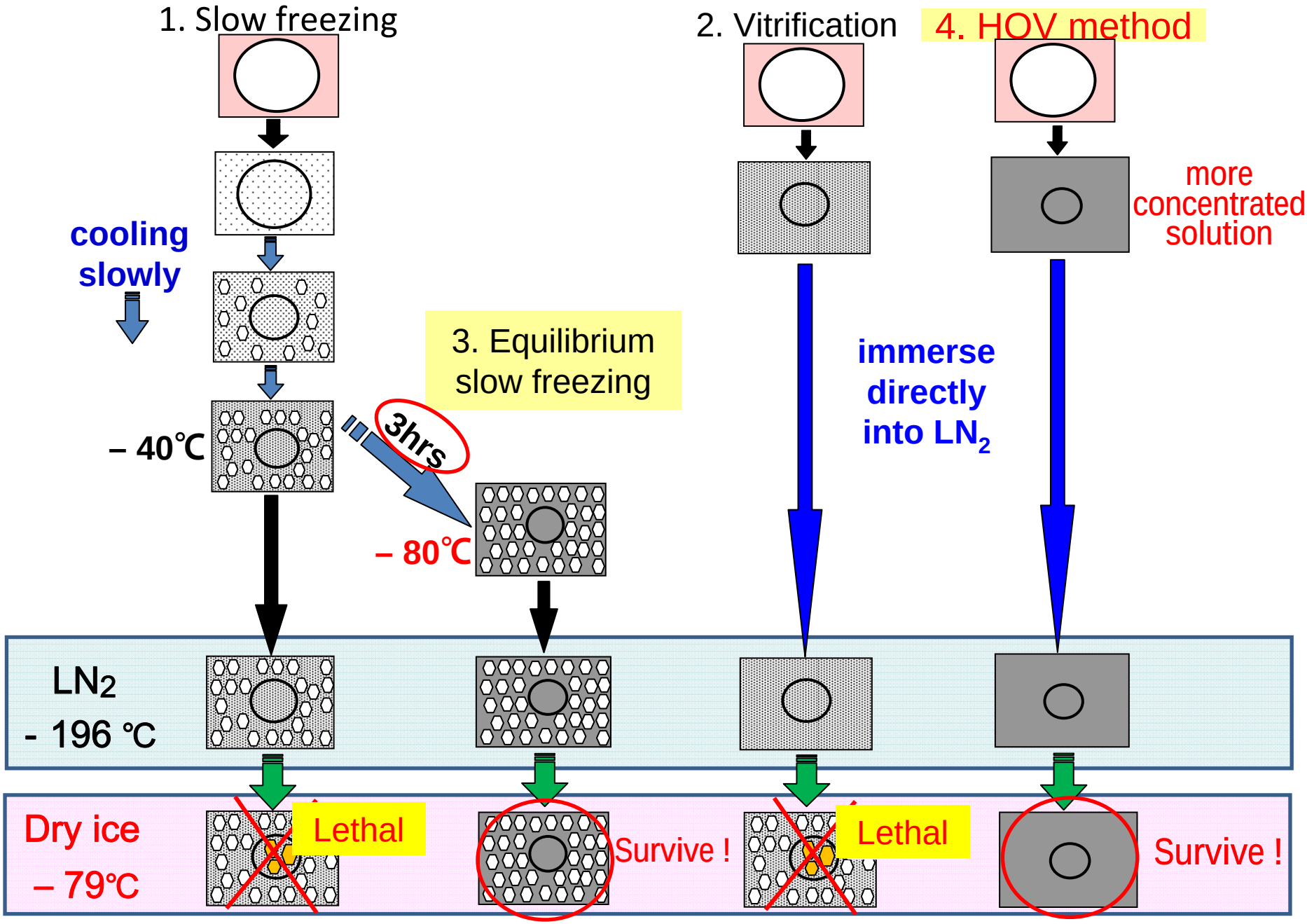
Transfer the entire volume to a dish with 1ml tip



Transfer embryos and wash in 50 μl of 0.25M sucrose-PB1

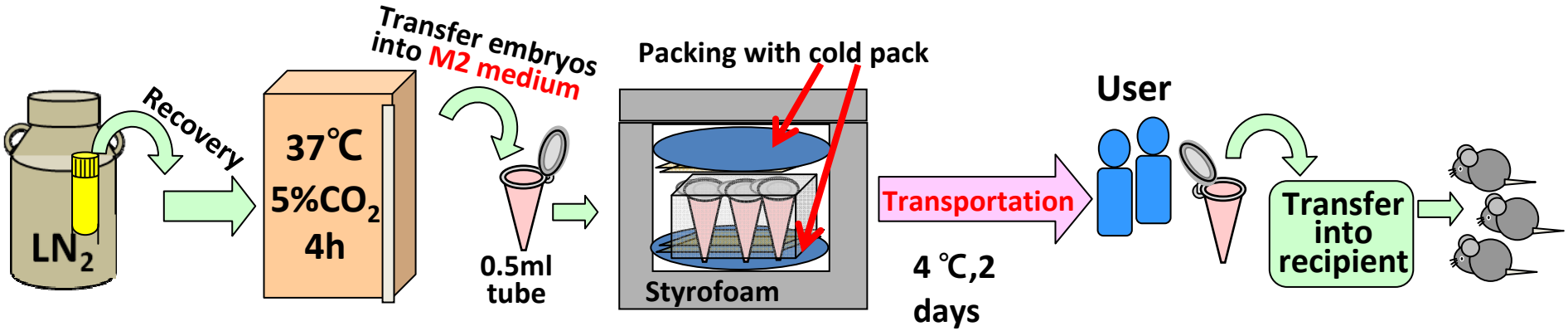
Culture in medium until embryo transfer

Summary of cryopreservation methods for mouse embryo



Exp.2 Transportation of embryos at 4-8℃

1. Protocol of transportation developed by Prof. Nakagata in Kumamoto Univ(CARD).



2. Results of domestic transportation

Transportation	Total No. of embryos	No. of tubes	No. of embryos		*In vivo* development		
			recovered (%)	normal (%)	pregnant (%)	Implantation sites (%)	offspring (%)
From CARD to BRC	120	3	120 (100)	120 (100)	3/4 (75)	25/40 (63)	16/40 (40)
From BRC to NIRS*	96	4	95 (99)	94 (99)	5/5 (100)	55/65 (92)	30/60 (50)

*: National Institute of Radiological Science

Results: After transportation for 2 days at refrigeration temperature, most of embryos were morphologically normal. And 40-50% of embryos developed into offspring by embryo transfer.→ This method is practically useful.

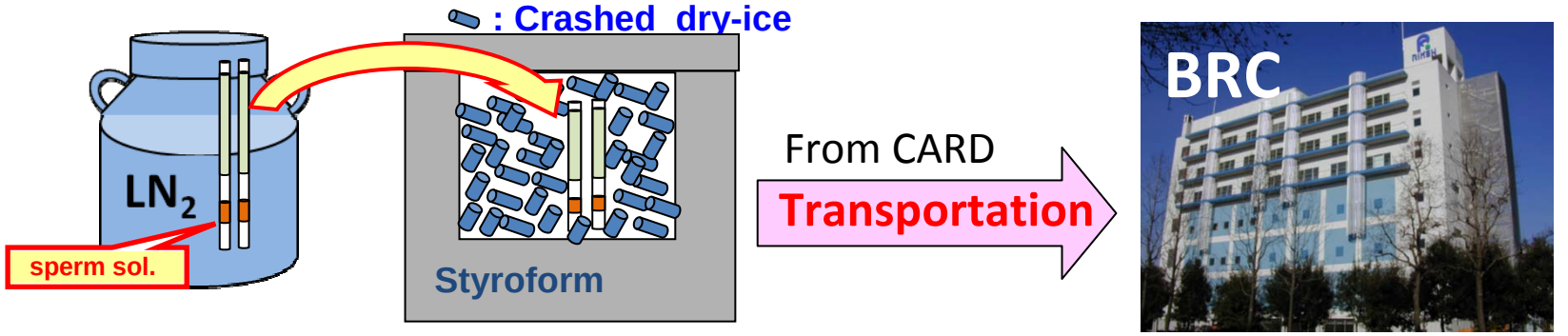
Exp.3 Transportation of spermatozoa at -80℃

1. Summary of transportation with frozen sperm at -80 ℃

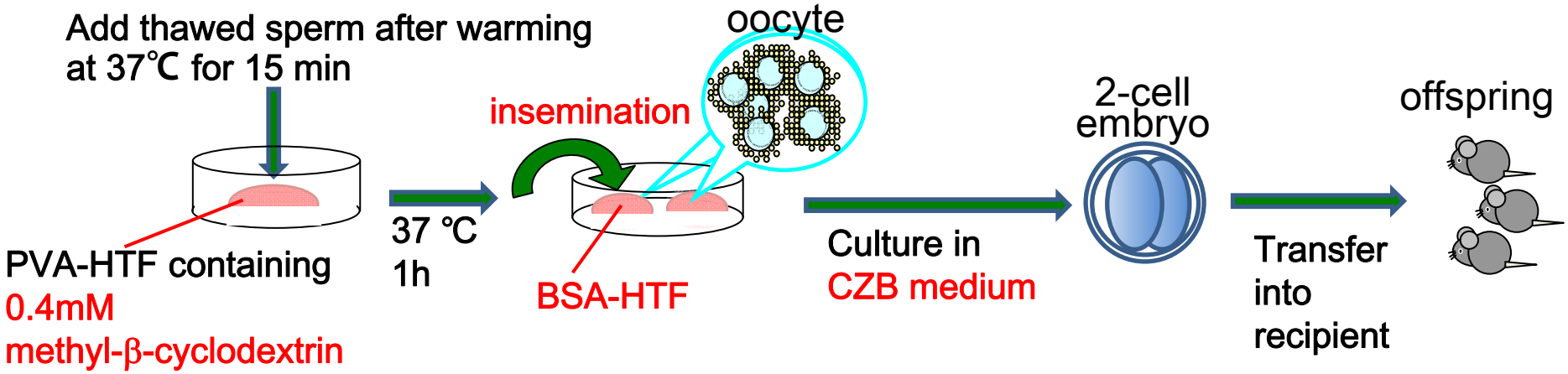
(1)Freezing of sperm in 18% raffinose and 3% skim milk solution with plastic straws

(Takeshima, Nakagata, Ogawa, Exp. Anim. 1991)

(2) Transportation of frozen sperm with dry-ice



(3) Production of live mouse by IVF and embryo transfer



Exp.3 Transportation of spermatozoa at -80℃

2. Results of IVF with transported frozen C57BL/6J sperm and development *in vivo*.

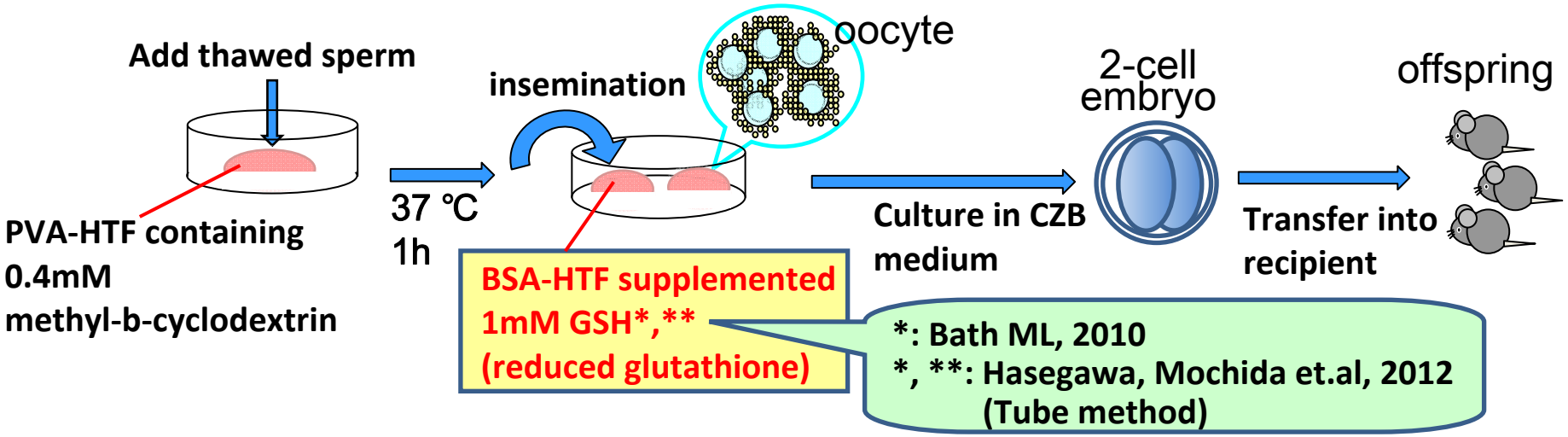
Sample No.	No. of oocytes		*In vivo* development		
	Inseminated	Fertilized (%)	Pregnant (%)	Implantation sites (%)	Offspring (%)
1	137	63 (46.0)	3/3	33/36 (92)	24/36 (67)
2	106	60 (56.6)	3/3	34/36 (94)	30/36 (83)
3	177	65 (36.7)	3/3	28/36 (78)	24/36 (67)
4	183	84 (45.9)	3/3	32/36 (89)	26/36 (72)
Total	603	46.3 ± 4.1%	12/12 (100)	86.0 ± 4.5%	72.0 ± 3.7%

Results: After transportation with dry-ice for 2 days, we successfully obtained offspring from frozen sperm by in vitro fertilization.
→ This transportation procedure is a practical method.

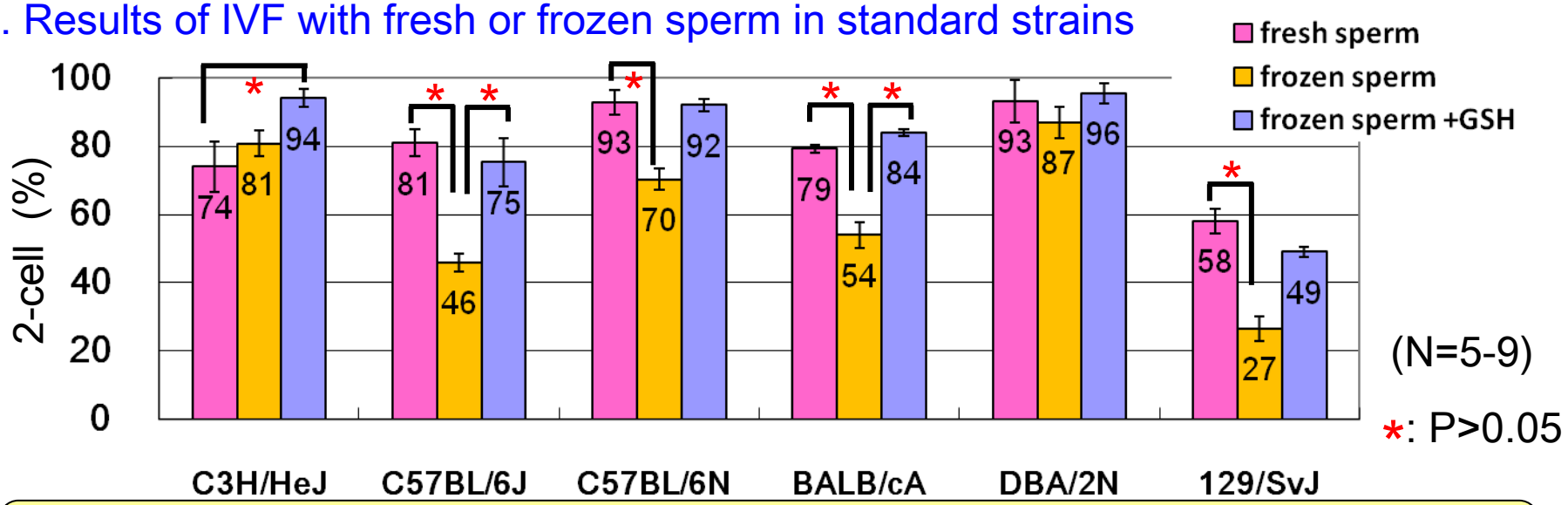
These results were reported at meeting in 2008.

Exp.3 Transportation of spermatozoa at -80℃

3. Established procedure of IVFwith frozen sperm



4. Results of IVF with fresh or frozen sperm in standard strains

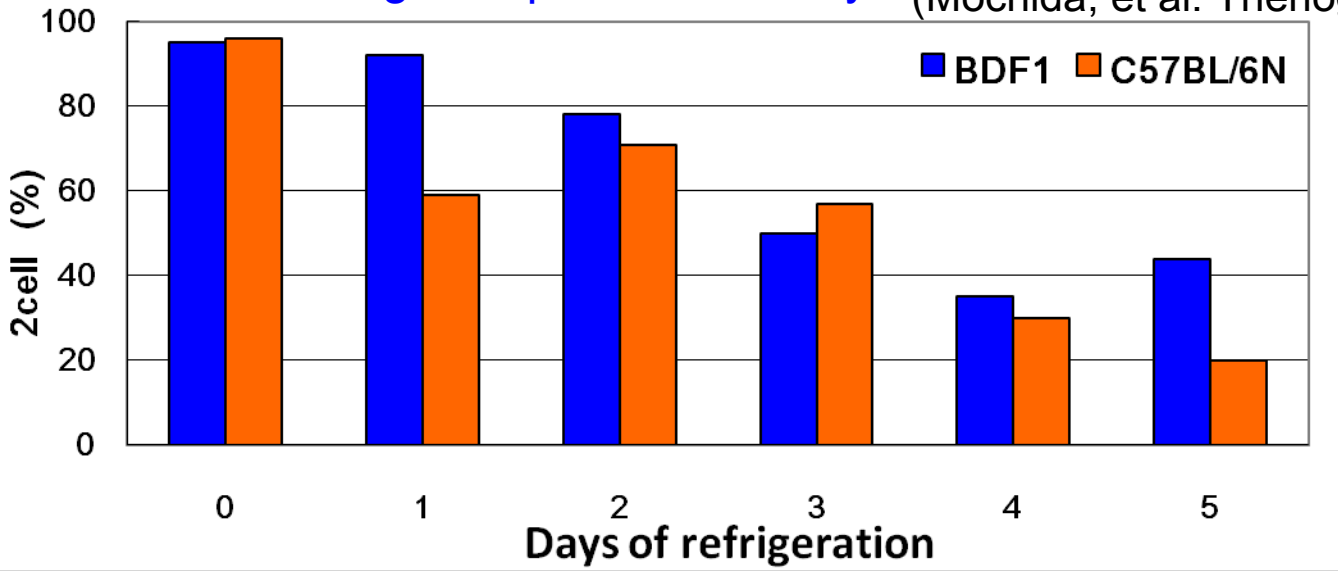


Results: **There was no differences between fresh and frozen (added GSH) group except C3H/HeJ strain.**

Exp.4 Transportation of spermatozoa at 4-8℃

1. Transportation of sperm within epididymides at refrigerated temperature

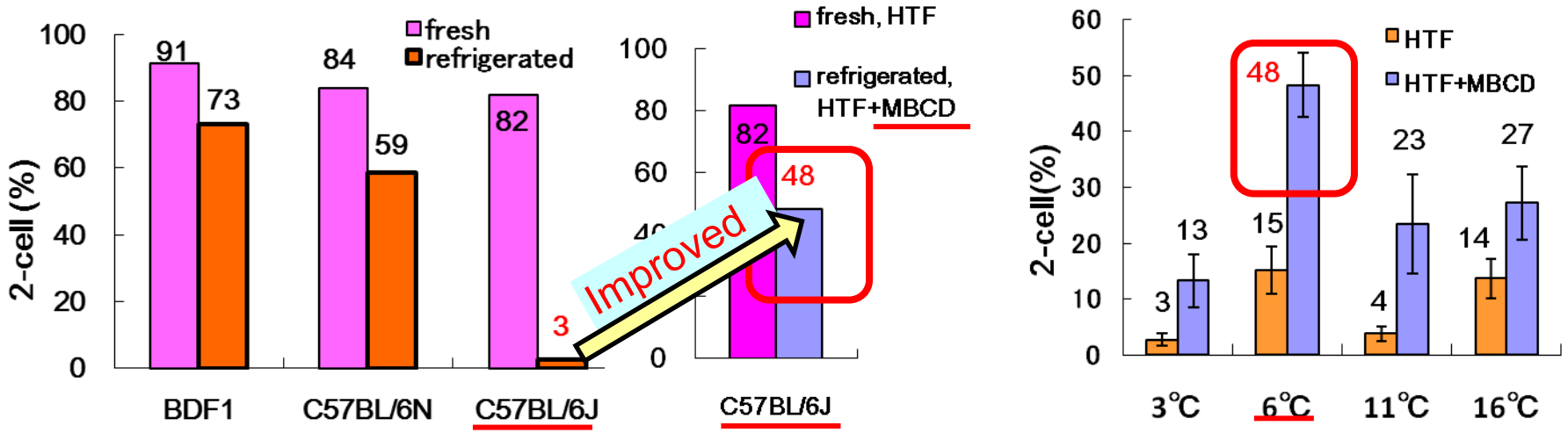
2. Results of IVF after storage of sperm until 5 days (Mochida, et al. Theriogenology, 2005)



Results: Embryos were obtained by IVF with refrigerated epididymides for 5 days, but the rates of fertilization decreased gradually.

Exp.4 Transportation of spermatozoa at 4-8℃

3. Results of IVF after refrigeration of C57BL/6J sperm for 2days



4. Practical results of IVF after transportation of sperm (C57BL/6J background strains)

	Strain No.						Total
	1	2	3	4	5	6	
Inseminated	70	22	46	10	31	36	
Fertilized	72	24	59	34	50	54	
(%)	97.2	91.7	78.0	29.4	62.0	66.7	70.8 ± 10.0%

1mM GSH were added in insemination medium

Results: After transportation of sperm with refrigerated epididymides for 2 days, we successfully obtained embryos even in B6J strain.
→ This transportation procedure has practically used in our center.

Cost of transportation

1. Frozen materials: applicable within 5days

- Dry-ice packages for both of embryo and sperm are safe, easy to carry and economical method.

Distance	Cost of transportation		
	Live mice (2-3 pairs)	dry shipper (round-trip)	dry-ice package
Domestic (600km)	$200~900	$40	$20
Intercontinental *	$2,000	$2500~3,500	$600~1,750

*: from Japan to U.S. or Europe

2. Unfrozen materials: applicable within 2days

- We have often used for only domestic transportation.
- The refrigeration package is remarkably economical method.

Distance	Cost of transportation		
	Live mice (2-3 pairs)	dry shipper (round-trip)	refrigeration package (round-trip)
Domestic (600km)	$200~900	$40	$40

Conclusion

- **Efficient transportation methods of embryos and spermatozoa at dry-ice temperature or under refrigeration were devised.**
- **HOV method is eminently applicable for routine embryo cryopreservation in many mouse facilities.**

Acknowledgements

We thank
Dr. Martin Fray (MRC Harwell)
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for performing the recovery test and invaluable discussions.

Our members of

Reproductive technology group in BRC

&

Ogura's Lab.
at the party of 10th anniv.

Please visit our HP

Thank you
for your attention !!