Aseptic and automatable vitrification of human embryonic stem cells using defined media

ULg - Glycomar

October 22th 2012
Introduction: objectives

- Definition of hESCs cryopreservation conditions:
  1. allowing recovery of live and biologically intact human embryonic cells (hESCs)
  2. working in aseptic conditions (EC directive 2004/23/EC)
  3. using chemically defined media without human & animal serum (mTeSR1®)
  4. compatible with automation
Definition of the optimal cryopreservation procedure

- Comparison of two methods of cryopreservation
  - “Conventional” Slow Freezing (SLF) in 1 ml cryotubes
  - Aseptic vitrification (Vit) in French straws
All conditions have been validated on:

- **mouse embryos:**
  - Submitted paper: (work performed on zygotes)
    Vitrification succeeds with lower intracellular concentration of cryoprotectants (ICCP) as compared to slow freezing, despite exposure to higher concentrations of cryoprotectant solutions

- **mESCs**
  - cf: BEST presentation @ Lisbon (6/12/2011)
Definition of the optimal cryopreservation procedure

Comparison of two methods of cryopreservation on hESCs

- «Conventional» Slow Freezing (SLF) in 1 ml cryotubes
  - mTeSR1®
  - Serum
  - DMSO

- Aseptic vitrification (Vit) in 0.16 ml French straws
  - mTeSR1®
  - Cryoprotectants
SLF as usually used for hESCs

- ~ 10^6 cells/ml
- 10% DMSO – 40% KO-SR in mTeSR1®
- In cryotubes of 1 to 2 ml
- Cooling rate: -1 to -2°C/min until -80°C, plunge in LN2

From the bench and from the literature:

**Advantages:**
- Easy !!!
- Universal

**Drawbacks:**
- Leaky to liquid N2 (˃˂ to EU Tissue and Cells Directive 2004/23/EC)
- Poor control of supercooling → impair cell viability
Vitrification of hESCs

- Vitrification = extreme increase of viscosity upon very high speed cooling & warming (~1200°C/min)
- Ultimately results in a solid amorphous state

From the bench and from the literature:

**Advantages**
- No ice crystal formation
- No need of specific device for cooling & warming

**Drawbacks**
- Use of high extracellular concentrations of cryoprotectants (but low intracellular [CPs])
Our vitrification procedure:

In defined & serum-free medium

- hESCs cultured in defined serum-free medium: mTeSR1®

Aseptic

- Sealed straw: no contact with LN2
  (in compliance with EC recommendations)
Aseptic vitrification method: cooling

1.: + 140 µL of Sol. 1 = 280 µL; **incomplete** equilibration

2.: + 280 µL of Sol. 2; **incomplete** equilibration
   Centrifugation @ 5000 g
   Supernatant (560µL) removed

3.: Pellet re-suspended in 160 µL of Sol. 3; **no** equilibration
   Fill in straw immediately; seal at both ends
   Direct plunge in LN2
Aseptically vitrified straw

hESCs straw design:

92 mm french straw
= 0.16 ml

Identification plug

Mechanically sealed with a plug

133 mm: compatible with international standards of straw storage

Straw melted with a thermosealer
Aseptic vitrification method: warming

1.
15 sec after thawing: + 1 mL of mTeSR1® = 2 mL of Suc 0.5M

2.
30 sec after thawing: + 2 mL of mTeSR1® = 4 mL of Suc 0.25M

3.
60 sec after thawing: + 4 mL of mTeSR1® = 8 mL of Suc 0.125M
500 g during 5 min
Supernatant removed, add 2 mL of mTeSR1®
Results: Post-vitrification hESCs characteristics

- Morphology of colonies
- Morphometric analysis
- Karyotype
- Immuno-histochemistry
- Teratoma formation
Morphology of colonies
3 & 7 d post-warming
Hoffman modulation contrast; x100

Slow freezing
Vitrification

3d post warming
7d post warming
Morphometric analysis

Vitrified hESCs, d5, x40, picture #393
Pictures taken from the center of the well
Morphometric analysis

Vitrified hESCs, d5, x40
Picture taken from the center of the well
Morphometric analysis:
Proliferation curves after warming of slow frozen vs vitrified cells
¼ well of a 6-well plate

hESCs proliferation curves after SF or V
Morphometric analysis:

Proliferation curves after warming of slow frozen vs vitrified cells

1/4 well of a 6-well plate

Problem solved now

hESCs proliferation curves after SF or V
Karyotype analysis post-vitrification: 46XX
Immuno-histochemistry

hESCs RCM1: p11 after vitrification (x10)
Hematoxilin - Eosine

Teratoma formation post-vitrification (x20)

Neuroepithelium
Endodermal epithelium
Smooth muscle
Glandular epithelium
Conclusions

- **Aseptic** vitrification of hESCs in **defined media** w/o animal / human serum

- Stepwise addition and dilution of cryoprotectants before cooling and after warming ➔ Automation

- Vitrified RCM1 cells maintain their **stem state**
Perspectives

- Multiple steps of vitrification
- Method should be tested on other hES cell lines
Thanks to:

- Pierre Vanderzwalmen
- Joëlle Piret
- Nadine Antoine
Thank you for your attention