Cryopreservation of fish oocytes: achievements and prospects

CRYOCYTE
EC-Project
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Why should we test fish oocytes?

- No consistent results with cryopreservation of fish embryos

* Xeno-transplantation of primordial stem cells (Takeuchi et al., 2004) requires time to maturation of host and is currently relevant to sibling/related species
Cryopreservation of fish embryos

- The main obstacles are:

1. Relatively large eggs
2. Contain yolk
3. Impermeable to cryoprotective (CPAs) agents
   - especially the syncytial layer
4. High water content- especially in hydrated marine pelagic eggs
5. Early embryonic developmental stages are sensitive to chilling
• Recent successful results in cryopreservation of oocytes from Mammalian species

• Why not test fish oocytes?
CRYOPRESERVATION OF FISH OOCYTES

Maturing oocyte

Cryopreservation Procedure
  
  Freezing
  
  thawing

  in vitro maturation
  
  Ovulation
  
  Fertilization

oocytes envelopes

CPA permeability

CPA Toxicity

Freezing

Cathepsins

Hydration

GVBD

Hormones
Cryopreservation of Oocytes

Cryopreservation methods (WP 7, WP 8)

<table>
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<tr>
<th>Zebrafish (ZF)</th>
<th>Gilthead seabream (GSB)</th>
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Biological Barriers

Oocyte envelopes (WP 1)

Hydration (WP 2)

Biological markers for Viability

Staining methods (WP 7)

Physiological (WP 3, markers) (WP 5, WP 6)
GVBD, Cathepsins, hydration

Genomic markers (WP 3)

Proteomic markers (WP 4)

Development of in vitro oocyte incubation procedures

In vitro maturation (WP 6)

Ovulation (WP 6)

Fertilization (WP 6)
Fish models

• Zebrafish
  *Danio (Brachydanio) rerio (ZF)*

• Gilthead seabream
  *Sparus aurata (GSB)*

Share the same ovarian reproductive strategy
  Daily spawners
  group synchronous oocyte development
Oocyte developmental stages

Gilthead seabream

Pelagophil

Benthophil

Zebrash

Germinal Vesicle Breakdown (GVBD)

500 μm
• What developmental stage should be selected for cryopreservation?

- End of vitellogenin uptake

- Before hydration in pellagic floating eggs (GSB)

- Oocyte envelope (zona-pellucida proteins) permeability
Time-course of oocyte hydration in *Sparus aurata*

Is water uptake a facilitated mechanism?

Fabra *et al.* (2005)
Changes in oocyte envelope structure during oocyte maturation in the GSB
Toxicity of cryoprotective agents (CPAs)

Permeability of CPAs

Cooling to cryogenic temperatures

Thawing

Evaluation of viability
Assessment of oocyte viability

- Staining – CFDA, Propidium iodide, Trypan blue, MTT (Thiazoly blue tetrazolium bromide)
- Functional- GVBD, Cathepsin activity
- Genomic markers
- Proteomic markers
Toxicity of CPAs

- DMSO - 2M (GVBD); 4M (MTT)
- Propanediol - 4M (MTT)
- Methanol - 6M (GVBD; MTT)
- Ethylene glycol - 6M (MTT)
Permeability of oocytes:
Internal concentration of $^{14}$C-Methanol in GSB oocytes

Incubated in 75% L-15

\[ M = \frac{(O-b)/n}{V} \]
\[ V = \frac{4}{3}\pi R^3 \]

Time: p < 0.0001
Concentration: p < 0.001
Time*Concentration: p = NS

In GSB: $1.16 \pm 0.59 M$ (after 15 min)
Large individual variation

In ZF: $0.39 \pm 0.04 M$ (after 60 min)

70-80% located within the oocyte
Comparison between GSB vitellogenic oocytes and embryos in methanol content

Differences between stages: \( p < 0.0001 \)
Oocytes > morula > gastrula or cells
Freezing protocol for GSB

GSB-OOC.1

Sampling temperatures

- 24°C
- -7°C
- -16°C, before seeding
- -16°C, after seeding
- -30°C
- -50°C
- -70°C

-5°C/min

-1°C/min

LN₂

-1°C/min

hold 5 min, seeding

hold 5 min

-1°C/min

-10°C/min

-70°C

-7°C

-5°C/min

24°C
Freezing of GSB oocytes

Survival of GSB oocytes after freezing with EG 3.0 M (in 75% L-15)

Visual percent of control

≤300 µm  400 µm  ≥600 µm
Survival of GSB oocytes

(≥ 600 µm)
Non-frozen, 24°C
15 min
Thawed from LN₂

0 hr

1 hr

2 hr

(≥ 250-300 µm)
Non-frozen, 24°C
15 min
Thawed from LN₂
Cathepsin B activity after freezing of GSB oocytes
Temperature treatments of stage 4 GSB oocytes

-70°C frozen methanol 4M

-70°C frozen L-15
Whole-mount *in situ* hybridization screening on oocytes using the identified most abundant maternal transcripts (SAGE Library) - ZF

Oocyte viability molecular signature (OVMS) assay

A: Cyclin B  B: DAZL  C: MeII  D: Lectomedin
Molecular Marker: DAZL

Control

After cryopreservation
CRYOPRESERVATION OF FISH OOCYTES

- Maturing oocyte
  - CPA permeability
  - CPA Toxicity
  - Freezing
- Cryopreservation Procedure
  - thawing
- in vitro maturation
  - Ovulation
  - Fertilization
- Cathepsins
- Hydration
- GVBD
- Hormones
Production of recombinant GSB LH (rLH) in the yeast expression system

In vitro oocyte maturation

Effects of hCG & rLH on final oocyte maturation (FOM)

Meiri, Rosenfeld et al.
Conclusions and prospects

- Oocyte envelopes are relatively permeable to CPAs
- Oocytes tolerate relatively high concentrations of CPAs
- Small size (but not mature) oocytes are viable after cryopreservation (MTT staining)
- Main obstacle: Lucent oocytes - Maintenance of the native structure of VITELLOGENIN - cooling rates? Sugars?
- Molecular and functional tools were developed for assessing in depth viability of oocytes and contributing to our knowledge on oocyte development and maturation
- In vitro maturation (Ovulation? Fertilization?) seems feasible
The CRYOCYTE Team