EMMA Workshop: May 8 2012.

Sperm freezing
-----MBCD and GSH approach-----

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動物資源開発研究部門 Center for Animal Resources and Development

Center for Animal Resources and Development

Topics

1. Introduction

2. Technology Development:

Sperm cryopreservation

Sperm preincubation

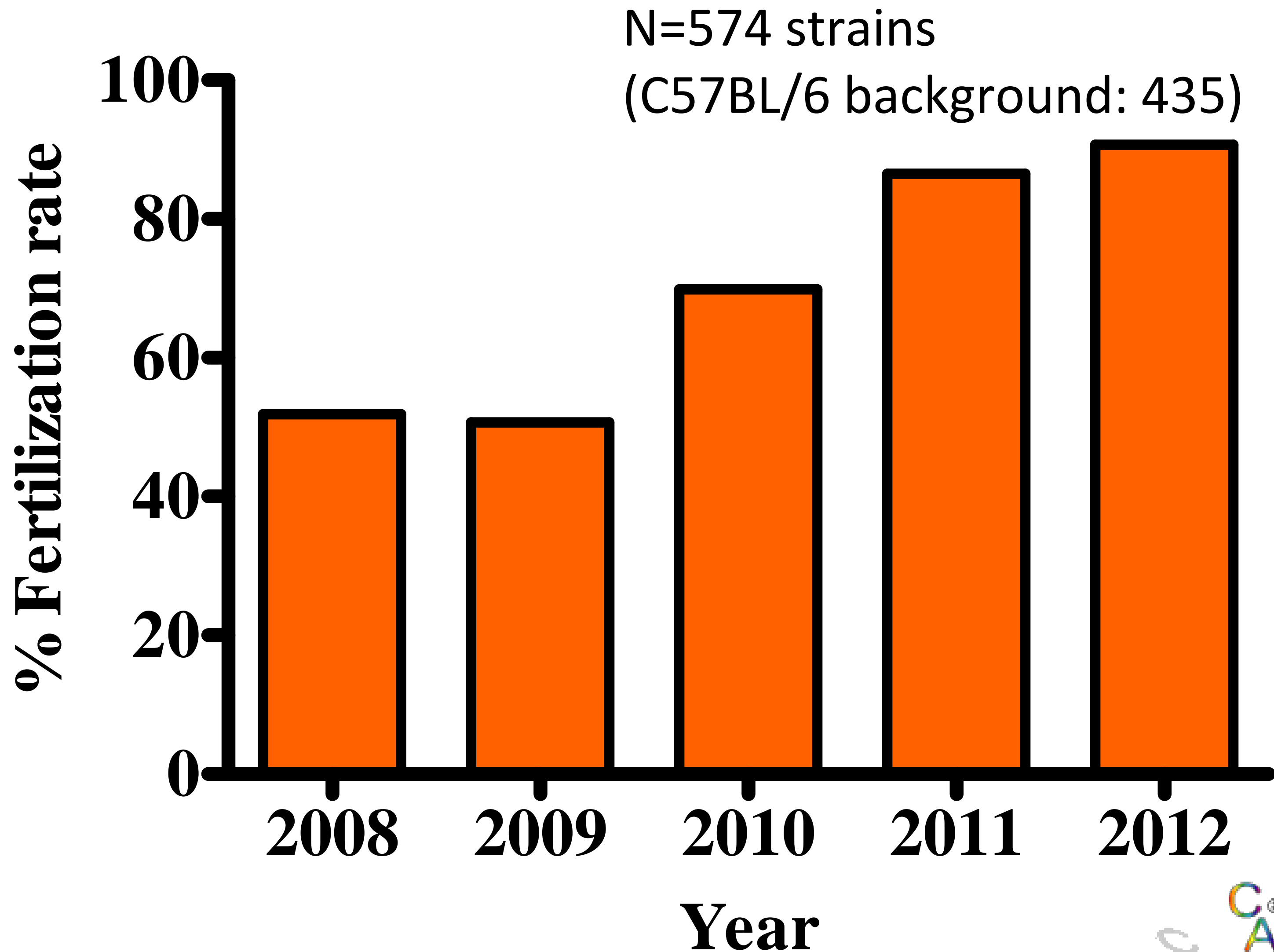
In vitro fertilization

3. Application of our technologies

Inbred and hybrid strains

Cryopreserved sperm prepared by various protocols

CARD Protocol:
Sperm cryopreservation and IVF



Technology Development

Prof. Nakagata

Mammalian Genome 11, 572–576 (2000).
DOI: 10.1007/s003350010109

Cryopreservation of mouse spermatozoa

N. Nakagata
Division of Reproductive Engineering, Center for Animal Resources & Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan

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BIOLOGY OF REPRODUCTION 78, 546–551 (2008)
Published online before print 28 November 2007.
DOI 10.1095/biolreprod.107.065359

Methyl-Beta-Cyclodextrin Improves Fertilizing Ability of C57BL/6 Mouse Sperm after Freezing and Thawing by Facilitating Cholesterol Efflux from the Cells[1]

Original Article

Combination medium of cryoprotective agents containing L-glutamine and methyl-β-cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6J mouse sperm

T Takeo and N Nakagata

BIOLOGY OF REPRODUCTION 85, 000–000 (2011)
Published online before print 20 July 2011.
DOI 10.1095/biolreprod.111.092536

Reduced Glutathione Enhances Fertility of Frozen/Thawed C57BL/6 Mouse Sperm after Exposure to Methyl-Beta-Cyclodextrin[1]

Toru Takeo and Naomi Nakagata[2]
Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan

Nakagata Method

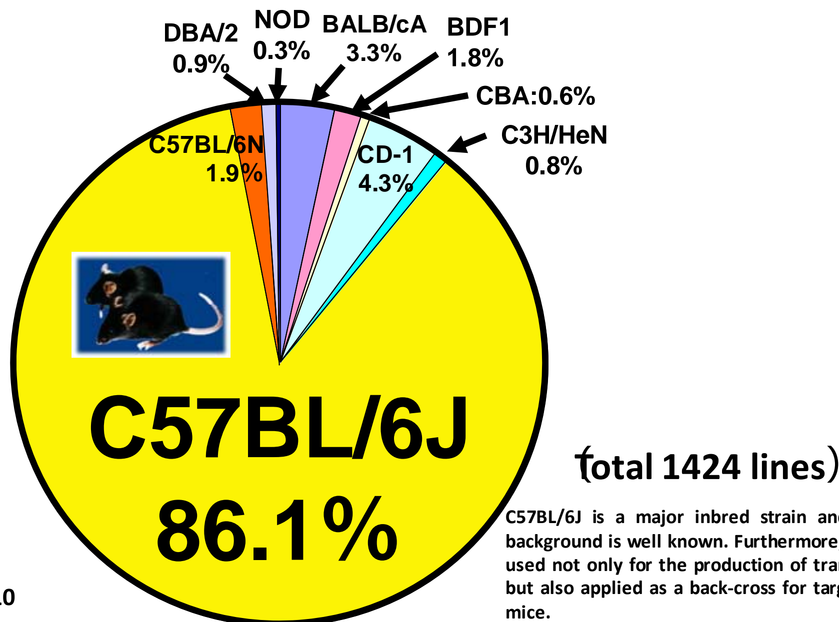
Methyl-β-Cyclodextrin (MBCD):
TYH with MBCD

Modified R18S3 (mR18S3):
R18S3+L-glutamine

Reduced glutathione (GSH):
mHTF + GSH

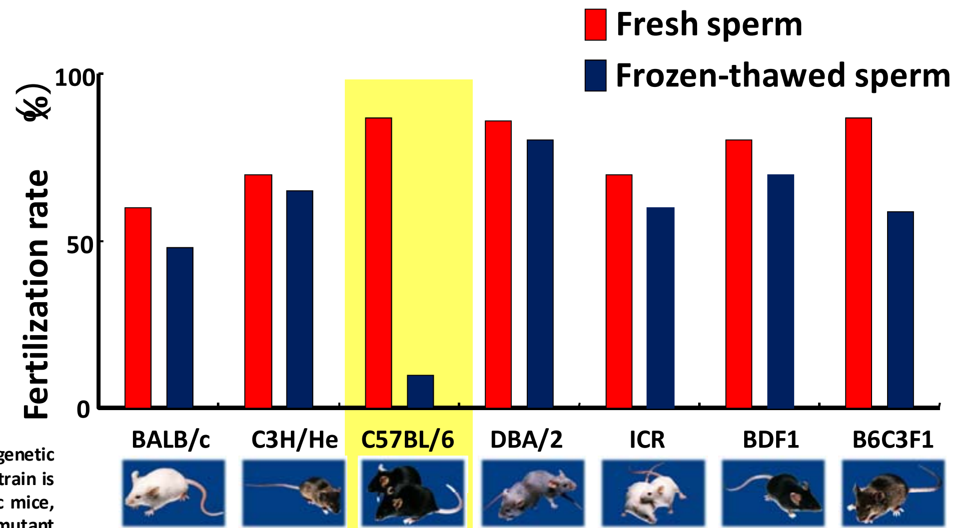
Mammal Genome 2000.
Biol Reprod 2008.
Lab Animal 2010.
Biol Reprod 2011.

C57BL/6 Mouse



2010

C57BL/6J is a major inbred strain and its genetic background is well known. Furthermore, this strain is used not only for the production of transgenic mice, but also applied as a back-cross for targeted mutant mice.



1. Major inbred strain
2. Most common background for genetically engineered mouse
3. Low fertility of frozen-thawed sperm

We have addressed the problem of low fertility of frozen-thawed C57BL/6 mouse sperm.

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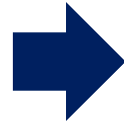
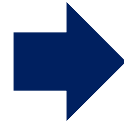
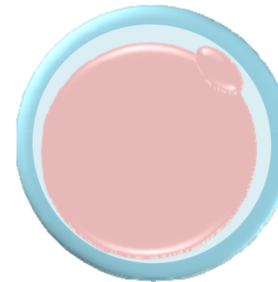
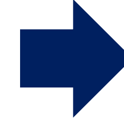
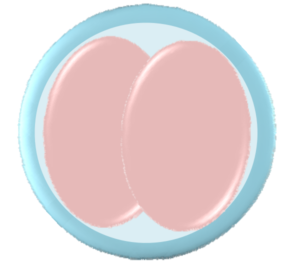
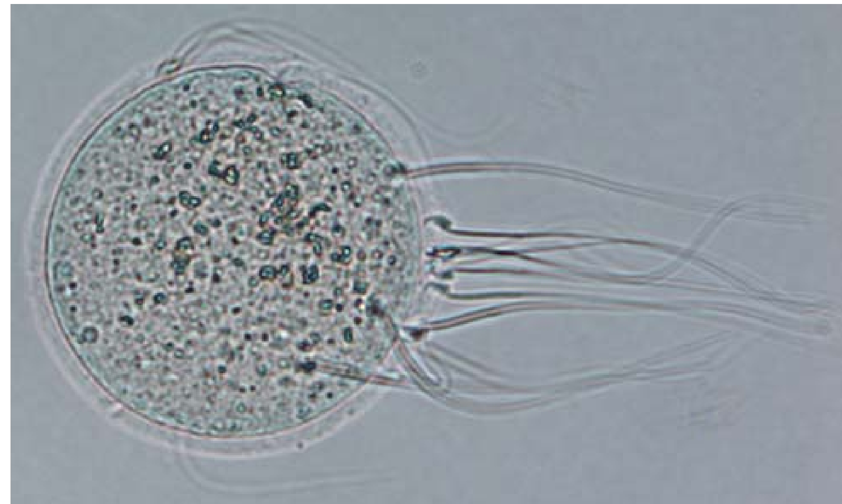
1. Technology Development

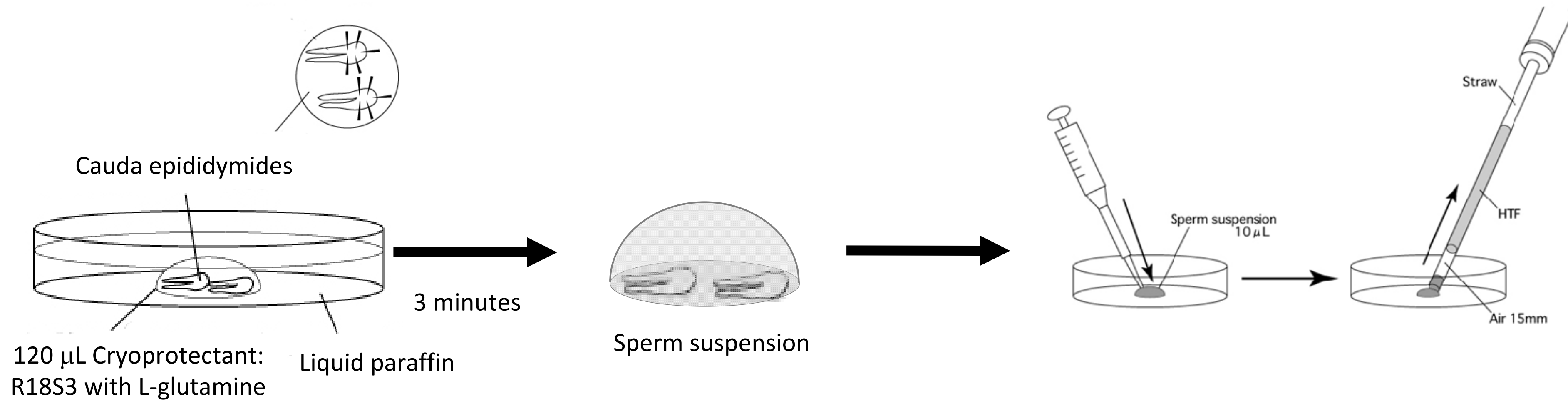
Cryopreservation: **Preincubation：** **Fertilization：**

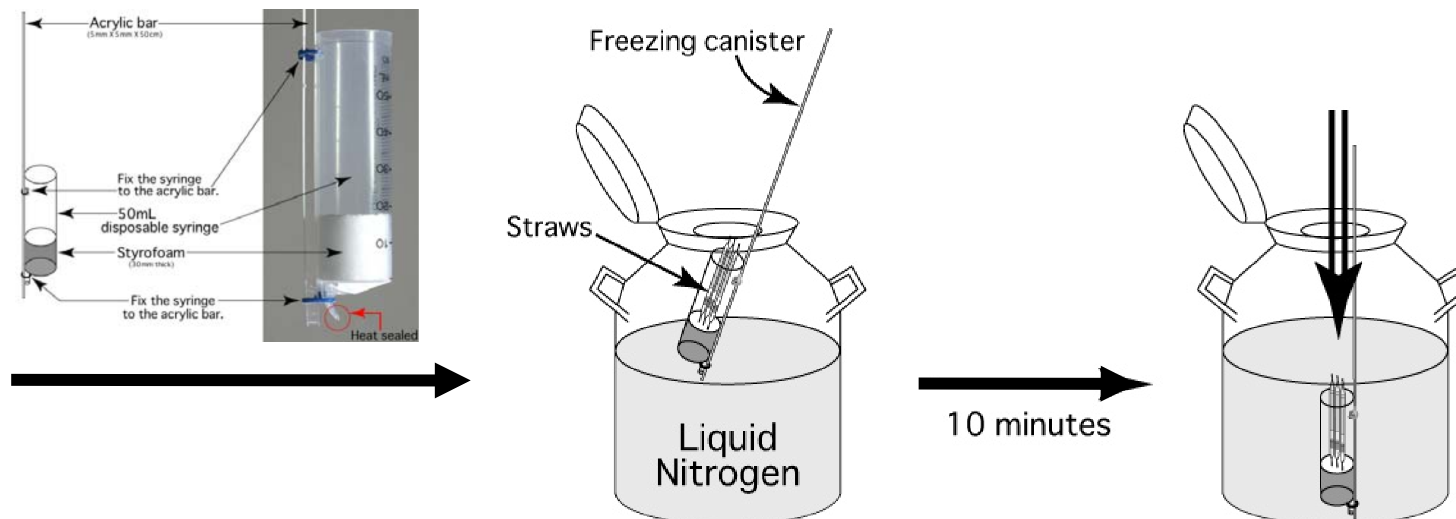




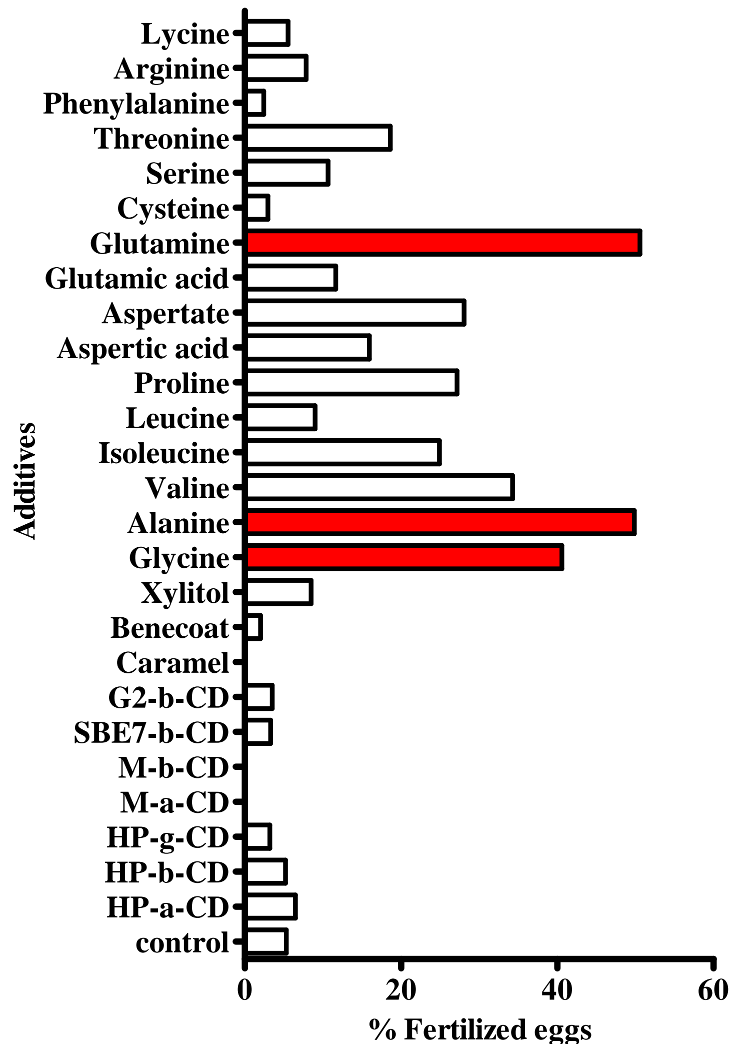
Sperm Cryopreservation





CARD online manual: http://card.medic.kumamoto-u.ac.jp/card/english/sigen/index.html

Cryoprotectant solution:
18 % raffinose pentahydrate + 3% skim milk solution (R18S3) + L-glutamine

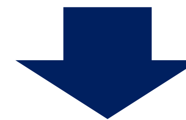


Screening

Sugars, cyclodextrins, amino acids in R18S3

Amino acids

glycine, alanine, glutamine

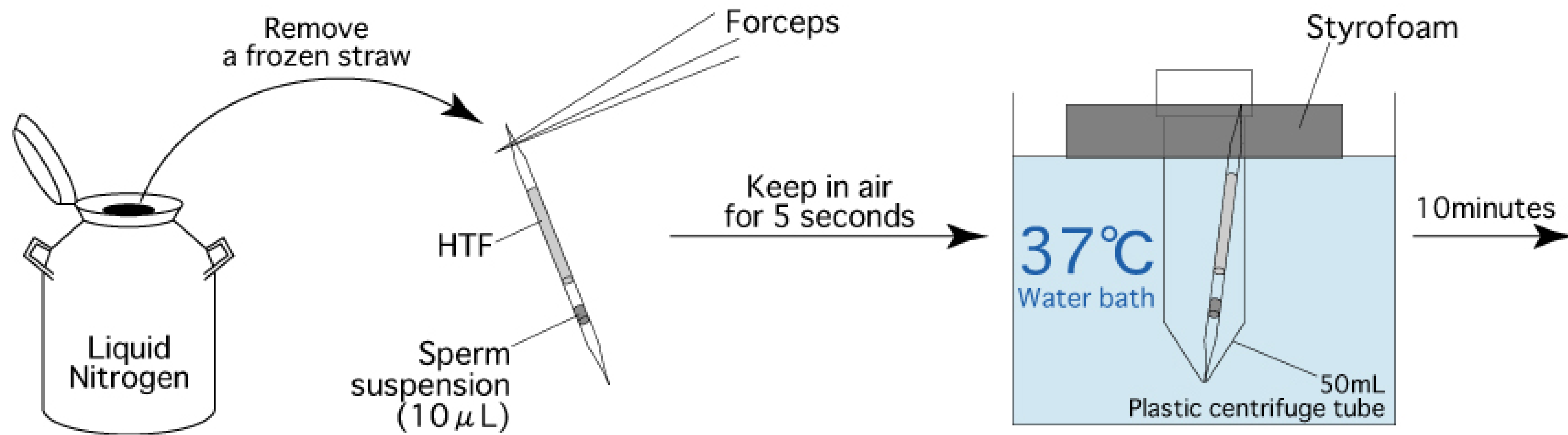


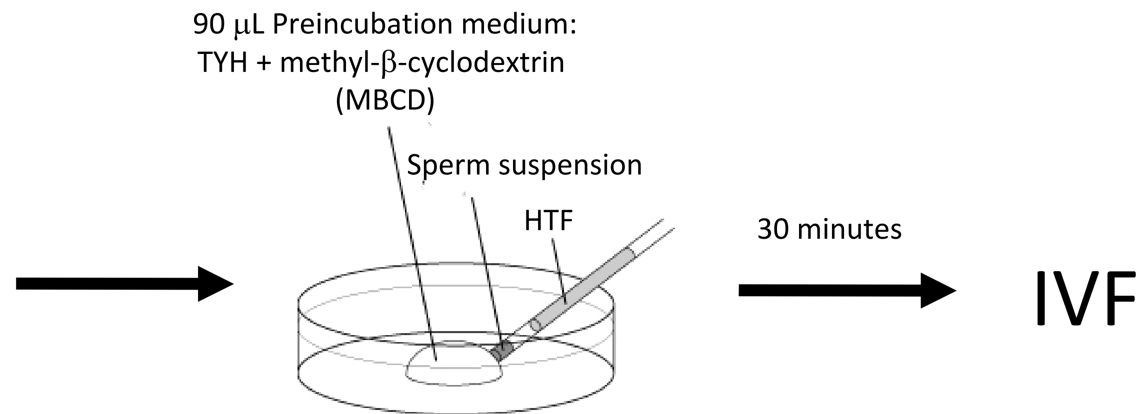
mR18S3:
R18S3 + L-glutamine

Unpublished data

Takeo and Nakagata, Lab Animal 2010.

Sperm preincubation





30 minutes

IVF

CARD online manual: http://card.medic.kumamoto-u.ac.jp/card/english/sigen/index.html

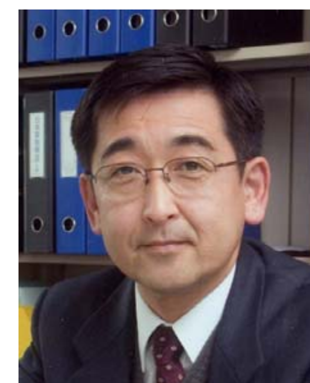
Sperm preincubation medium: TYH with methyl-β-cyclodextrin (MBCD)

Prof. Toyoda ：
Reproductive Biology

TYH:
Toyoda
Yokoyama
Hoshi

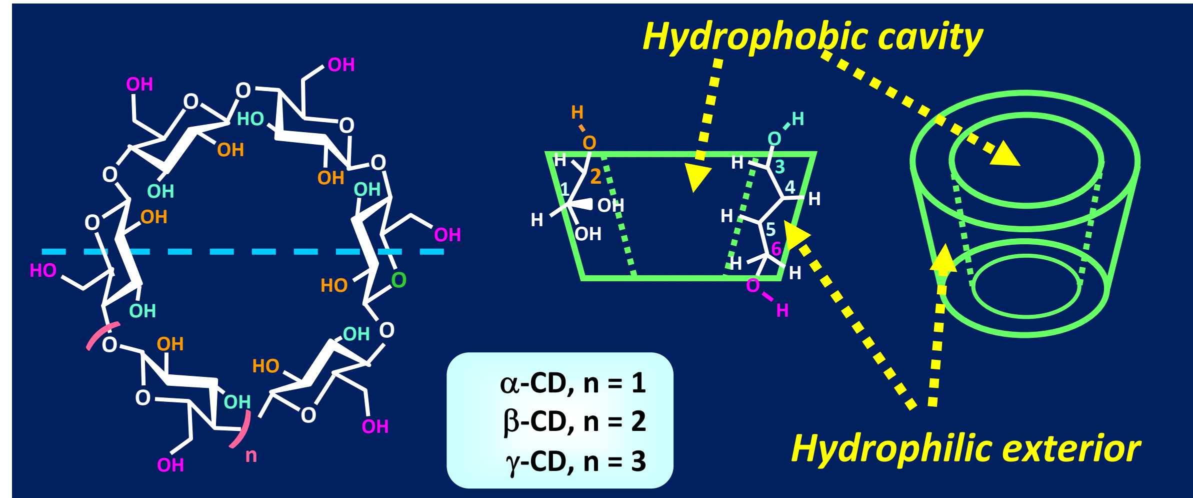
Toyoda Y et al. 1971: J. Anim. Reprod. 16: 147-151.

Component	mg/100 mL
NaCl	697.6
KCl	35.6
Glucose	100
Sodium pyruvate	5.5
$CaCl_2/2H_2O$	25.1
$MgSO_4/7H_2O$	29.3
KH_2PO_4	16.2
$NaHCO_3$	210.6
Potasium penicillin G	7.5
Streptomysin sulfate	5.0
Polyvinylalcohol	100



Prof. Irie:
Cyclodextrin Science
for Physical Pharmaceutics

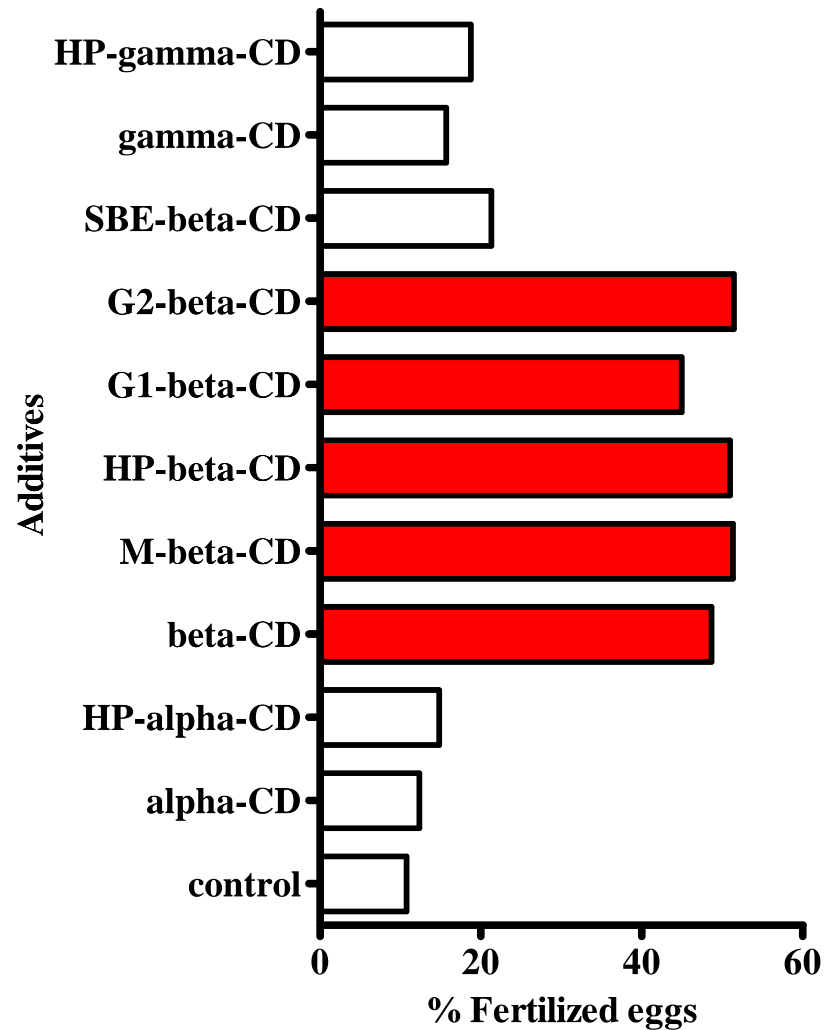
Cyclodextrins



Compound	n	R_1	R_2	R_3	D.S. [a]	M.W. [b]	Solubility (g/dL) [c]	Hemolytic activity (mM) [d]
α-CyD	6	-H	-H	-H	-	972	14.5	14
β-CyD	7	-H	-H	-H	-	1135	1.85	5.7
HP-α-CyD [e]	6		-H or $-CH_2CH(CH_3)OH$		4.1	1210	> 50	> 50
HP-β-CyD [e]	7		-H or $-CH_2CH(CH_3)OH$		4.8	1413	> 50	14.5
DM-α-CyD [f]	6	$-CH_3$	-H	$-CH_3$	12	1141	> 50	6.0
DM-β-CyD [f]	7	$-CH_3$	-H	$-CH_3$	14	1331	57	1.3
M-β-CyD [g]	7		-H or $-CH_3$		12.6	1311	> 50	2.8

a) Average degree of substitution, b) Molecular weight, c) In water at 25 ,
d) The concentration of CyDs to induce 50% hemolysis of rabbit erythrocytes,
e) 2-Hydroxypropyl-CyDs, f) 2,6-Di-*O*-methyl-CyDs, g) Methyl-β-CyD.

Nature β-CD and β-CD derivatives enhance fertility of frozen-thawed sperm



Unpublished data

Screening

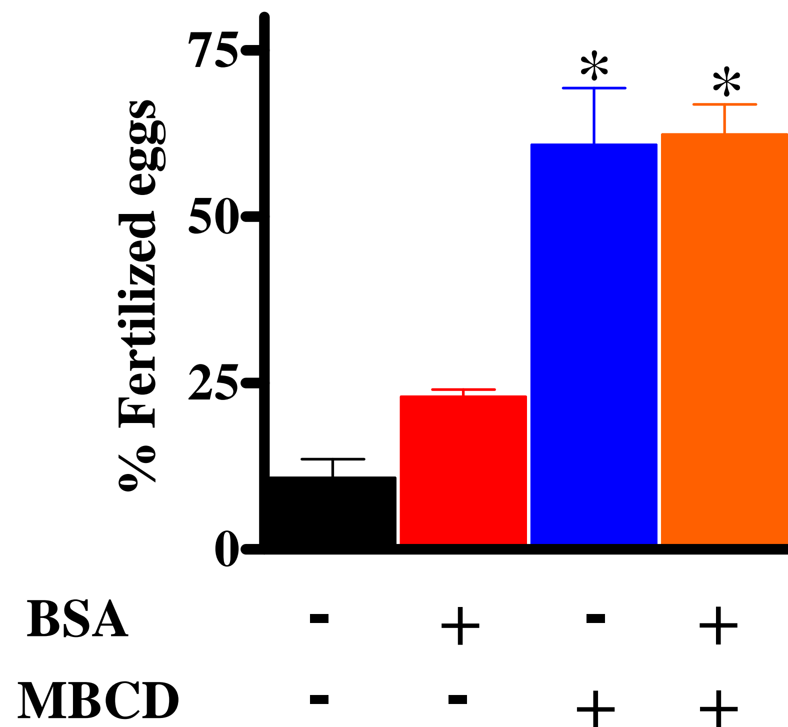
Nature CDs, various CD derivatives in TYH

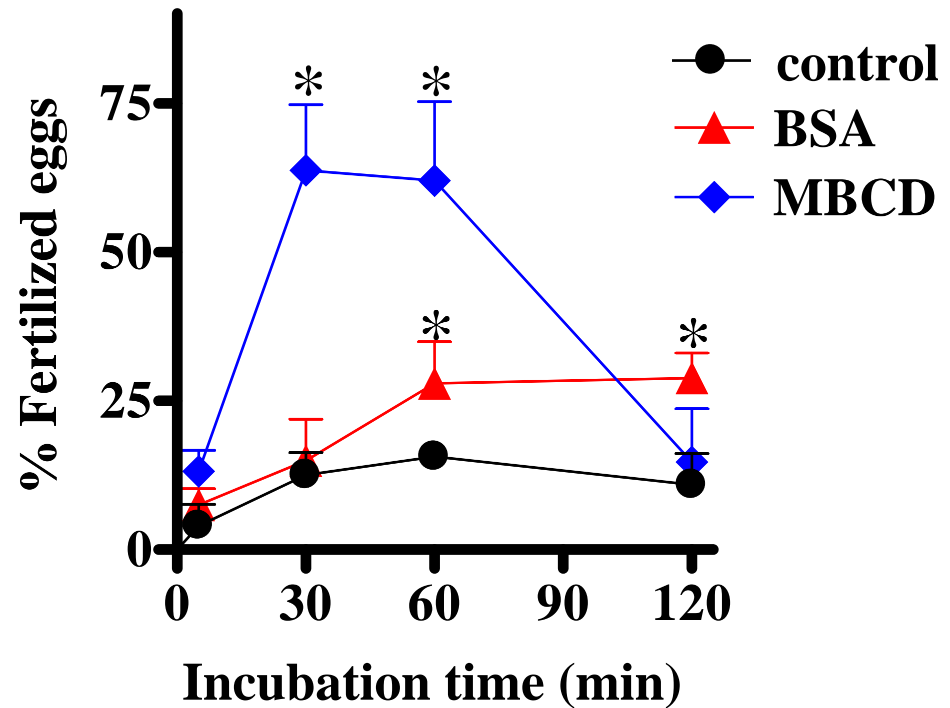
β-CDs

BCD, MBCD, HPBCD, G1BCD, G2BCD

Sperm preincubation:
TYH + MBCD

MBCD activates fertility of frozen-thawed C57BL/6 mouse sperm

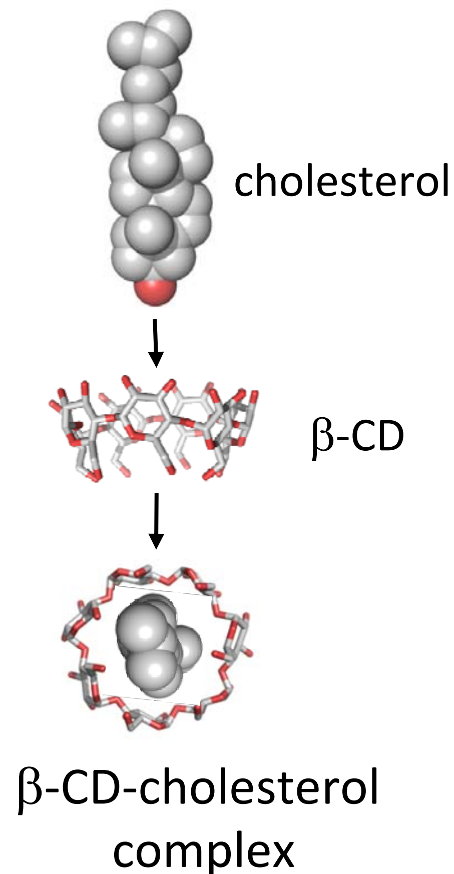




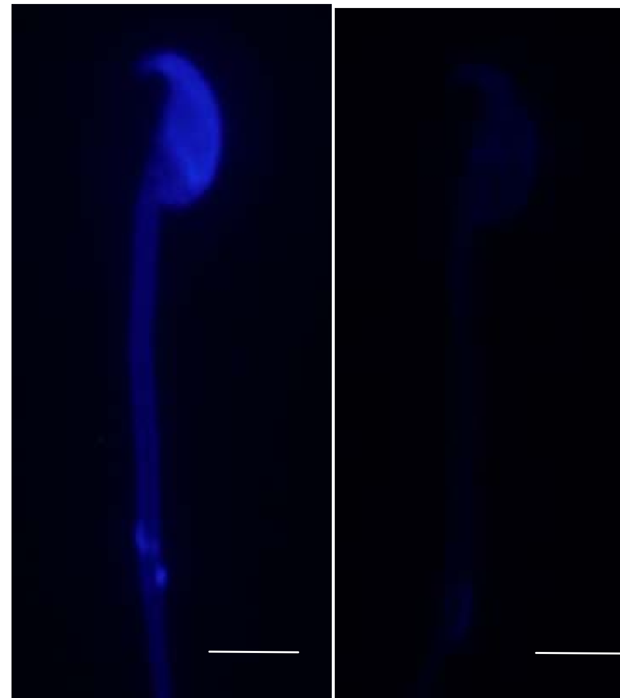
Takeo et al, Biol Reprod 2008.

MBCD facilitates cholesterol efflux from sperm membrane

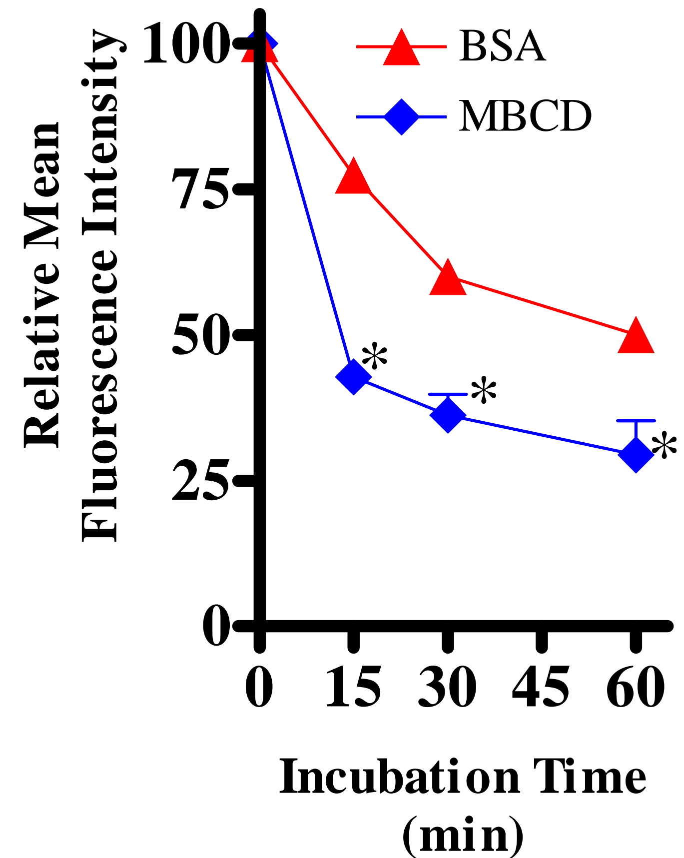
CD-cholesterol complex



Membrane cholesterol

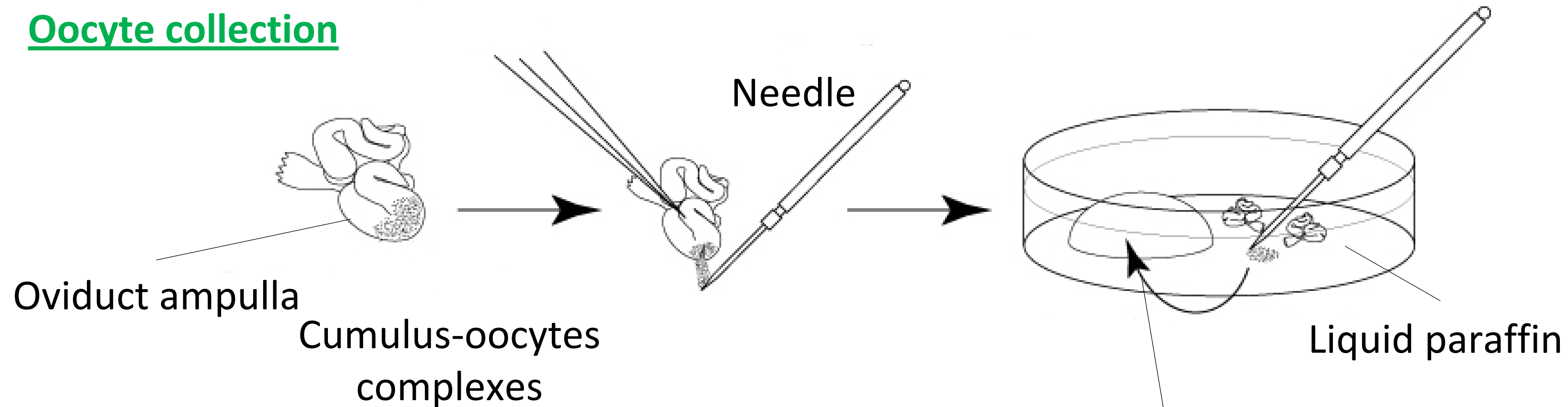


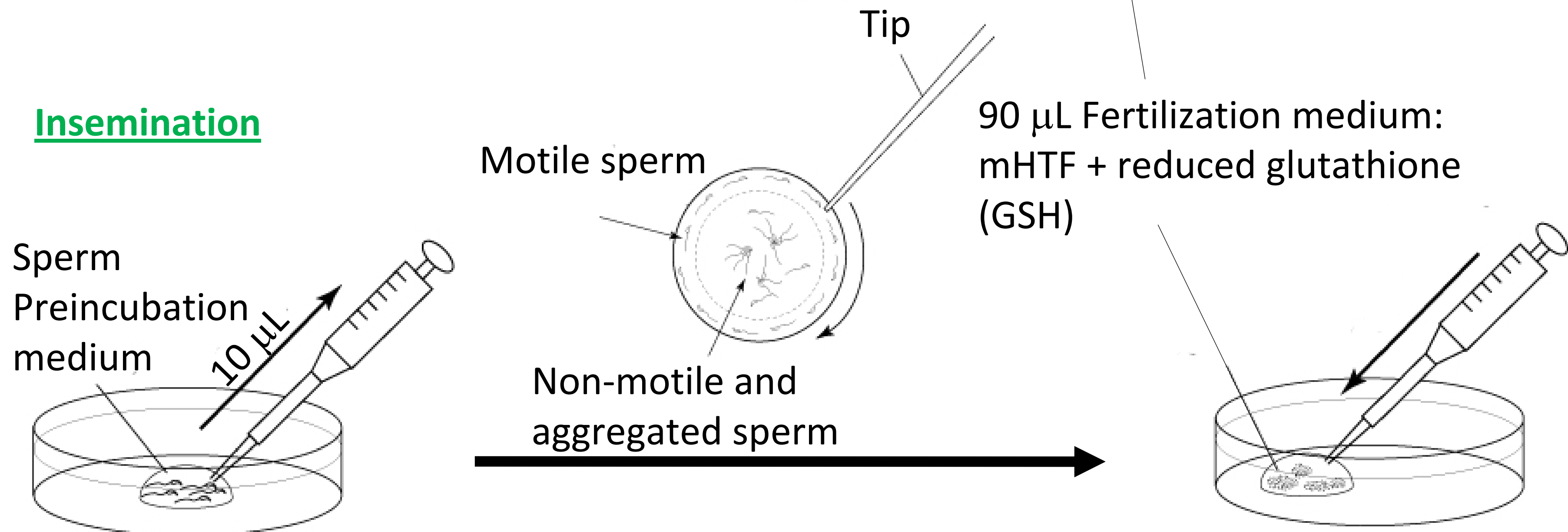
Cholesterol reduction in sperm membrane



Takeo et al, Biol Reprod 2008.

In vitro fertilization





CARD online manual: http://card.medic.kumamoto-u.ac.jp/card/english/sigen/index.html

Fertilization medium: mHTF with reduced glutathione (GSH)

Component	mg/100 mL
NaCl	593.8
KCl	35.0
Glucose	50.0
$CaCl_2$	57.0
$MgSO_4/7H_2O$	4.9
KH_2PO_4	5.4
$NaHCO_3$	210.0
Sodium pyruvate	3.7
Sodium lactate (μL)	340.0
Penicillin G potassium salt	7.5
Streptomycin sulfate	5.0
Bovine serum albumin	400.0
Reduced glutathione	30.7

Antioxidants improve the fertility of frozen-thawed sperm

OPEN ACCESS Freely available online — PLoS one

Conserving, Distributing and Managing Genetically Modified Mouse Lines by Sperm Cryopreservation

G. Charles Ostermeier, Michael V. Wiles, Jane S. Farley, Robert A. Taft

Ostermeier, PLoS ONE, 2008.

OPEN ACCESS Freely available online — PLoS one

Inhibition of In Vitro Fertilizing Capacity of Cryopreserved Mouse Sperm by Factors Released by Damaged Sperm, and Stimulation by Glutathione

Mary L. Bath

Bath M, PLoS One, 2010.

Oxidative stress is related to the reduction in fertility

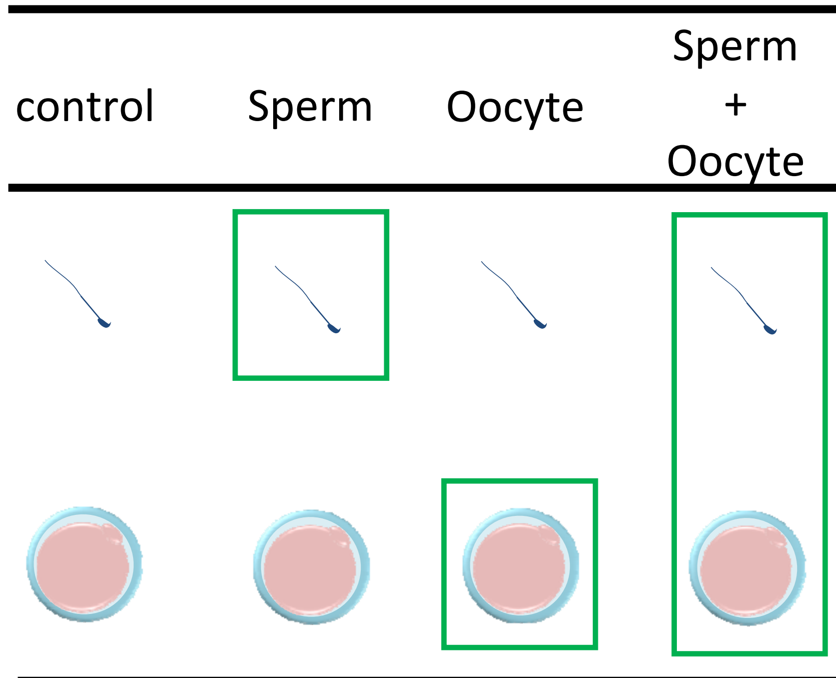
Fertilization is facilitated by the treatment of GSH to oocytes

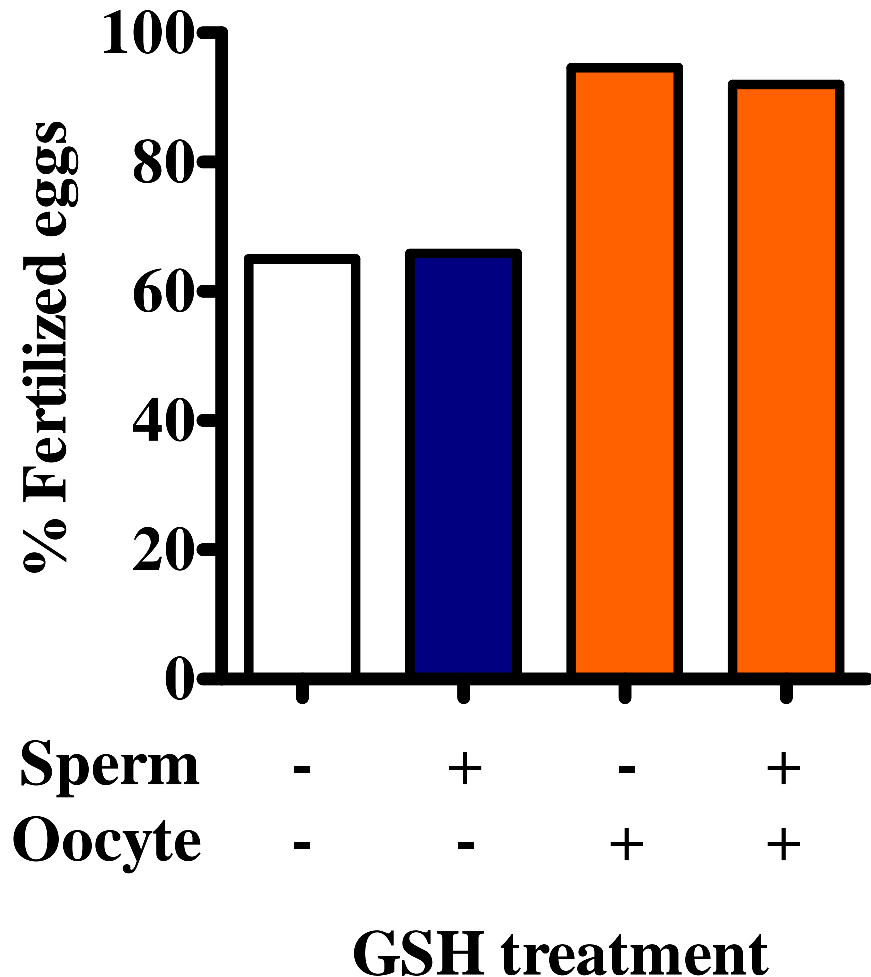
Medium

Sperm preincubation:
TYH + MBCD or TYH + MBCD + **GSH**

Fertilization:
mHTF or mHTF +**GSH**

GSH treatment



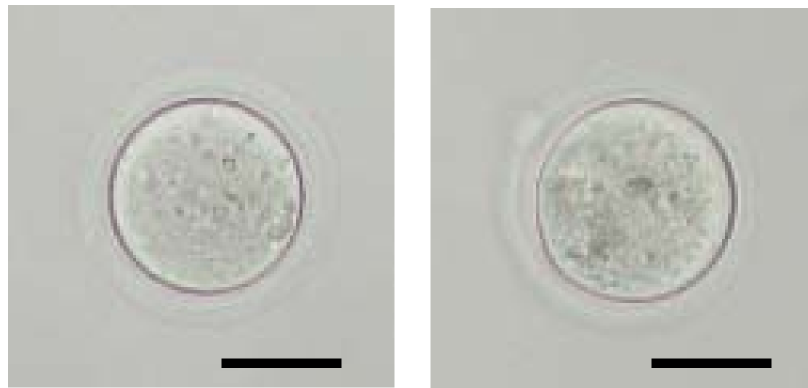


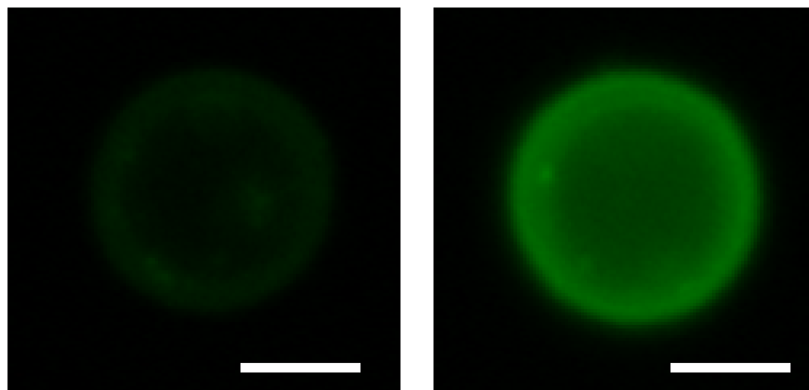
Takeo and Nakagata, Biol Reprod 2011.

GSH dissects disulfide bonds in ZP proteins and promotes the expansion of ZP

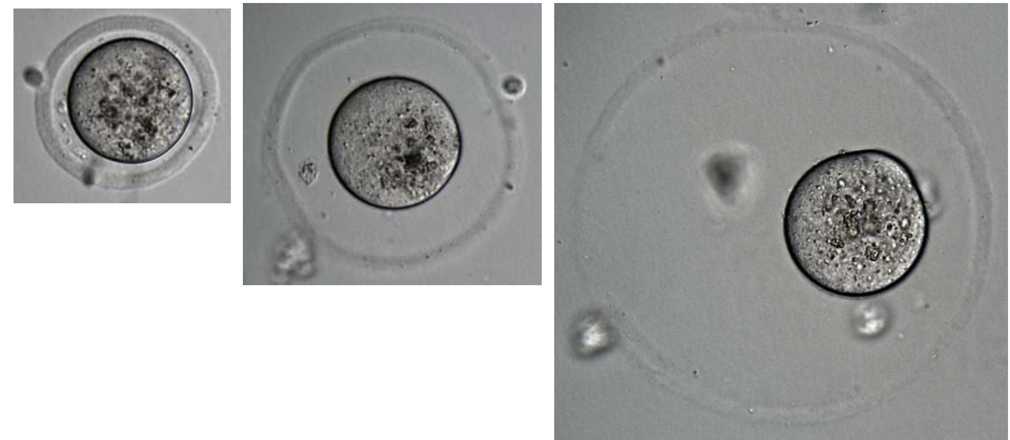
Visualization of -SH

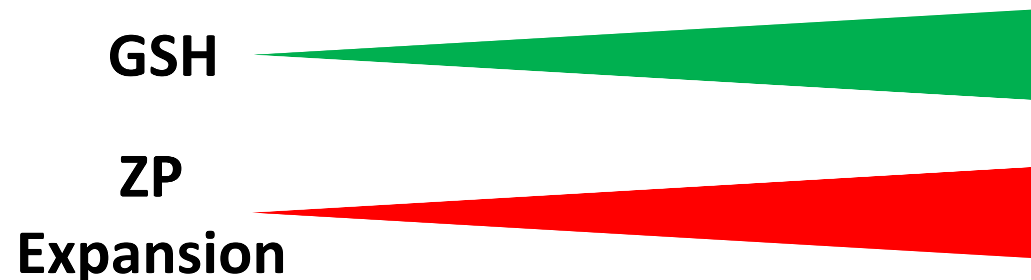
ZP expansion





GSH (-) **GSH (+)**

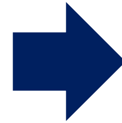




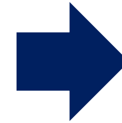
Takeo and Nakagata, Biol Reprod 2011.

CARD protocol:
Sperm cryopreservation and IVF

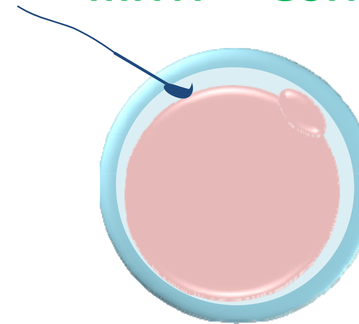
Cryopreservation:
R18S3 + L-glutamine

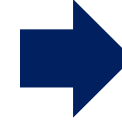


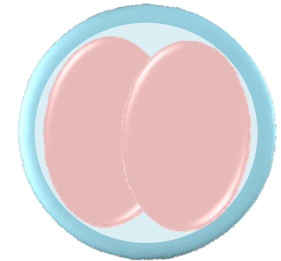
Preincubation：
TYH + MBCD

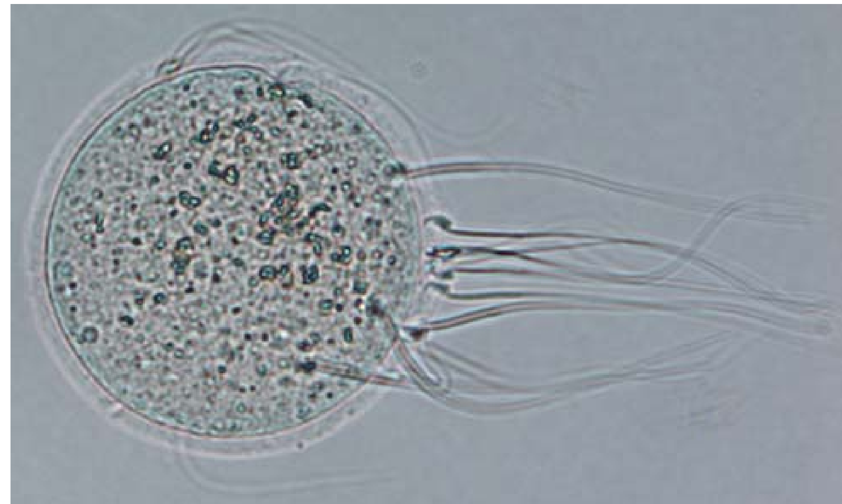


Fertilization：
mHTF + GSH









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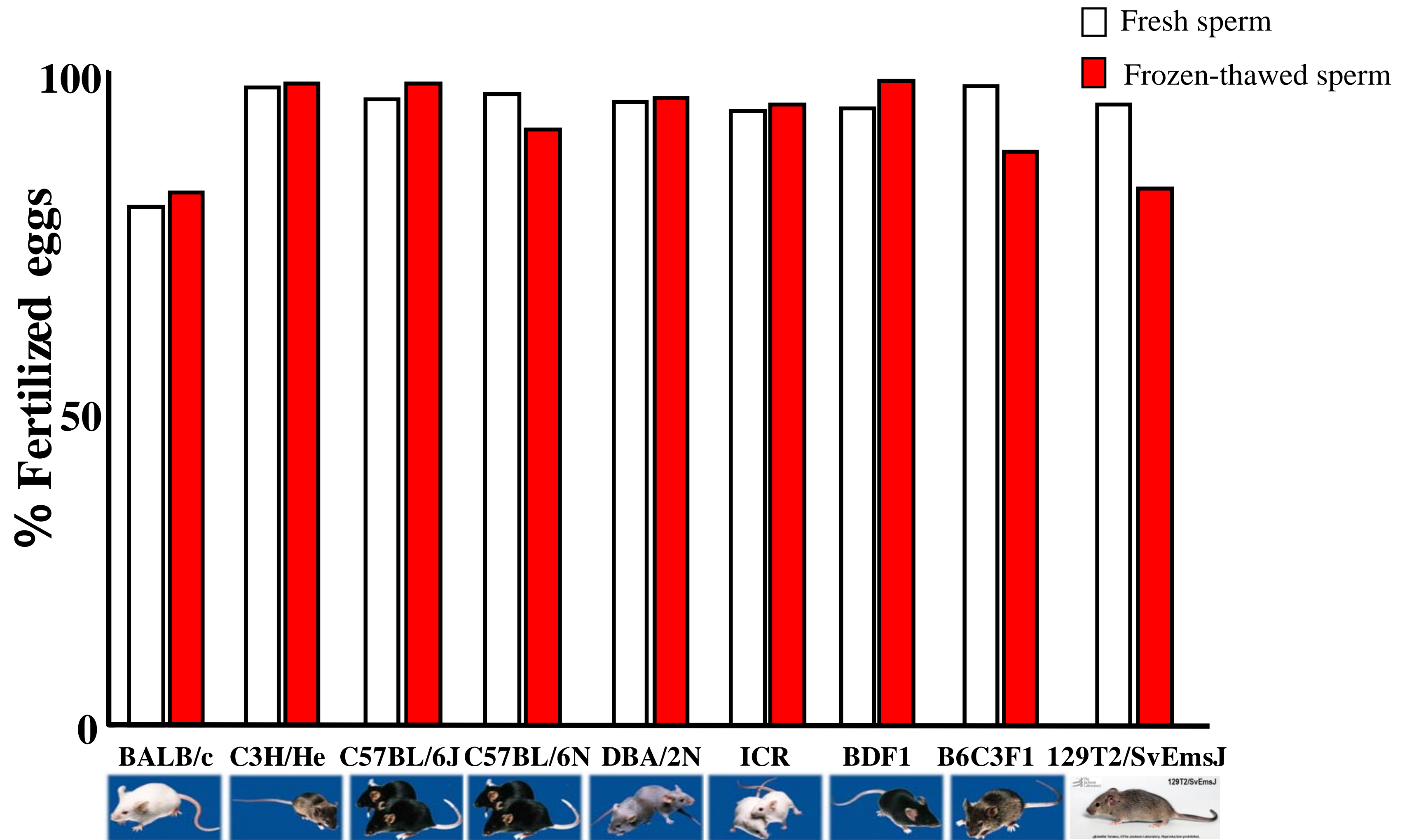
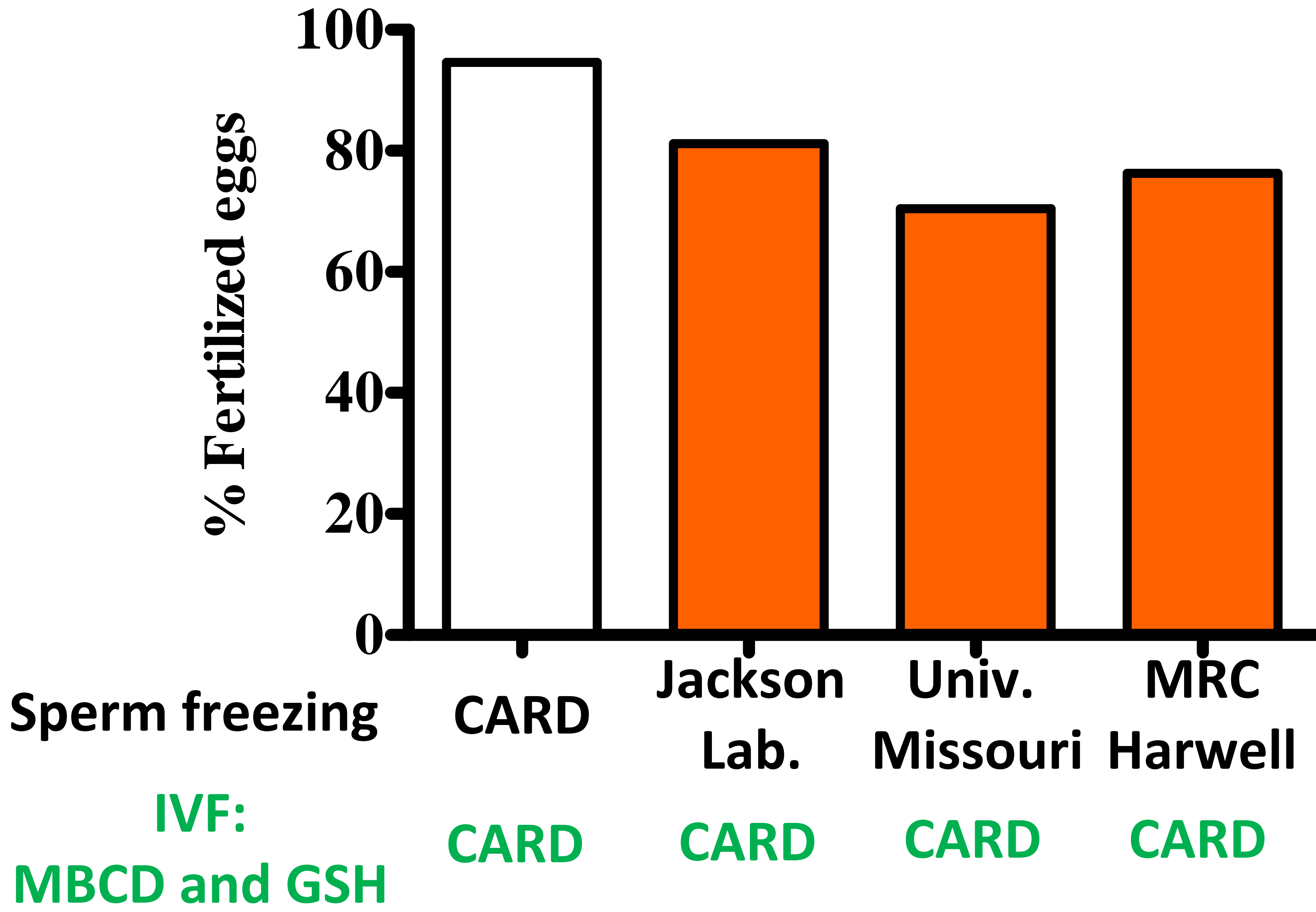
Cryopreserved sperm prepared by various protocols

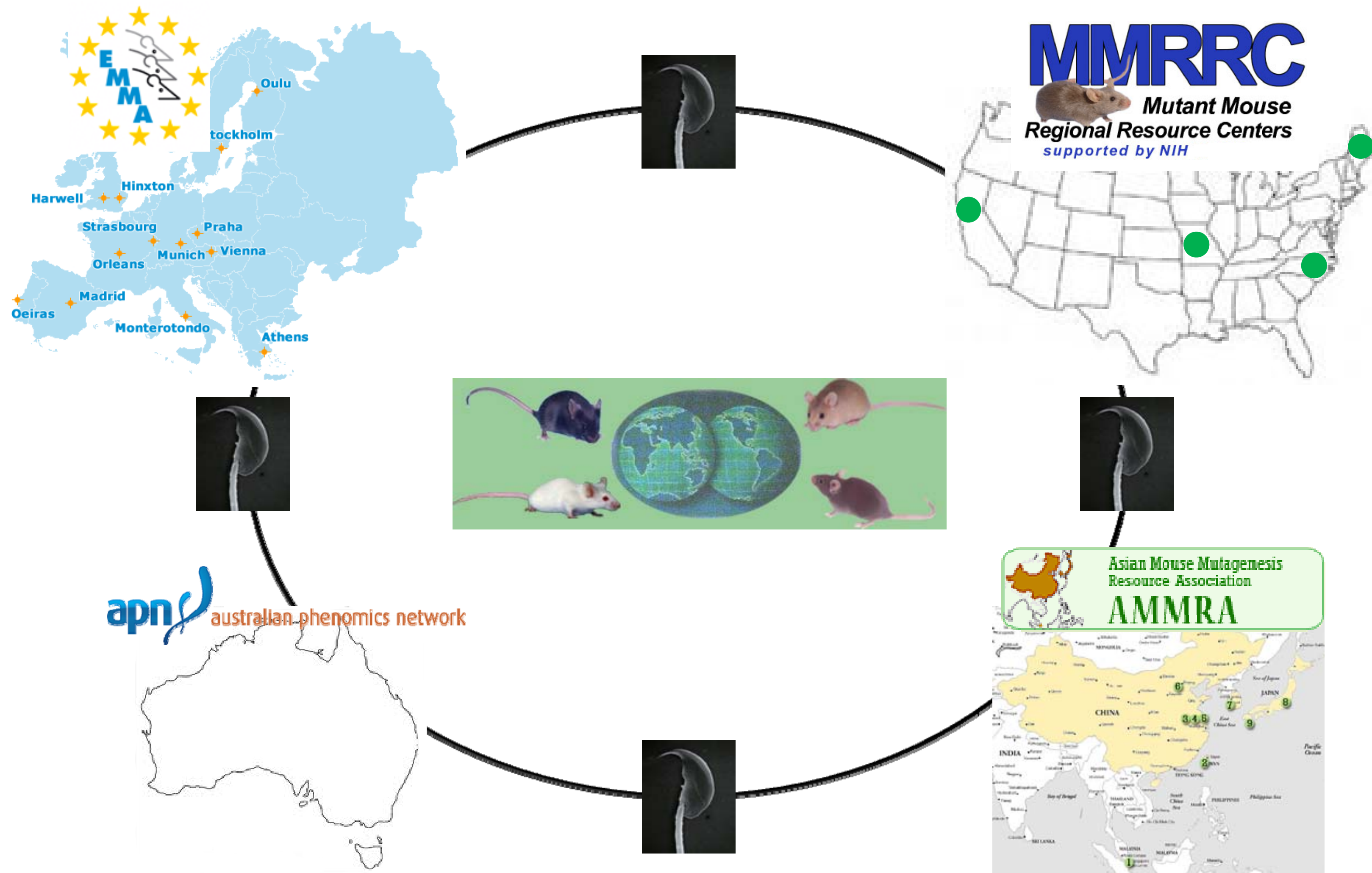
Our protocol overcomes the strain-dependency of recovery rate in frozen-thawed sperm



Our IVF protocol is applicable to cryopreserved sperm produced by different protocols



We can use sperm cryopreservation for archiving and distributing the samples in mouse bank community.



Acknowledgements

European Mouse Mutant Archive: EMMA

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Prof. Irie

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ありがとう