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# Sperm freezing

## -----MBCD and GSH approach-----

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Center for  
Animal  
Resources and  
Development

# Topics

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## **1. Introduction**

## **2. Technology Development:**

Sperm cryopreservation

Sperm preincubation

In vitro fertilization

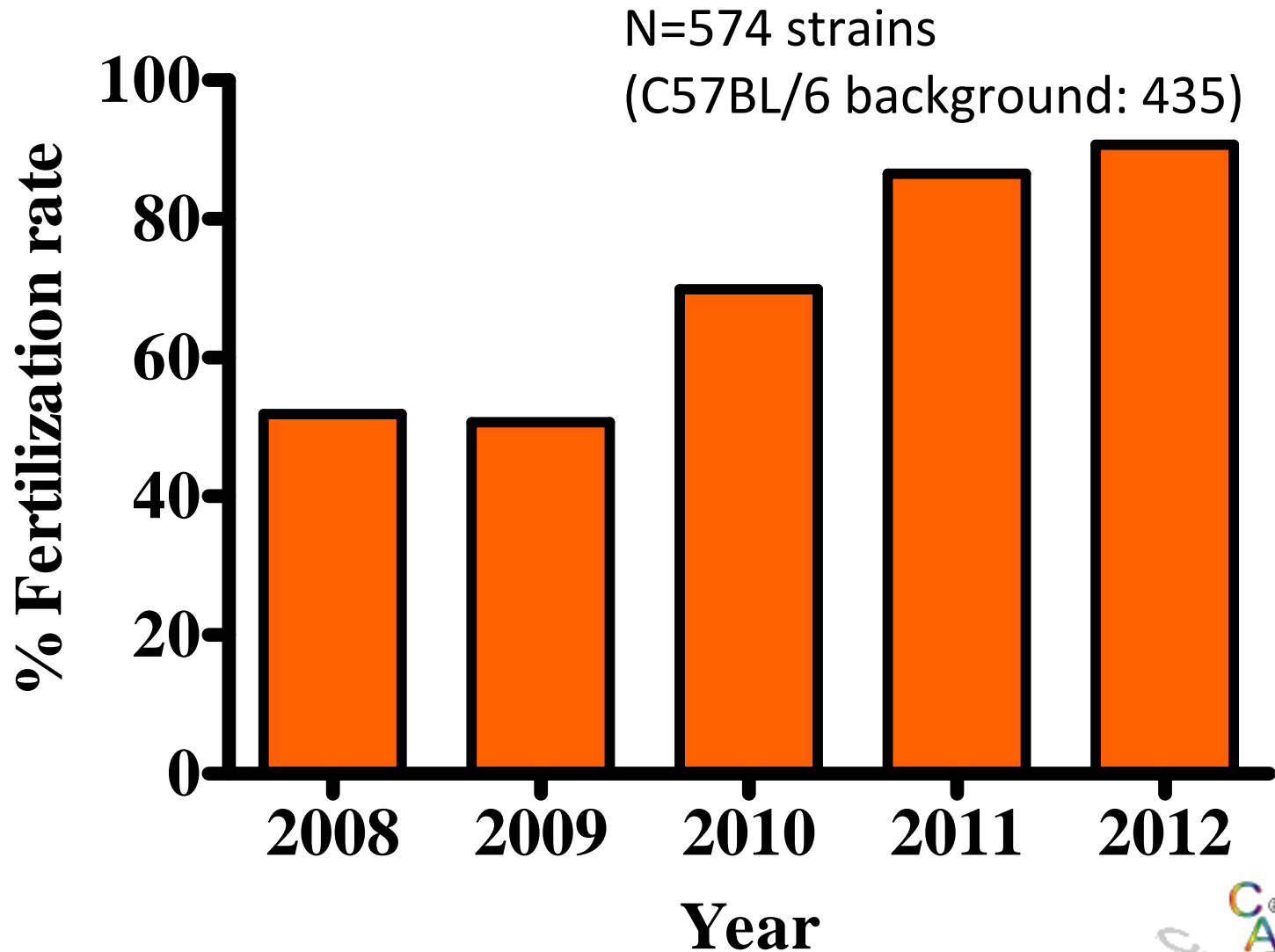
## **3. Application of our technologies**

Inbred and hybrid strains

Cryopreserved sperm prepared by various protocols

# CARD Protocol: Sperm cryopreservation and IVF

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# Technology Development



Prof. Nakagata

Mammalian Genome 11, 572–576 (2010).  
DOI: 10.1007/s10033-010-1010-9

## Cryopreservation of mouse spermatozoa

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Received: 16 December 1999 / Accepted: 17 December 1999

**Abstract.** Recently, it has become easier to produce transgenic mice by using embryonic stem (ES) cells. However, the number of ES cells is limited, and the production of ES cells is a laborious and expensive process. In this study, we describe a method for the cryopreservation of mouse spermatozoa to maintain genetic diversity. This chapter also describes a detailed procedure for the cryopreservation of mouse spermatozoa.

### Introduction

Recently, a large number of transgenic mice have been produced by using ES cells (Iwano and Hatanaka 1988; Bedell et al. 1990; Herzig et al. 1991; Herzig et al. 1992). However, the number of ES cells is limited, and the production of ES cells is a laborious and expensive process. In this study, we describe a method for the cryopreservation of mouse spermatozoa to maintain genetic diversity. This chapter also describes a detailed procedure for the cryopreservation of mouse spermatozoa.

Since that time, numerous mouse sperm cryopreservation methods have been reported (Mokretan 1981; Inoue et al. 1997; So 1998). Despite the many different methods, the *in vitro* fertilization (IVF) and *in vivo* fertilization (IVF) rates are low. Moreover, these rates are not high enough to be used as a practical alternative to the

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## Nakagata Method

## Methyl- $\beta$ -Cyclodextrin (MBCD): TYH with MBCD

## Modified R18S3 (mR18S3): R18S3+L-glutamine

## Reduced glutathione (GSH): mHTF + GSH

### Original Article

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

T Takeko and N Nakagata

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

### Abstract

Recently, a vast number of genetically engineered mice have been produced. However, the number of ES cells is limited, and the production of ES cells is a laborious and expensive process. In this study, we describe a method for the cryopreservation of mouse spermatozoa to maintain genetic diversity. This chapter also describes a detailed procedure for the cryopreservation of mouse spermatozoa.

**Keywords:** Mouse sperm cryopreservation, Laboratory Animals 2010; 44: 132–137. DOI: 10.1007/s10033-010-1010-9

Sperm cryopreservation is a useful tool for maintaining genetic diversity. However, the number of ES cells is limited, and the production of ES cells is a laborious and expensive process. In this study, we describe a method for the cryopreservation of mouse spermatozoa to maintain genetic diversity. This chapter also describes a detailed procedure for the cryopreservation of mouse spermatozoa.

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Published online before print: 20 July 2011.  
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## Reduced Glutathione Enhances Fertility of Frozen/Thawed C57BL/6 Mouse Sperm after Exposure to Methyl- $\beta$ -Cyclodextrin<sup>1</sup>

Toru Takeko and Naomi Nakagata<sup>2</sup>

Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan

### ABSTRACT

Sperm cryopreservation is useful for the effective storage of genetic resources derived from genetically engineered mice. However, freezing the sperm of C57BL/6 mice, the most commonly used genetic background for genetically engineered mice, considerably reduces its fertility. We previously reported that methyl- $\beta$ -cyclodextrin (MBCD) dramatically improved the fertility of frozen/thawed C57BL/6 mouse sperm. Recently, it was reported that exposing sperm to reduced glutathione may alleviate oxidative stress in frozen/thawed mouse sperm, thereby enhancing *in vitro* fertilization (IVF); however, the mechanism underlying this effect is poorly understood. In the present study, we examined the combined effects of methyl- $\beta$ -cyclodextrin and reduced glutathione on the fertilization rate of IVF with frozen/thawed C57BL/6 mouse sperm and the characteristic changes in the zona pellucida induced by reduced glutathione. Adding reduced glutathione to the fertilization medium increased the fertilization rate. Methyl- $\beta$ -cyclodextrin and reduced glutathione independently increased fertilization rates, and their combination produced the synergistic effect. We found that reduced glutathione increased the amount of free thiol in the zona pellucida and promoted zona penetration. Finally, 2-oxoethyl cysteine produced by IVF with the addition of reduced glutathione developed normally and produced live offspring. In summary, we have established a novel IVF method using methyl- $\beta$ -cyclodextrin during sperm preincubation and reduced glutathione during the IVF procedure to enhance fertility of frozen/thawed C57BL/6 mouse sperm. **Keywords:** sperm cryopreservation, fertilization, *in vitro* fertilization (IVF), zona pellucida

### INTRODUCTION

The cryopreservation of mouse sperm is a simple and useful method to address a vast number of genetically engineered mice [1]. Sperm cryopreservation saves space, reduces costs related to the upkeep of each mouse lineage, minimizes genetic drift, and facilitates the transport of mice between research facilities [2–4]. Therefore, sperm cryopreservation has been

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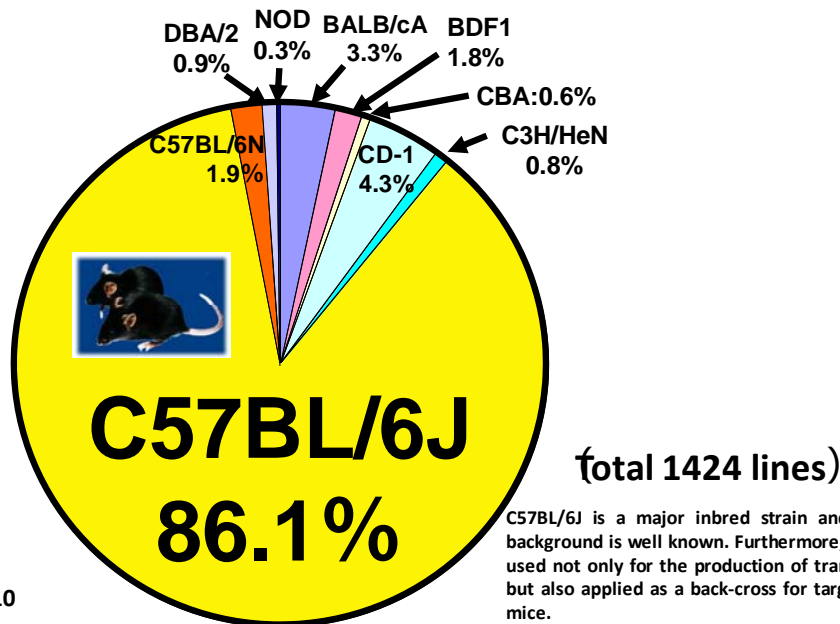
In addition, we discovered that preincubation with methyl- $\beta$ -cyclodextrin (MBCD) markedly increases the *in vitro* fertility of frozen/thawed sperm [19]. MBCD is a substituted cyclic heptasaccharide consisting of  $\alpha$ -(1,4)-glucopyranose units with a hydrophilic outer surface and a central hydrophobic cavity [20]. Its unique structure gives MBCD the ability to form inclusion complexes with many lipophilic agents. During sperm preincubation, MBCD penetrates cholesterol efflux from the plasma membranes of sperm, inducing capacitation [21, 22]. Cholesterol efflux is much faster and more efficient using MBCD than efflux using bovine serum albumin, which accounts for the effectiveness of MBCD at increasing sperm fertility [19].

Recently, the addition of reduced glutathione (GSH) to a fertilization medium was reported to increase the fertility of frozen/thawed sperm of various strains of mice [23]. GSH is a biological antioxidant that protects mammalian sperm against the loss of DNA integrity and motility through oxidative stress [24, 25]. However, the mechanism by which GSH improves

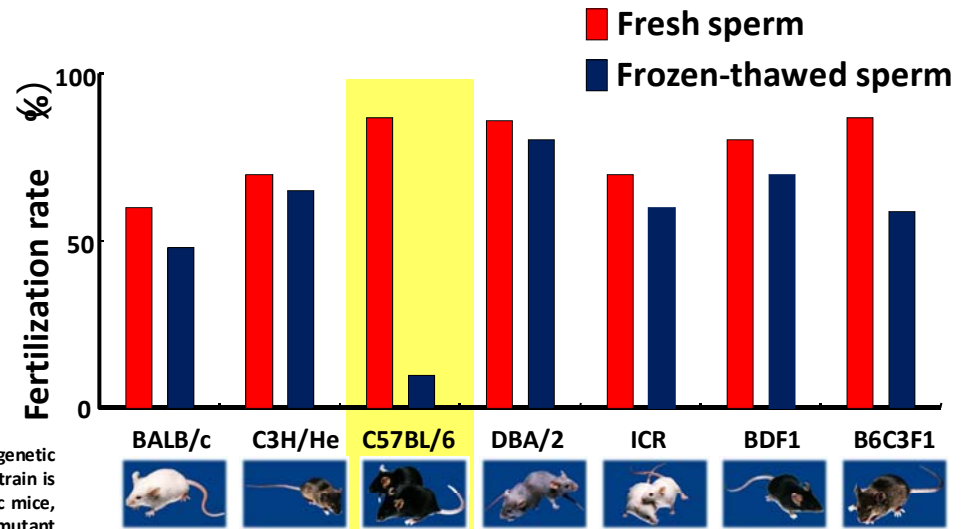
Mammal Genome 2000.  
Biol Reprod 2008.  
Lab Animal 2010.  
Biol Reprod 2011.



# C57BL/6 Mouse



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.



2010

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.

# Topics

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## 1. Introduction

## 2. Technology Development:

Sperm cryopreservation

Sperm preincubation

In vitro fertilization

## 3. Application of our technologies

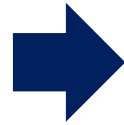
Inbred and hybrid strains

Cryopreserved sperm prepared by various protocols

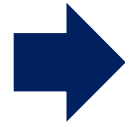
# 1. Technology Development

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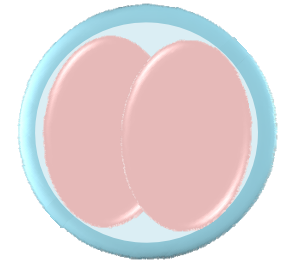
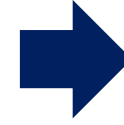
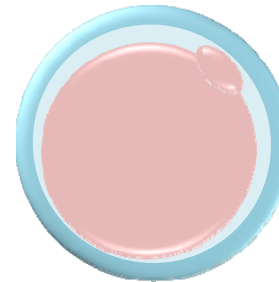
**Cryopreservation:**



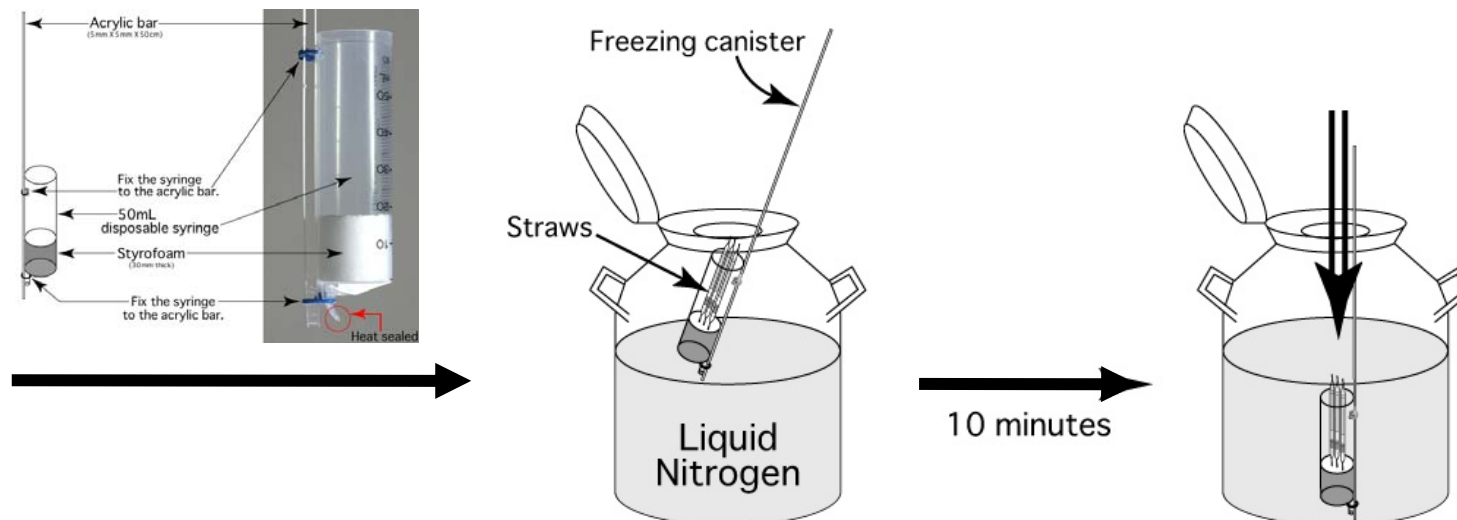
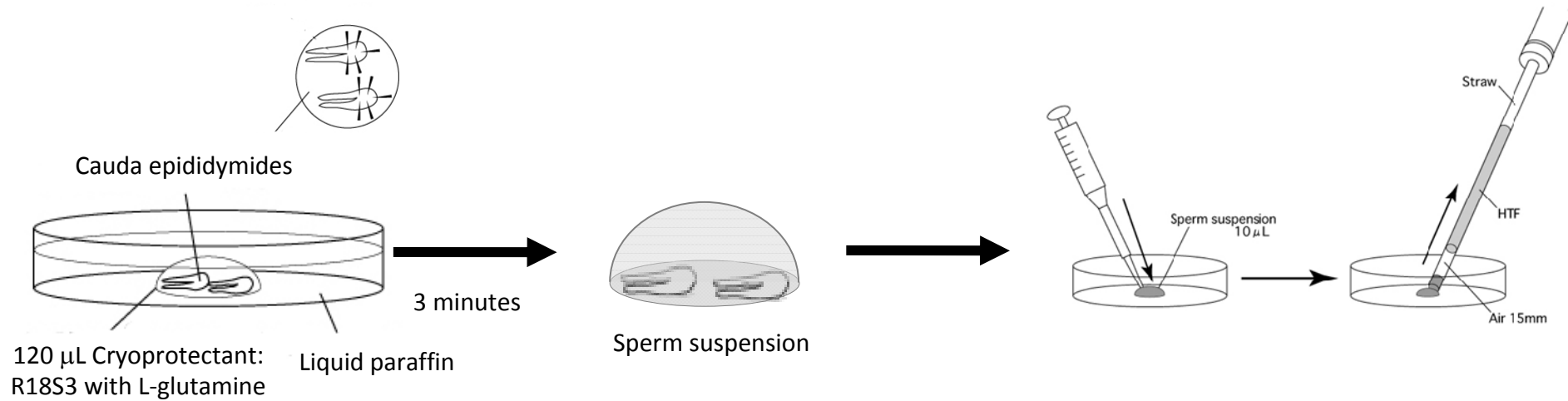
**Preincubation :**



**Fertilization :**

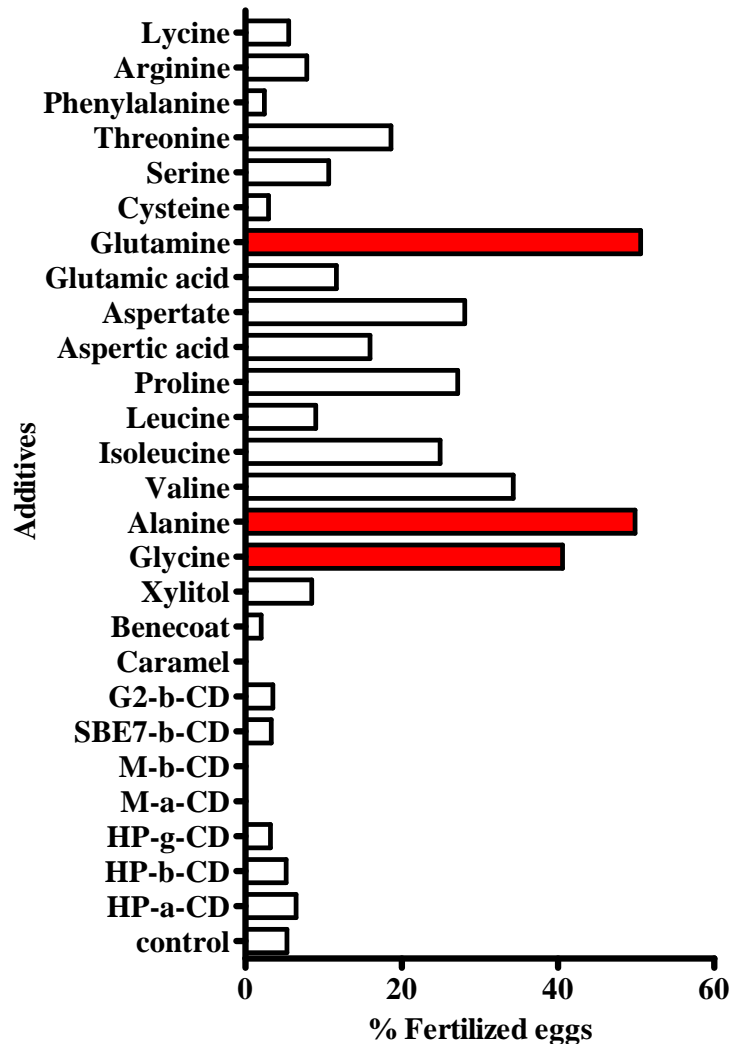


# Sperm Cryopreservation





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



**Screening**

Sugars, cyclodextrins, amino acids  
in R18S3



**Amino acids**

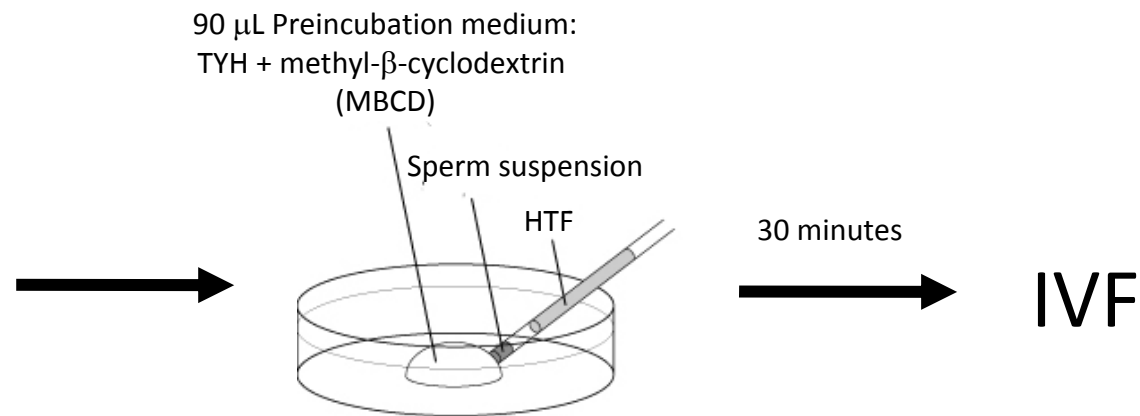
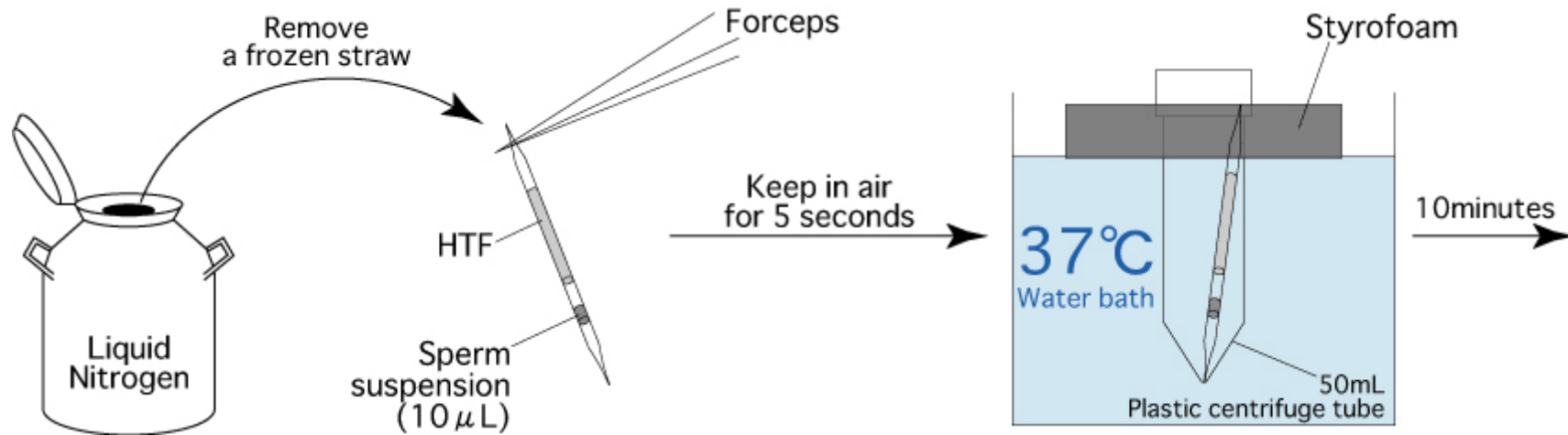
glycine, alanine, glutamine



mR18S3:

R18S3 + L-glutamine

# Sperm preincubation



# Sperm preincubation medium: TYH with methyl- $\beta$ -cyclodextrin (MBCD)



Prof. Toyoda :  
Reproductive Biology

**TYH:**  
**T**oyoda  
**Y**okoyama  
**H**oshi

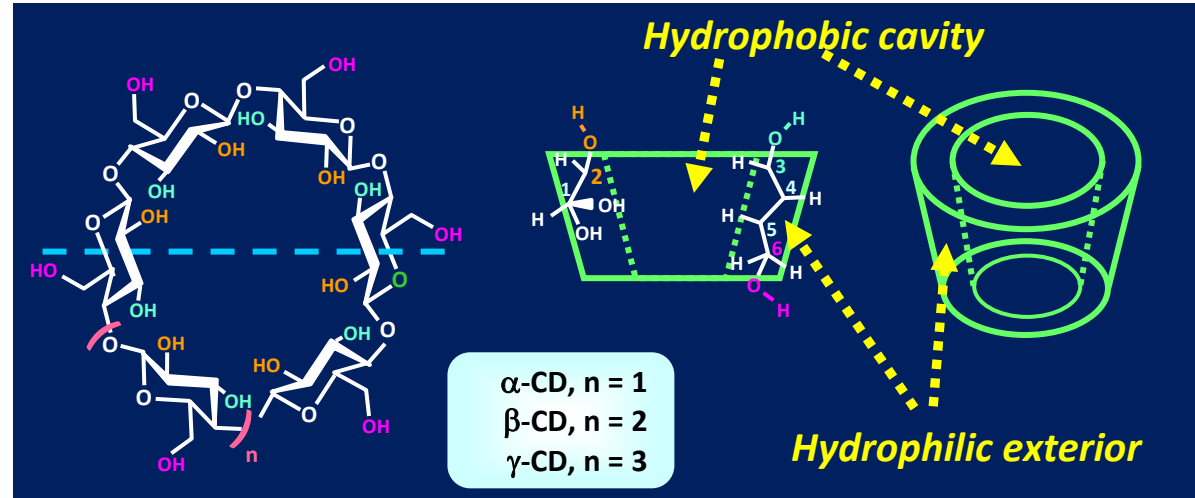
Toyoda Y et al.  
1971: J. Anim.  
Reprod. 16:  
147-151.

Component	mg/100 mL
NaCl	697.6
KCl	35.6
Glucose	100
Sodium pyruvate	5.5
CaCl <sub>2</sub> /2H <sub>2</sub> O	25.1
MgSO <sub>4</sub> /7H <sub>2</sub> O	29.3
KH <sub>2</sub> PO <sub>4</sub>	16.2
NaHCO <sub>3</sub>	210.6
Potassium penicillin G	7.5
Streptomycin sulfate	5.0
Polyvinylalcohol	100



Prof. Irie:

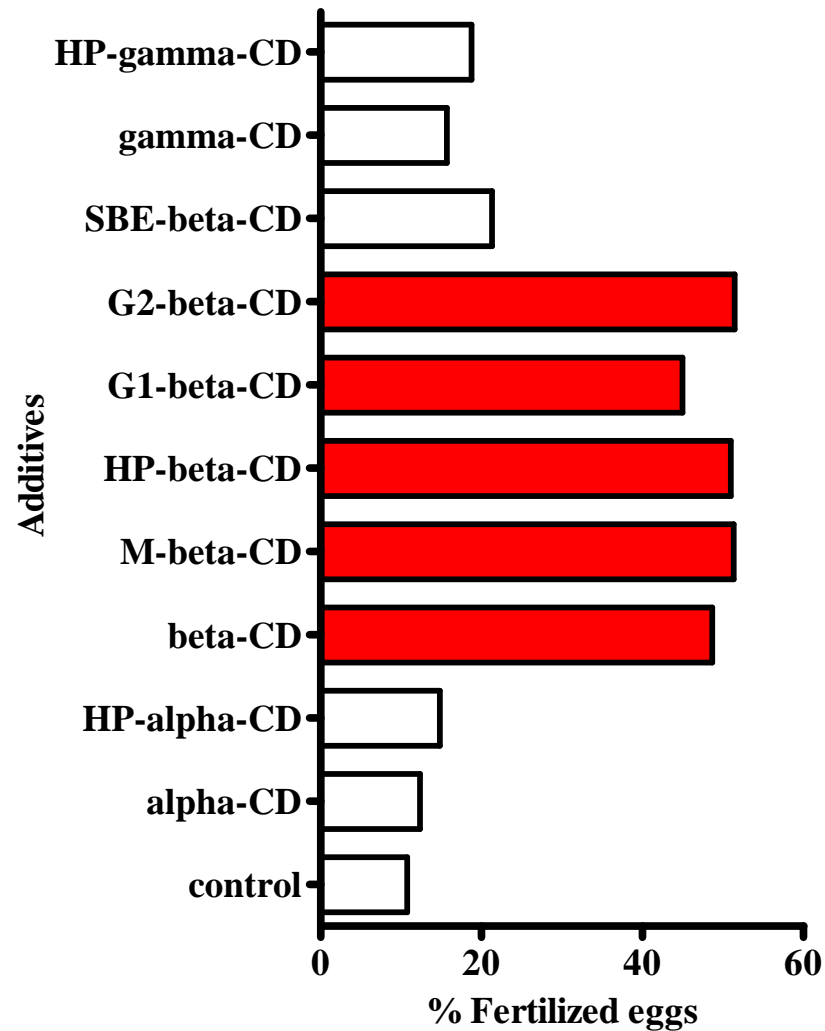
# Cyclodextrins



Compound	n	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	D.S. <sup>a)</sup>	M.W. <sup>b)</sup>	Solubility (g/dL) <sup>c)</sup>	Hemolytic activity (mM) <sup>d)</sup>
$\alpha$ -CyD	6	-H	-H	-H	-	972	14.5	14
$\beta$ -CyD	7	-H	-H	-H	-	1135	1.85	5.7
HP- $\alpha$ -CyD <sup>e)</sup>	6	-H or -CH <sub>2</sub> CH(CH <sub>3</sub> )OH			4.1	1210	> 50	> 50
HP- $\beta$ -CyD <sup>e)</sup>	7	-H or -CH <sub>2</sub> CH(CH <sub>3</sub> )OH			4.8	1413	> 50	14.5
DM- $\alpha$ -CyD <sup>f)</sup>	6	-CH <sub>3</sub>	-H	-CH <sub>3</sub>	12	1141	> 50	6.0
DM- $\beta$ -CyD <sup>f)</sup>	7	-CH <sub>3</sub>	-H	-CH <sub>3</sub>	14	1331	57	1.3
M- $\beta$ -CyD <sup>g)</sup>	7	-H or -CH <sub>3</sub>			12.6	1311	> 50	2.8

a) Average degree of substitution, b) Molecular weight, c) In water at 25°C,  
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- $\beta$ -CyD.

# Nature $\beta$ -CD and $\beta$ -CD derivatives enhance fertility of frozen-thawed sperm



Unpublished data

## Screening

Nature CDs, various CD derivatives in TYH



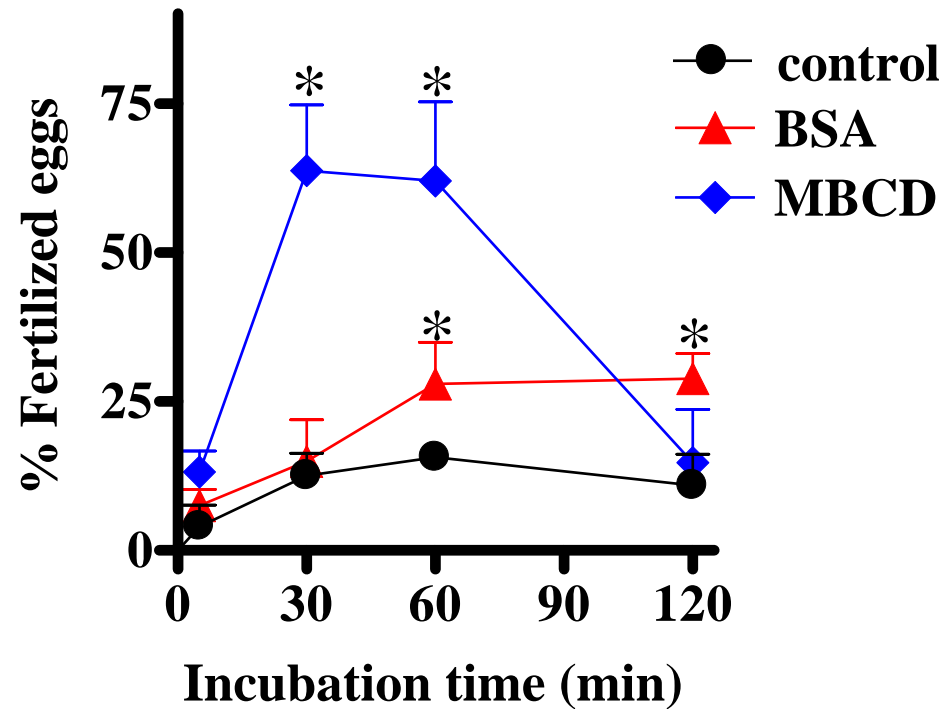
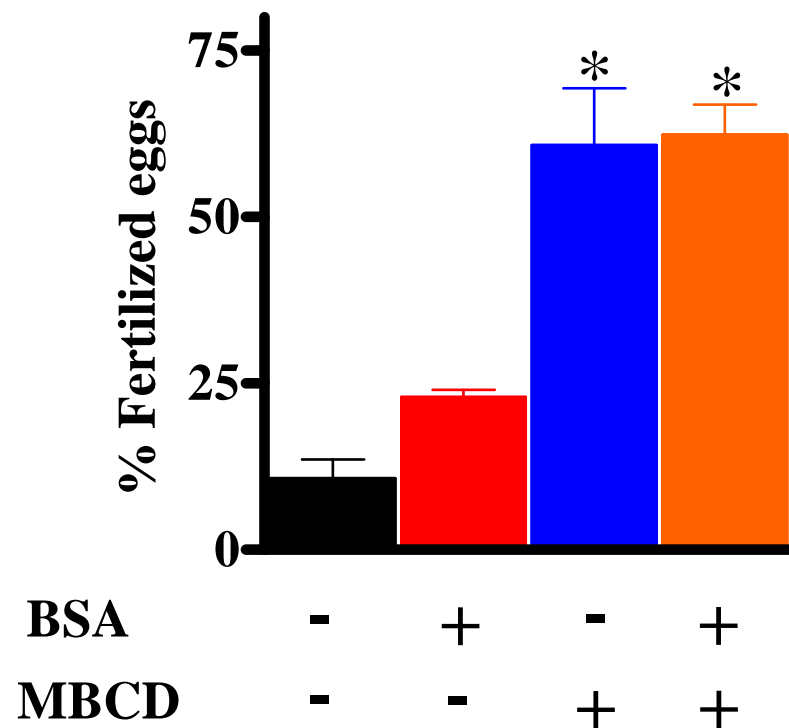
## $\beta$ -CDs

BCD, MBCD, HPBCD, G1BCD, G2BCD



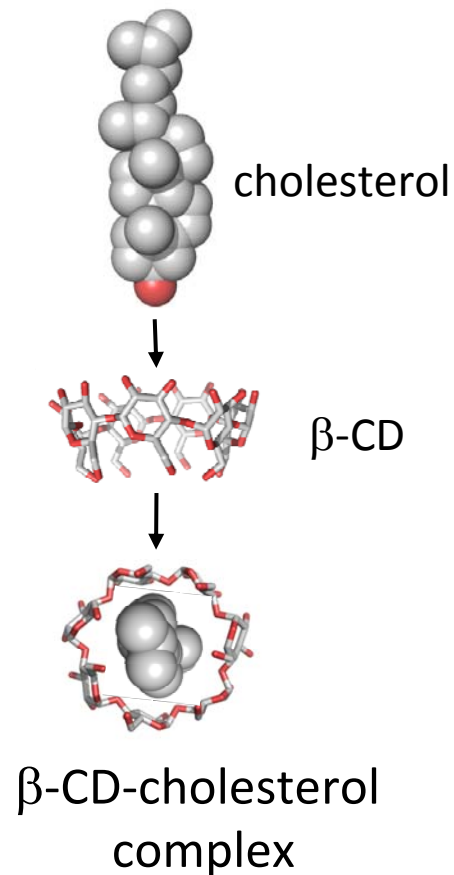
Sperm preincubation:  
TYH + MBCD

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

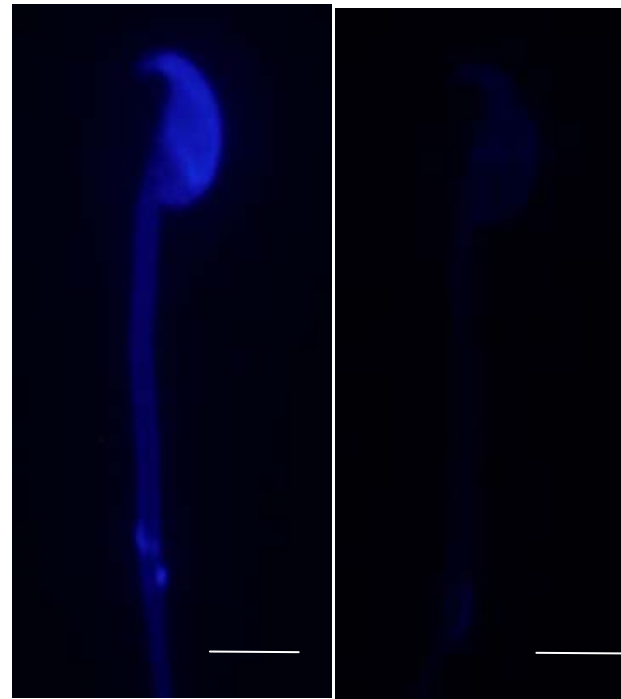


# MBCD facilitates cholesterol efflux from sperm membrane

CD-cholesterol complex



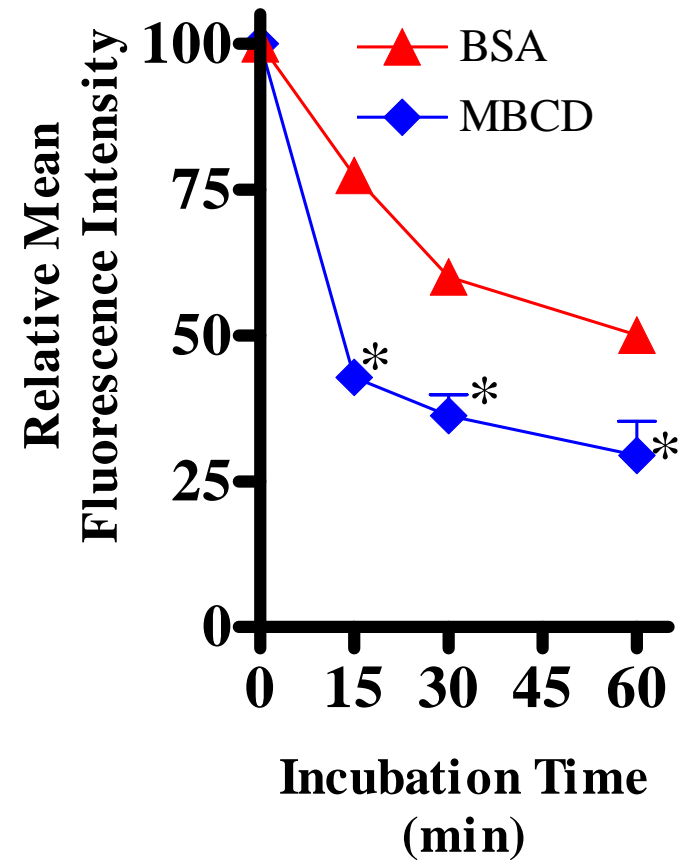
Membrane cholesterol



control

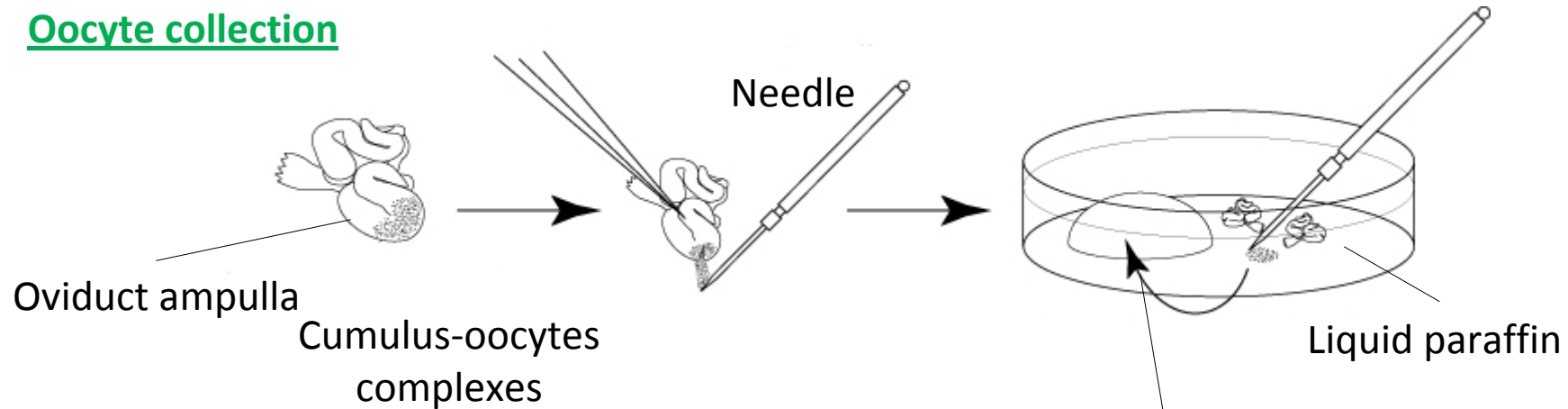
MBCD treated

Cholesterol reduction in sperm membrane

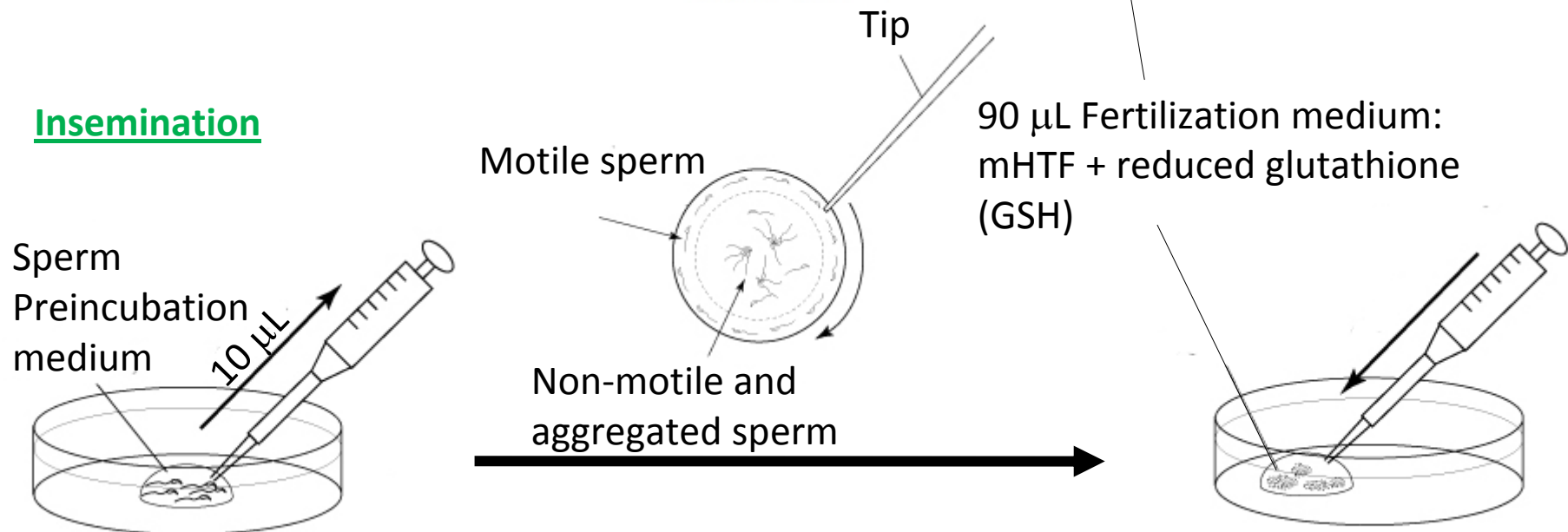


# *In vitro* fertilization

## Oocyte collection



## Insemination





# Fertilization medium: mHTF with reduced glutathione (GSH)

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Component	mg/100 mL
NaCl	593.8
KCl	35.0
Glucose	50.0
CaCl <sub>2</sub>	57.0
MgSO <sub>4</sub> /7H <sub>2</sub> O	4.9
KH <sub>2</sub> PO <sub>4</sub>	5.4
NaHCO <sub>3</sub>	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

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

G. Charles Ostermeier<sup>1,2\*</sup>, Michael V. Wiles<sup>1,2</sup>, Jane S. Farley<sup>2</sup>, Robert A. Taft<sup>2\*</sup>

**1** Technology Evaluation and Development, The Jackson Laboratory, Bar Harbor, Maine, United States of America, **2** Reproductive Sciences, The Jackson Laboratory, Bar Harbor, Maine, United States of America

### Abstract

**Background:** Sperm from C57BL/6 mice are difficult to cryopreserve and recover. Yet, the majority of genetically modified (GM) lines are maintained on this genetic background.

**Methodology/Principal Findings:** Reported here is the development of an easily implemented method that consistently yields fertilization rates of 70±5% with this strain. This six-fold increase is achieved by collecting sperm from the vas deferens and epididymis into a cryoprotective medium of 18% raffinose (w/v), 3% skim milk (w/v) and 477 μM monothiolglycerol. The sperm suspension is loaded into 0.25 mL French straws and cooled at 37±1°C/min before being plunged and then stored in LN<sub>2</sub>. Subsequent to storage, the sperm are warmed at 2,232±162°C/min and incubated in *in vitro* fertilization media for an hour prior to the addition of oocyte cumulus masses from superovulated females. Sperm from 735 GM mouse lines on 12 common genetic backgrounds including C57BL/6J, BALB/cJ, 129S1/SvEvM, FVB/NJ and NOD/Stat1 were cryopreserved and recovered. C57BL/6J and BALB/cJ fertilization rates, using frozen sperm, were slightly reduced compared to rates involving fresh sperm; fertilization rates using fresh or frozen sperm were equivalent in all other lines. Developmental capacity of embryos produced using cryopreserved sperm was equivalent, or superior to, cryopreserved IVF-derived embryos.

**Conclusions/Significance:** Combined, these results demonstrate the broad applicability of our approach as an economical and efficient option for archiving and distributing mice.

**Citation:** Ostermeier GC, Wiles MV, Farley JS, Taft RA (2008) Conserving, Distributing and Managing Genetically Modified Mouse Lines by Sperm Cryopreservation. PLoS ONE 3(7): e4292. doi:10.1371/journal.pone.0020792

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**Competing Interests:** The following authors G. Charles Ostermeier, Michael V. Wiles, Jane S. Farley, and Robert A. Taft are inventors on a patent application containing some subject matter that is described in this manuscript; the application is assigned to The Jackson Laboratory and managed by The Jackson Laboratory office of technology transfer.

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\* These authors contributed equally to this work.

### Introduction

Embryo cryopreservation is an effective strategy for managing mouse lines. Its adoption has been limited by the cost, time and the number of animals required. This is especially true for those lines where embryo yields are low, e.g. BALB/c. Cryopreserving sperm is an attractive alternative. However, its widespread use has been limited by the challenge of efficiently recovering cryopreserved sperm from some commonly used inbred strains [1]. In our experience (Table 1) and in that of others [2–5], the impaired fertility associated with cryopreserved mouse sperm is dependent on genetic background, with sperm from the C57BL/6 background being particularly sensitive. Yet, this strain is one of the most commonly used for creating and maintaining genetically modified (GM) lines. More than 75% of the 670 mouse lines submitted to The Jackson Laboratory's Repository from January 2004 to January 2005 were maintained on a predominantly C57BL/6J background. Further, The National Institutes of Health are using C57BL/6 embryonic stem (ES) cells to create a resource containing null mutations in every gene in the mouse genome [6].

Thus, it is critical that an effective and efficient method of cryopreserving and recovering C57BL/6 sperm be developed.

Since mouse sperm survive cryopreservation with reasonable success [2], the key to an efficient sperm cryopreservation and recovery scheme is maintaining post-thaw fertilization capacity. In mice, sperm develop the capacity to fertilize oocytes during transit through the female reproductive tract. As reviewed by Vicenzi et al. [7], sperm fertilization ability is associated with plasma membrane reorganization and increases in intracellular calcium levels and in Reactive Oxygen Species (ROS). Because cryopreservation modifies aspects of sperm function associated with fertilization capacity [7], perhaps these processes can be modulated to increase the fertility of cryopreserved mouse sperm. Thus, the objective of this work was to develop economical processes to cryopreserve C57BL/6 sperm that retain or enhance fertilizing capacity.

Because variable cooling and warming rates have been shown with some methods [10], our effort began by defining reproducible procedures for cryopreserving and thawing mouse sperm. Our methods were then refined to enhance the ability of cryopreserved

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

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Division of Molecular Genetics of Cancer, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

### Abstract

**Background:** In vitro fertilization (IVF) of eggs by frozen and thawed C57BL/6J mouse sperm is inhibited by dead sperm and enhanced by preincubation of the sperm in calcium-free medium. In other species, the presence of sperm killed by freezing and thawing has been associated with the generation of hydrogen peroxide.

**Methodology/Principal Findings:** The proportion of eggs fertilized by cryopreserved C57BL/6J mouse sperm was increased significantly by increasing the volume of fertilization medium in which sperm and eggs were coincubated. Enhanced fertilization occurred even though the concentration of potentially fertile sperm was decreased fivefold. This suggested that if a putative soluble factor was inhibiting fertilization, dilution of that factor, but not the sperm, should increase the fertilization rate. This was achieved by coincubation of the gametes in cell culture inserts (Transwell<sup>®</sup>) that during incubation were transferred progressively to wells containing fresh fertilization medium. Fertilization rates using inserts were high (66.6±2.4% versus 27.3±2.8% in wells alone). On the assumption that the soluble factor could be H<sub>2</sub>O<sub>2</sub>, reduced glutathione was added to the fertilization medium. This enhanced fertilization rate significantly (76.8±2.0% versus 21.2±1.9%), while addition of oxidized glutathione did not (52.7±2.5% with reduced glutathione; 44.5±8.8% with oxidized glutathione; 47.8±12.1% with no glutathione). Positive effects of reduced glutathione on IVF were also seen with frozen 129S1, FVB, and C3H sperm, and sperm from two lines of genetically modified C57BL/6J mice.

**Conclusions/Significance:** IVF in cell culture inserts and addition of glutathione to fertilization medium significantly increased the proportion of eggs fertilized by cryopreserved mouse sperm from four inbred strains, suggesting that reactive oxygen species generated during fertilization inhibit fertilization. The modified IVF techniques developed here enhance the feasibility and efficiency of using cryopreserved sperm from genetically modified lines of inbred mice.

**Citation:** Bath ML (2010) Inhibition of In Vitro Fertilizing Capacity of Cryopreserved Mouse Sperm by Factors Released by Damaged Sperm, and Stimulation by Glutathione. PLoS ONE 5(2): e9387. doi:10.1371/journal.pone.0020837

**Editor:** Suzanne M. Rutherford, Fred Hutchinson Cancer Research Center, United States of America

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### Introduction

The capacity of frozen and thawed mouse sperm to fertilize eggs *in vitro* appears to be inhibited by the presence of damaged sperm in the fertilization milieu [1]. Consequently, sperm suspension from strains prone to sperm damage after cryopreservation, such as C57BL/6J (>80% damaged sperm) fertilize relatively few eggs (<20%), while those from strains producing few damaged sperm, such as DBA/2 (<1% damaged sperm) fertilize a high proportion of eggs (>90%) [2]. Despite damage, a subpopulation of C57BL/6J sperm retains the potential to fertilize a high percentage of eggs. This potential is realized if sperm are incubated in calcium-free medium [1,3], in medium containing methyl-oxo-ethyl-oxalacetate (MBO) [4], or in medium containing a mix of MBO and poly reducing agents [5], before transfer of selected motile sperm to the fertilization milieu.

In the current study, instead of selecting motile sperm, the effect of reducing the concentration of molecules released into the

fertilization milieu during fertilization was investigated by incubating the sperm and eggs in cell culture inserts, without pre-incubation. Medium in the well below the inserts acted as a sink into which soluble factors could diffuse, to be diluted and removed from contact with sperm and eggs by subsequent transfer of inserts at intervals to wells containing fresh medium. This procedure resulted in high fertilization rates and suggested that a factor released into the fertilization milieu could be inhibiting fertilization.

Rotone sperm contain an aromatic amino oxidase that becomes active after sperm death [6], producing hydrogen peroxide, which reduces the lifespan of motile sperm, and which effect is eliminated by catalase, an antioxidant that converts hydrogen peroxide to water. Equine sperm damaged by 3 cycles of flash-freezing also generate increased amounts of H<sub>2</sub>O<sub>2</sub> compared to fresh sperm [7]. This suggested that mouse sperm damaged by freezing and thawing might release hydrogen peroxide into the fertilization milieu, inhibiting fertilization.

Ostermeier, PLoS ONE, 2008.

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

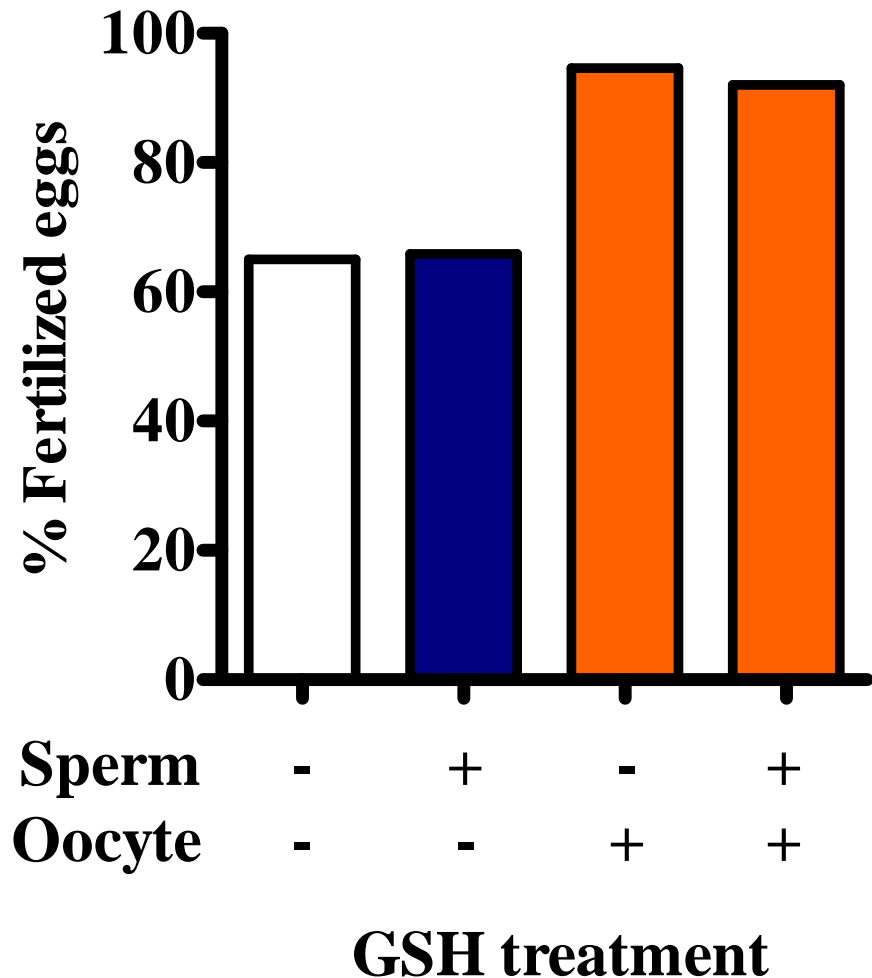
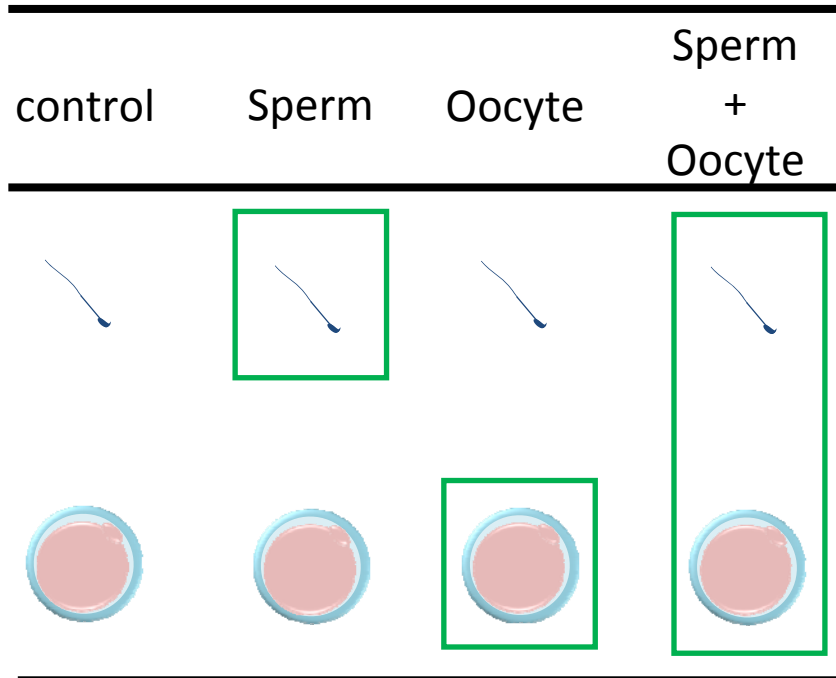
Sperm preincubation:

TYH + MBCD or TYH + MBCD + GSH

Fertilization:

mHTF or mHTF + GSH

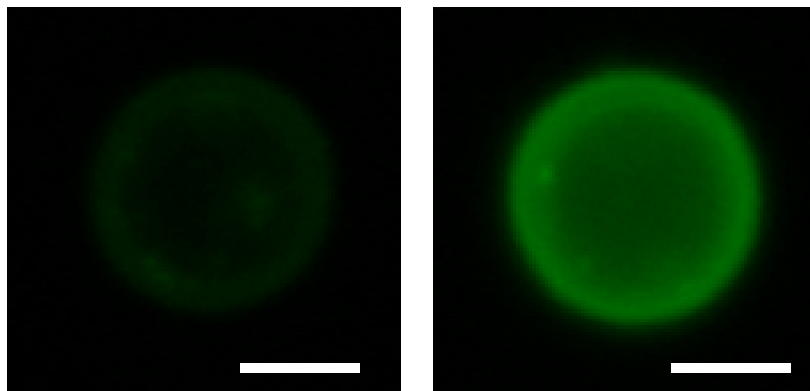
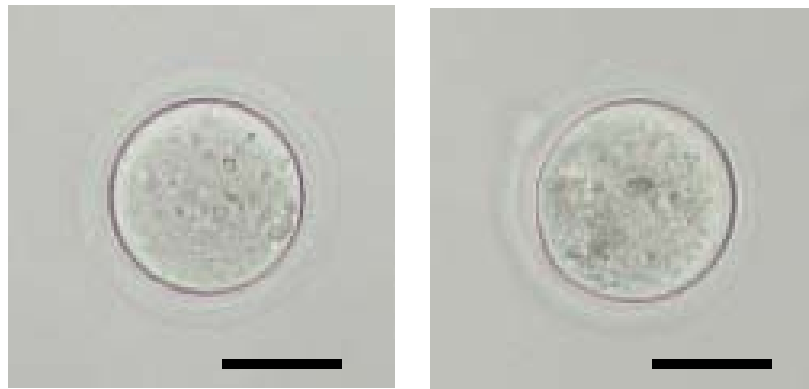
## GSH treatment



Takeo and Nakagata, Biol Reprod 2011.

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

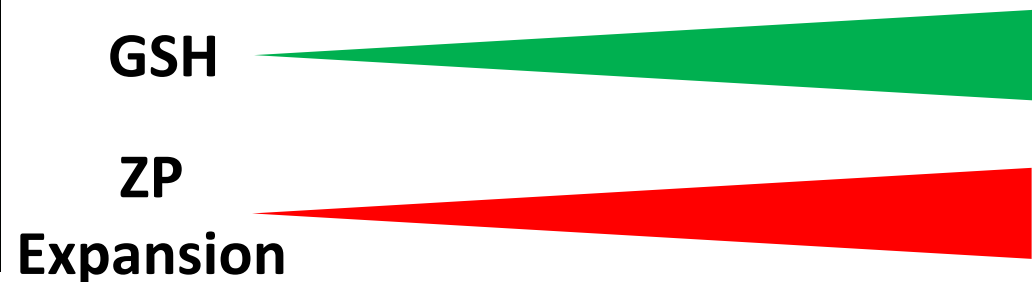
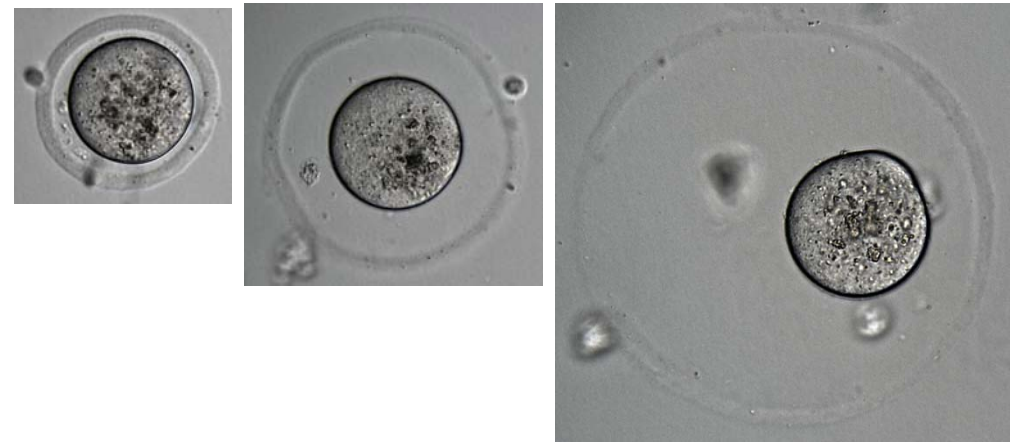
## Visualization of -SH



**GSH (-)**

**GSH (+)**

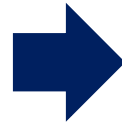
## ZP expansion



# CARD protocol: Sperm cryopreservation and IVF

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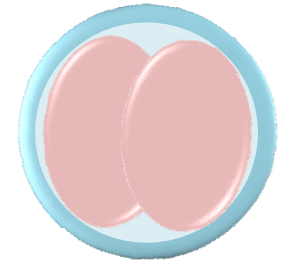
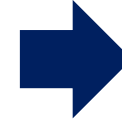
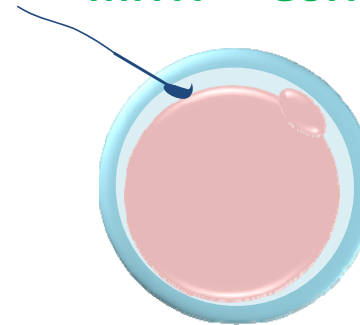
**Cryopreservation:**  
R18S3 + L-glutamine



**Preincubation :**  
TYH + MBCD



**Fertilization :**  
mHTF + GSH



# Topics

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## 1. Introduction

## 2. Technology Development:

Sperm cryopreservation

Sperm preincubation

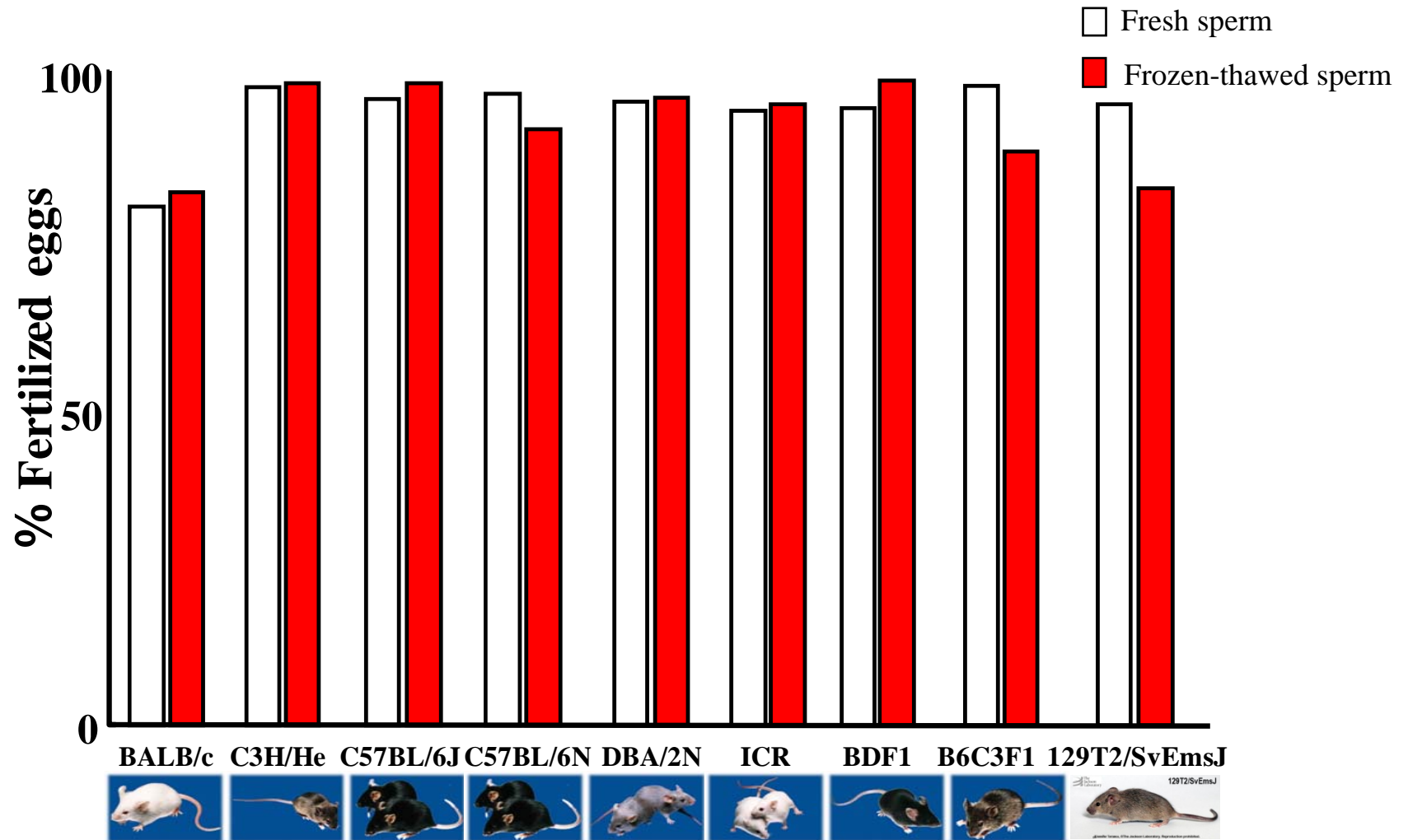
In vitro fertilization

## 3. Application of the technologies

Inbred and hybrid strains

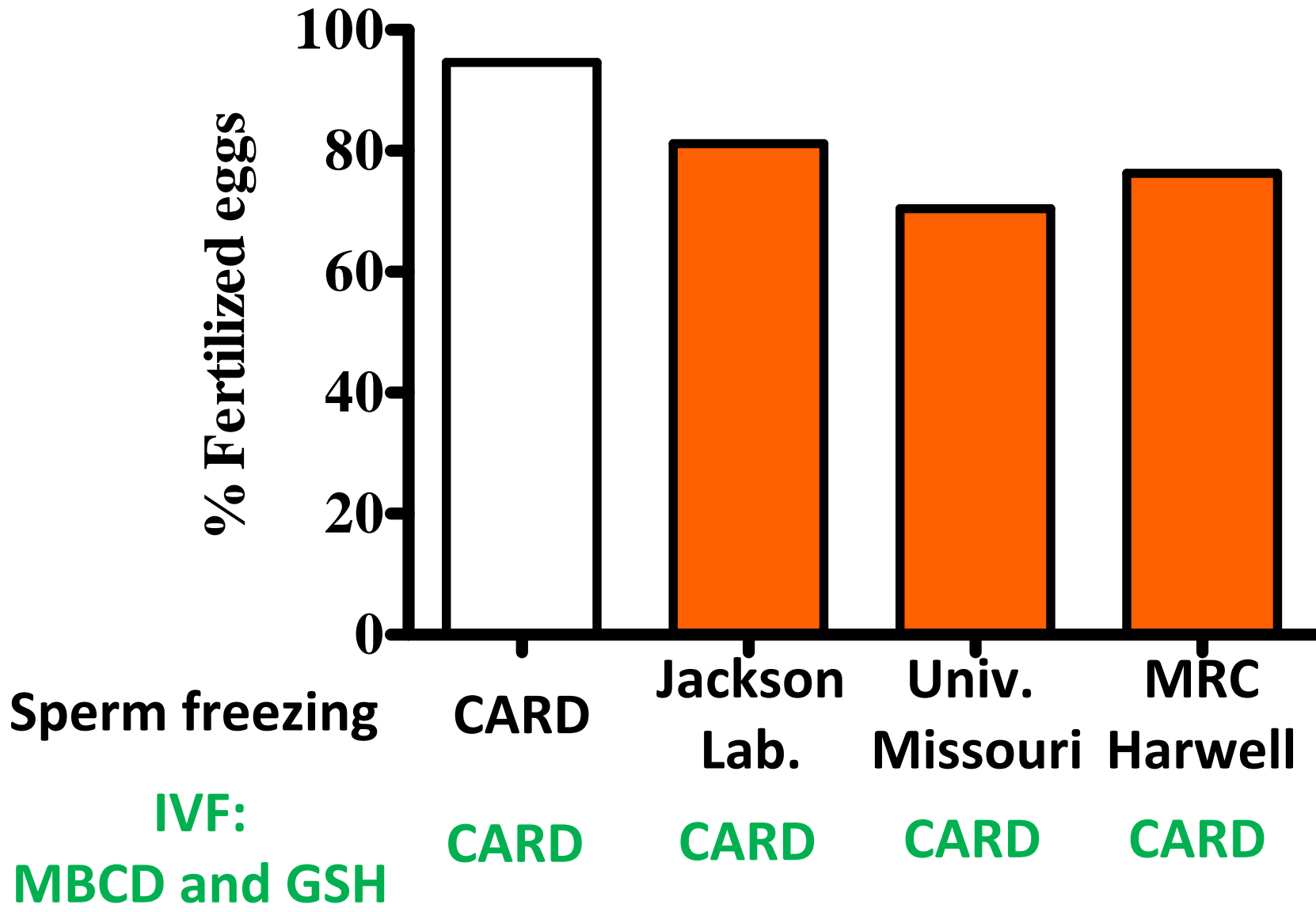
Cryopreserved sperm prepared by various protocols

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



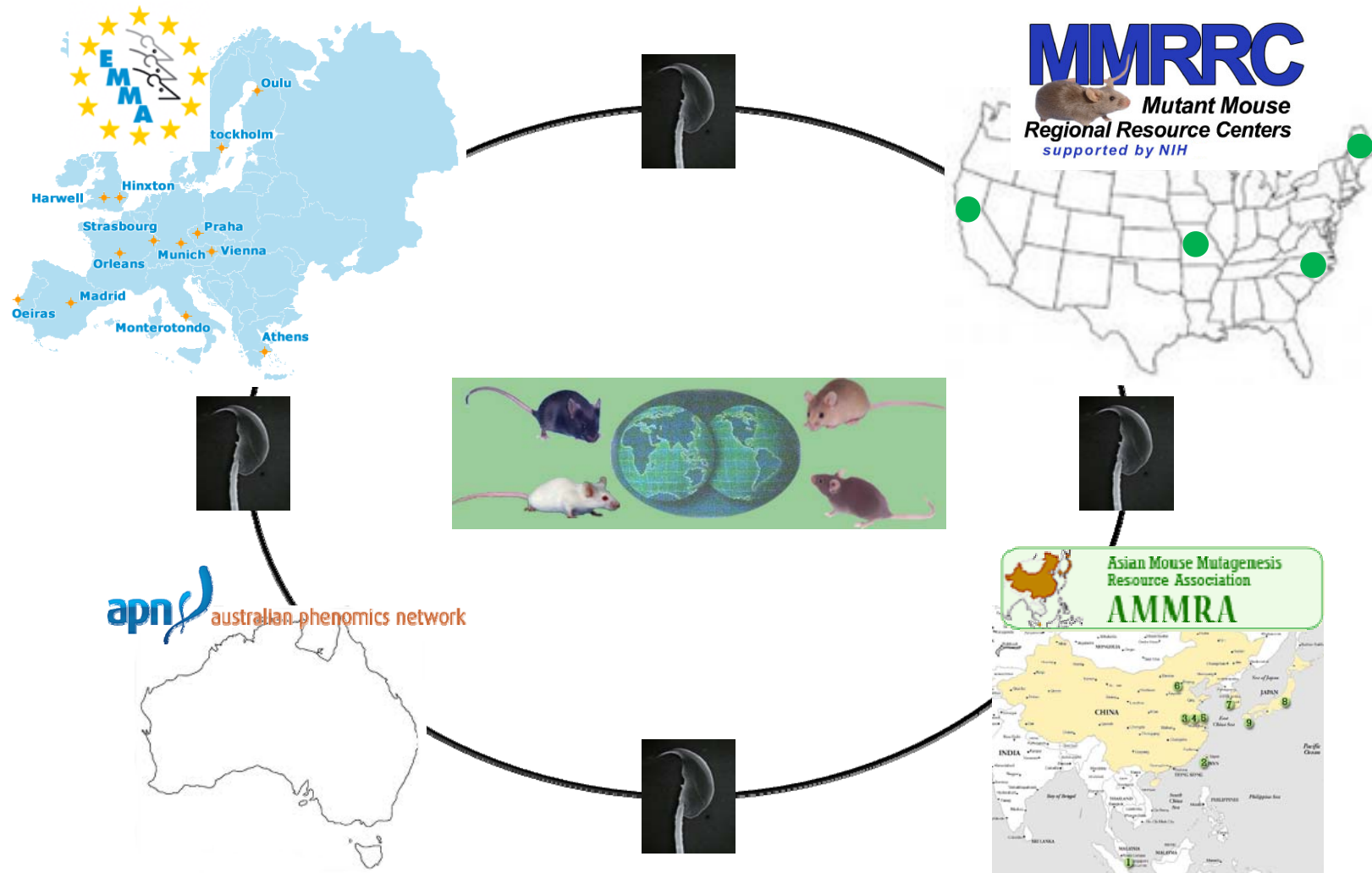
# Our IVF protocol is applicable to cryopreserved sperm produced by different protocols

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



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## European Mouse Mutant Archive: EMMA



Gracias

Thank you

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ありがとう

