# FACTORS THAT AFFECT IN VITRO FERTILIZATION USING CRYOPRESERVED MOUSE SPERM

**Sue Bath**
**Melbourne, Australia**

EMMA Cryopreservation Workshop
Madrid, Spain
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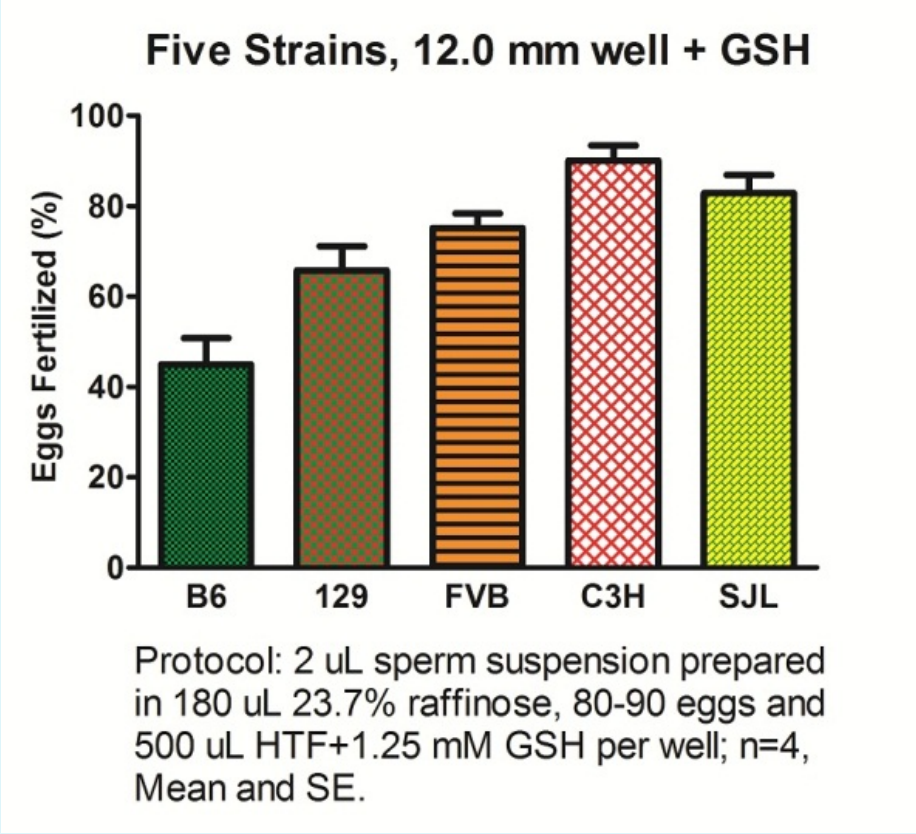
# Outline

- **Intrinsic Factors**
  - Strain of mouse
    - Sperm
    - Eggs
- **Extrinsic Factors**
  - Cryoprotectant
  - Sperm Freezing
  - Sperm Thawing
  - Incubator
  - Sperm Preincubation
  - Superovulation Issues
  - Fertilization Conditions
  - Outcome Assessment

# Mouse Strain

- Sperm
  - C57BL/6J vs other strains; form vs function?




- Variation in genetically engineered mice

# Mouse Strain

- **Eggs**
  - Superovulation issues
    - Protocol
    - 129 substrains
    - Strain variation in number of eggs
    - Age of female (B6 and other strains)

Methods later with Extrinsic Factors

# Extrinsic Factors

# Cryoprotectant

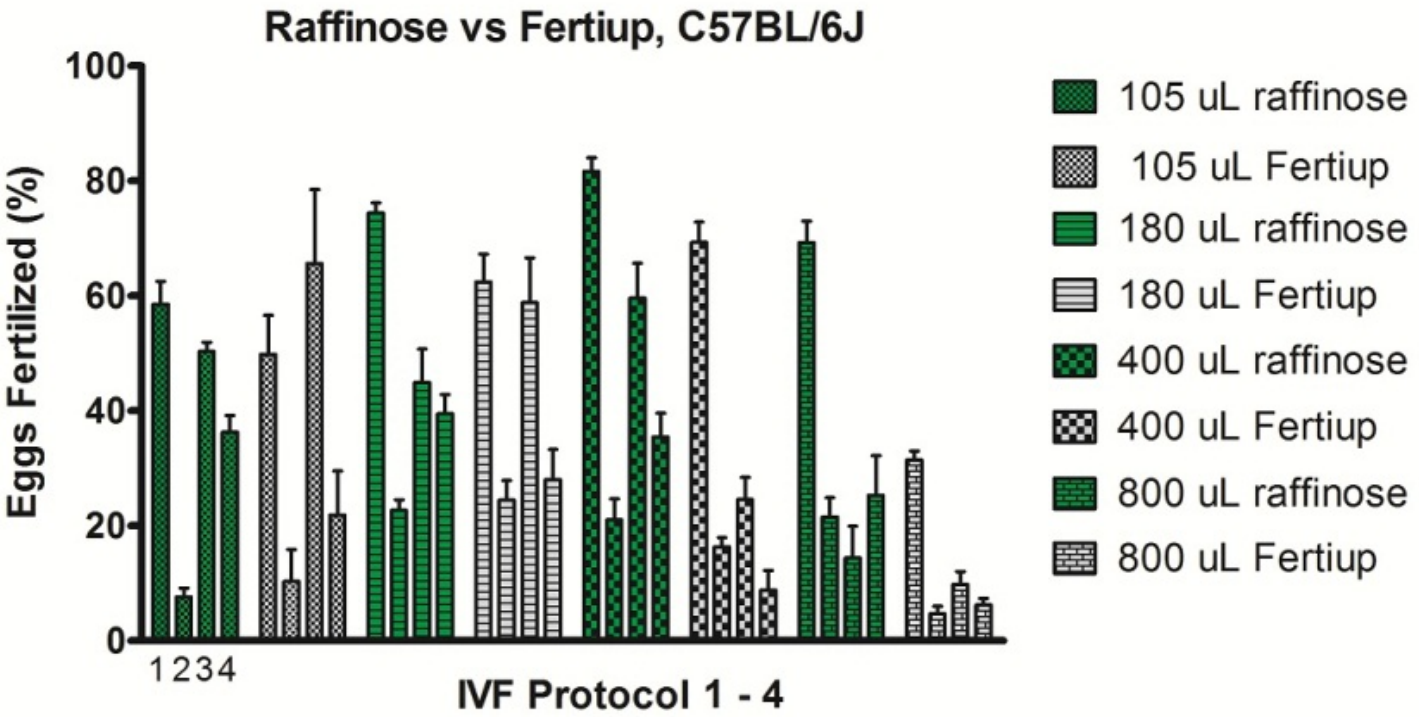
IVF rates above 60% have been achieved by C57BL/6J sperm frozen in:

- Raffinose (18-24%), optimal 23-24 % for B6; other strains no difference between 21 and 24%
- Raffinose (18%) + 3% skim-milk powder (R18S3)
- R18S3 + 100mM L-glutamine (same as Fertiup?)
- R18S3 + 477 uM monothioglycerol

Is one better than the other?

- Not known, as comparisons have not been done except in the case of 23.7% raffinose and Fertiup.
- L-glutamine stability?

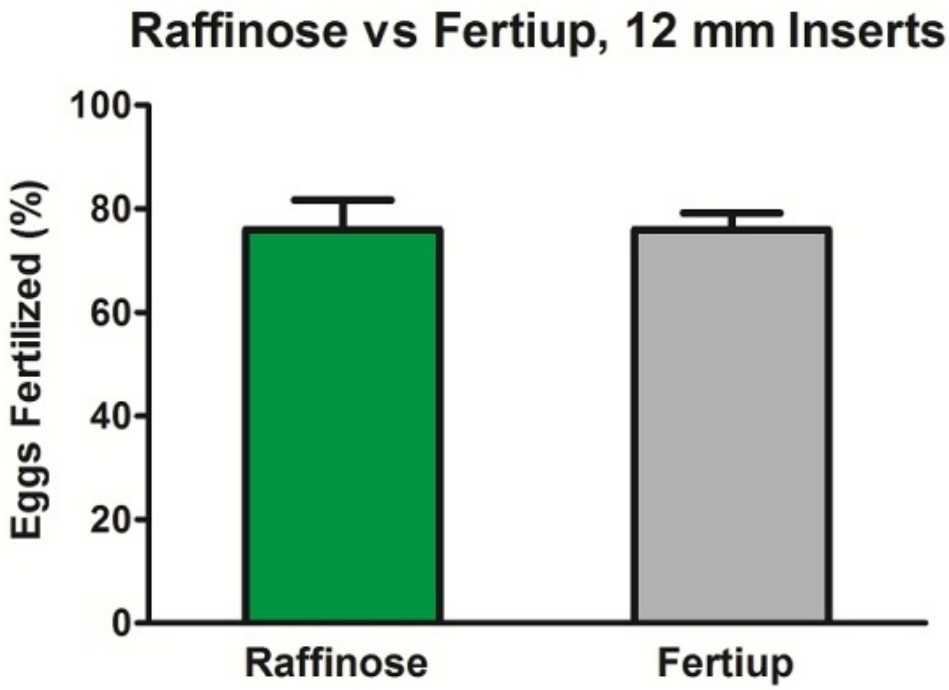
# Cryoprotectant




1. 96-well, 100 uL HTF + GSH
2. 96-well, - GSH
3. 48-well, 500 uL HTF + GSH
4. 48-well, - GSH

2 uL sperm suspension prepared in 23.7% raffinose,
80-90 eggs per well, n = 4, Mean and SE.

# Cryoprotectant




Protocol: 7.5 uL sperm suspension prepared in 180 uL 23.7% raffinose, 90-100 eggs, 500 uL HTF per insert; C57B/6J; n = 3, Mean and SE.

# Sperm Freezing

**Volume of Cryoprotectant**

•Sperm from 2 epididymides in 105 uL to 1.0 mL cryprotectant

•120-180 uL preferred if sperm are to be preincubated before transfer of motile sperm

**Freezing and Storage**

•Straws or conical-base cryovials

•Initial freezing in liquid nitrogen vapor

•Rate of cooling: 37$^{O}$C - 143$^{O}$C/min

•Store below -140$^{O}$C (ie. below the glass transition temperature)

# Sperm Thawing

- Rapid, at >2000$^{O}$C/min
- Use water bath at *37$^{O}$C* or 53/54$^{O}$C for straws, and 50$^{O}$C for tubes.
  - If above 37$^{O}$C, a short time (seconds) in air is required, allowing evaporation of $LN_2$ from interior of tube/straw, and a ring of frost to form around the outside of the tube/straw before placing it in the water bath.
    - Time in the water bath (straws 6 sec.; vials 30 seconds) needs to be precise.

# Incubator

Rapid recovery of atmosphere after opening door

# Sperm Preincubation

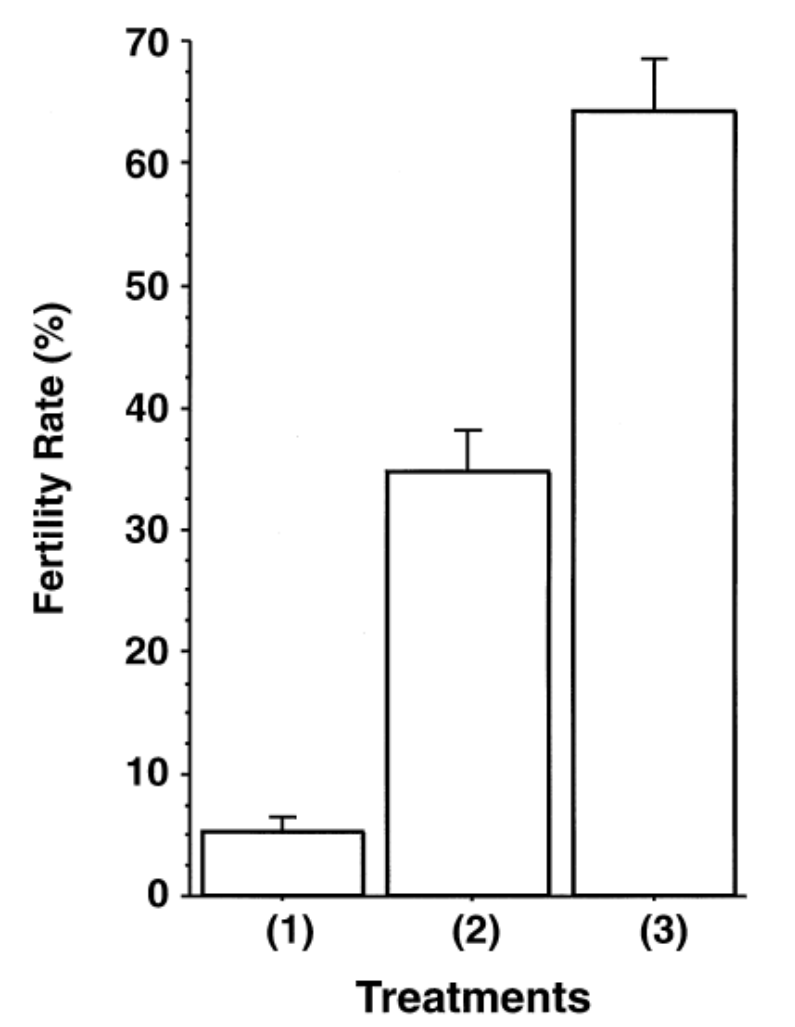
**Why Preincubate?**

- To allow time for capacitation
  - Promote efflux of cholesterol (MBCD)
  - Reduce proportion of acrosome-reactive sperm and possibly promote tyrosine phosphorylation ($Ca^{++}$ free)
- Selection of motile sperm for IVF

# Sperm Preincubation

**Is Preincubation Necessary?**

- For B6 – Yes
- Generally helpful for other strains; no down side




1. No preincubation
2. Remove dead sperm
3. Preincubate in Ca++ free medium

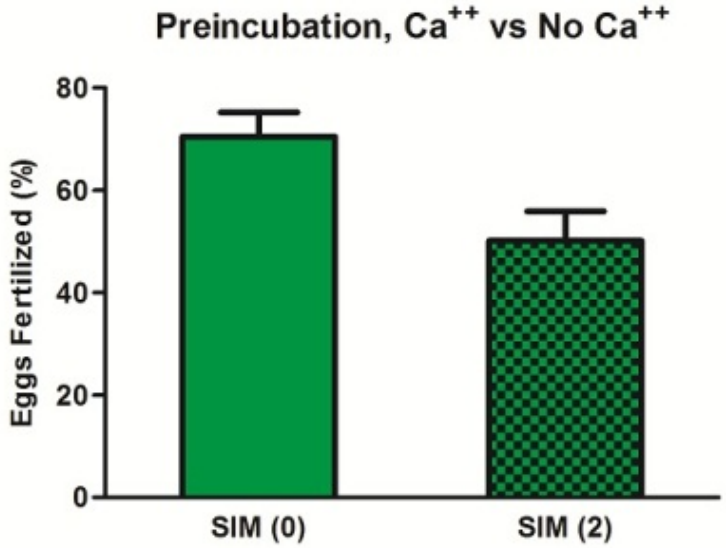
# Sperm Preincubation

## Sperm Preincubation Medium

- HEPES buffered medium, no added calcium
- Fertilization medium (HTF)
- TYH + MBCD
- HTF + MBCD
- TYH (no added $Ca^{++)}$
- TYH (no BSA or added $Ca^{++}$), more data needed to confirm
- TYH (no BSA, low [$Ca^{++}$]130 uM)

# Sperm Preincubation

## Sperm Preincubation Medium




Protocol: Sperm preincubated in HEPES buffered calcium - free SIM (0) or SIM (2). After 30 min 6 uL of medium containing motile sperm was transferred to a 200 uL drop of fertilization medium containing 80-90 eggs and overlaid with mineral oil; Sperm prepared in 105 uL 19% raffinose; C57BL/6J; n = 8, Mean and SE.

# Superovulation Issues

- Genetic differences in number and quality of eggs
- Needs optimising for each strain
- Some evidence suggesting s/c injections may be better than i/p
- Too much PMSG may result in polyploidy leading to a reduced proportion of pups born after embryo transfer

# Superovulation Issues

**Improving Egg Numbers and Quality**

- For strains other than C57BL/6, older egg donors (70-75 day-old) may be better
- 129 substrains (129T2 and 129S1,others?), inject 70-75 day-old females with PMSG and hCG 54 h apart and collect eggs 14 h later, or inject with 20 mg/kg GnRH agonist 24 h before PMSG and follow 55 h later with hCG to improve egg fertility/quality. Increases egg number and quality of 3-month-old BALB/c mice; other strains?
- Inject beta estradiol with PMSG
- Inject inhibin neutralizing antiserum; follow with hCG injection in 48 h. Problem: access to antiserum

# Fertilization Conditions

**Fertilization Medium**

- HTF or modified version containing increased [$Ca^{++}$]
- HTF or mHTF containing reduced glutathione (GSH)
- MEM has been used

**IVF Method**

- In drops of medium under oil
- In Multiwell plates with no oil
- In Transwells (Corning), no oil
- Volume of fertilization medium (final) from 100-510 uL
- Eggs with/without cumulus
- Time for co-incubation of sperm and eggs, 5-6 hours
- Sperm/uL varies; minimum seems to be 150 motile sperm/uL with minimal dead/non motile sperm and with no GSH in the fertilization medium

# Outcome Assessment

Important to assess both:

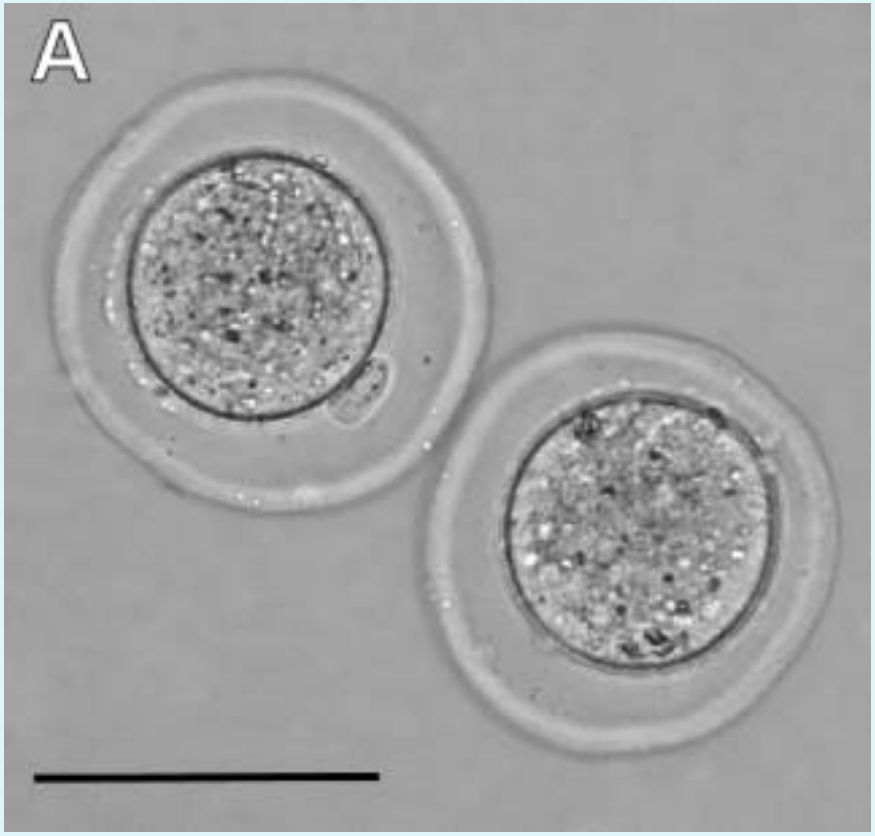
- fertilization rate
- proportion of embryos transferred producing live pups

# Conclusions

Methods are now available to overcome IVF problems using cryopreserved sperm from even the most difficult strains (eg. C57BL/6J)

**Major improvements/most robust methods:**

- Preincubation of thawed sperm in medium containing MBCD
- Transfer of motile sperm to HTF containing GSH for fertilization




**Discussion?**