



Kiev, 2007

Витрификация гамет и эмбрионов: системы и среды



Лев Левков

Fertility Unit, Karolinska University Hospital,
Stockholm, Sweden

Из истории криоконсервации гамет и эмбрионов

- Первая попытка замораживания спермы:
Spallanzani, 1776
- Открытие криозащитных свойств *глицерола*:
Polge et al., 1949, (замораживание спермы, 1951)
- Замораживание эмбрионов мышей: Whittingham, 1972
- Беременность из замороженных эмбрионов человека:
Trounson & Mohr, 1983 (*глицерол*)

Криоконсервация

- **Ооциты**
- **Эмбрионы**
- **Бластоцисты**
- **Сперматозоиды**
- **Овариальная ткань**
- **Тестикулярная ткань**

Самая длительная в истории криоконсервация эмбрионов:

12 лет

IVF Unit, Hadassah University Hospital, Israel

Ravel et al.,

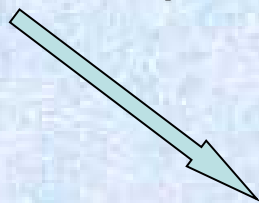
²⁰⁰⁴
Hum Reprod, V 19, No. 2, 328-329

Результат переноса размороженных эмбрионов:

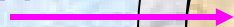
Всего перенесено:	4 эмб.	Редукция:	1 эмб.;
Имплантировались:	3 эмб.	Рождение:	двойня

Механизм действия криопротекторов

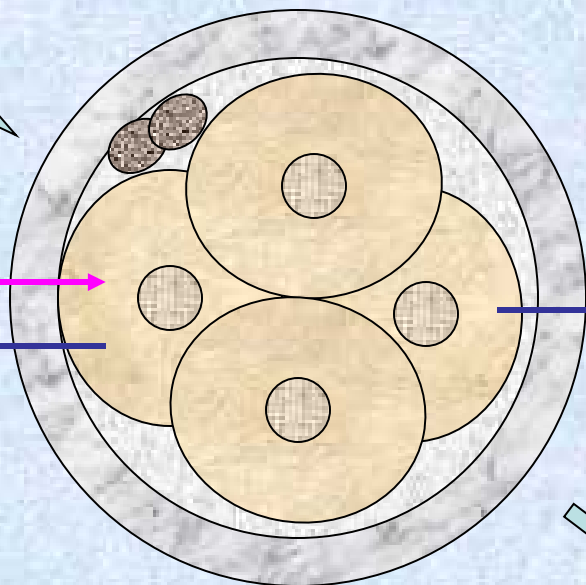
дегидратация



PrOH

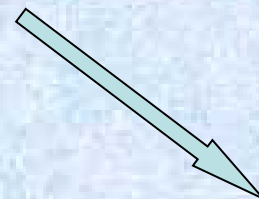


H₂O



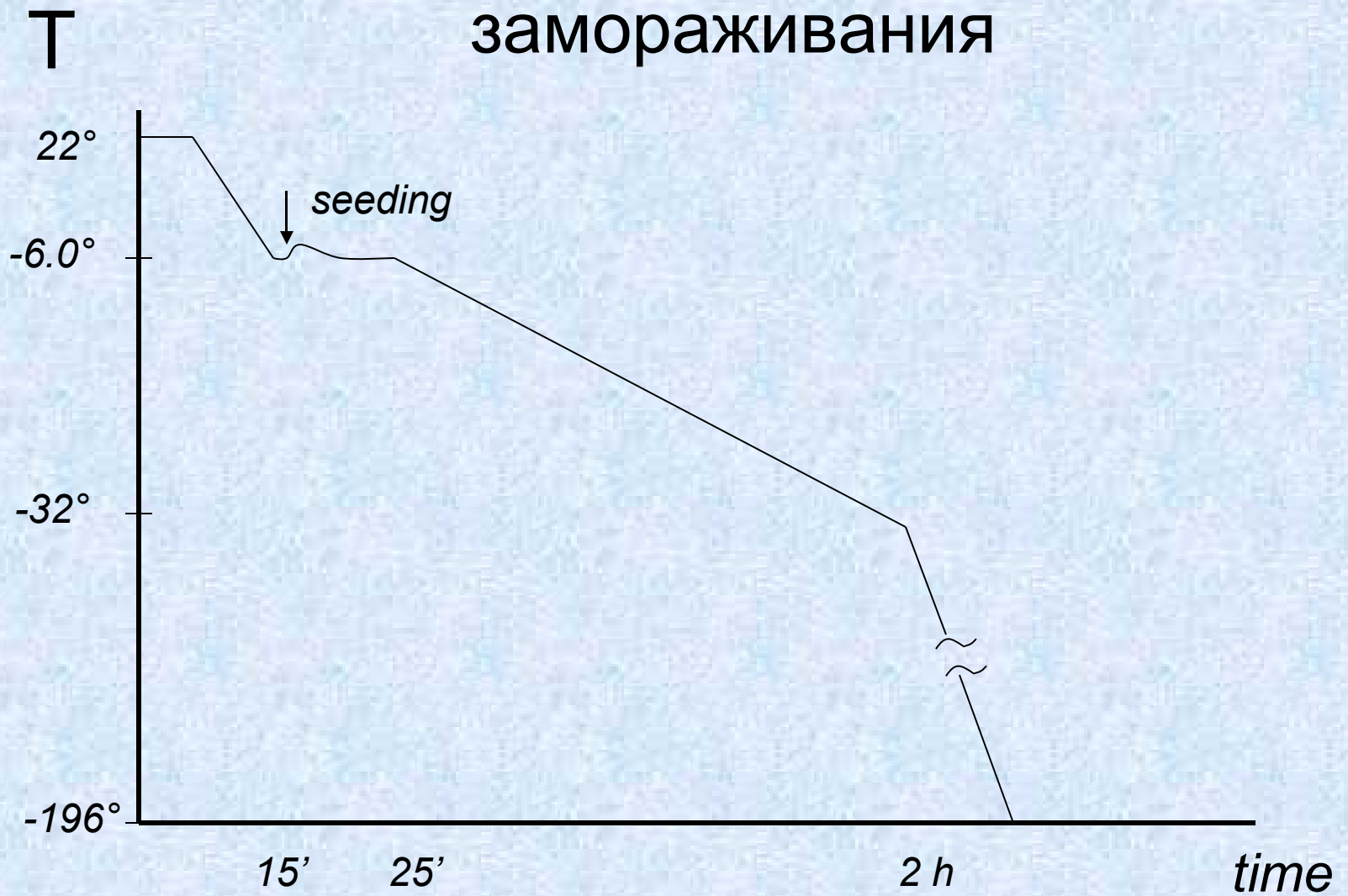
сахароза

H₂O



Vitrification

Протокол медленного замораживания



Vitrification

Is the fastest freezing method

It allows to avoid cristallization of water

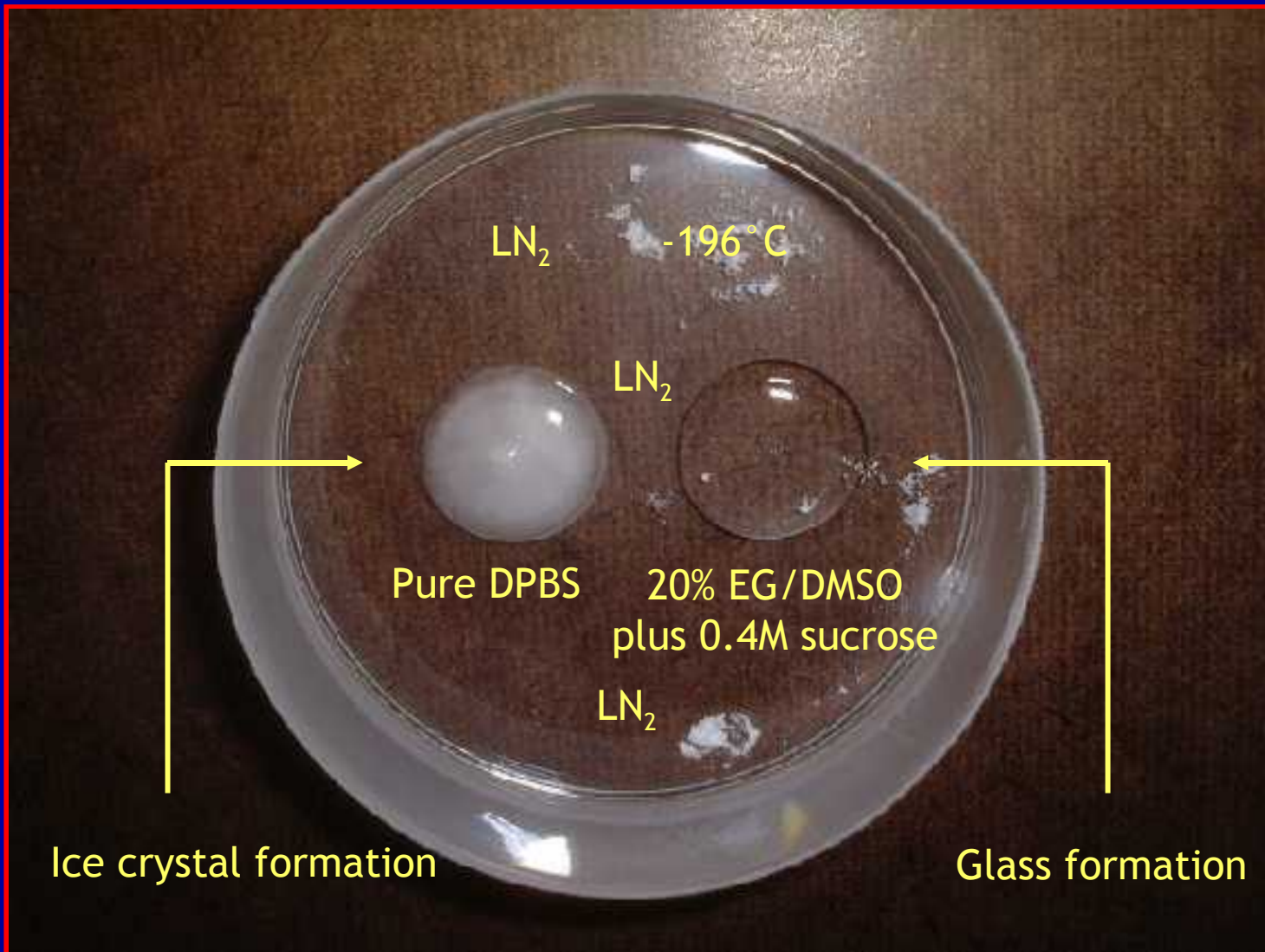


Liquid phase

The diagram consists of two ovals connected by a downward-pointing arrow. The top oval is light blue and contains the text 'Liquid phase'. The bottom oval is light brown and contains the text 'glass like phase'. The arrow is light green and points from the top oval to the bottom oval.

"glass like" phase

VITRIFICATION TEST



Liebermann et al.

Oocyte Vitrification

The earliest report: **1999 by Kuleshova and co investigators.**

Another live birth study from Korea (Yoon *et al.*, 2000). Yoon and coworkers summarized their entire experience with oocyte cryopreservation in 2003.

Both sets of investigators used similar protocols for vitrification.

Oocyte Vitrification

Ethylene glycol (EG) and sucrose were the cryoprotectants in the base medium PBS, supplemented with either 10 mg/ml HSA (**Kuleshova *et al.*, 1999**) or 10% FBS (**Yoon *et al.*, 2003**).

Kuleshova *et al.* used three **increasing concentrations of EG** (10, 20, 40%), adding 0.6 mol/l sucrose in the final dilution for vitrification.

Yoon limited the dilution **steps to two** with **1.5 mol/l EG** followed by **5.5 mol/l EG** and 1 mol/l sucrose for the final solution.

Warming

Kuleshova et al.,
0.4 mol/l Sucrose followed by
0.25 mol/l and finally
0.125 mol/l.

Yoon et al. began at a much higher concentration of
sucrose, 1 mol/l, followed by decreasing dilutions
0.5, 0.25, & 0.125 mol/l.

Warming

Survival rates: 65 and 69%

Fertilization rates: 72 and 45%

Kuleshova *et al.*: **one live birth** from the 17 oocytes frozen.

The larger study by Yoon *et al.* reported **six live births** (seven infants) from their **5-year experience**.

Vitrification methods

Cryoprotectants:

EG (ethylene glycol)

DMSO (dimethylsulphoxide)

Freezing

I. 40% (v/v) Ethyleneglycol & 0.3M/l sucrose

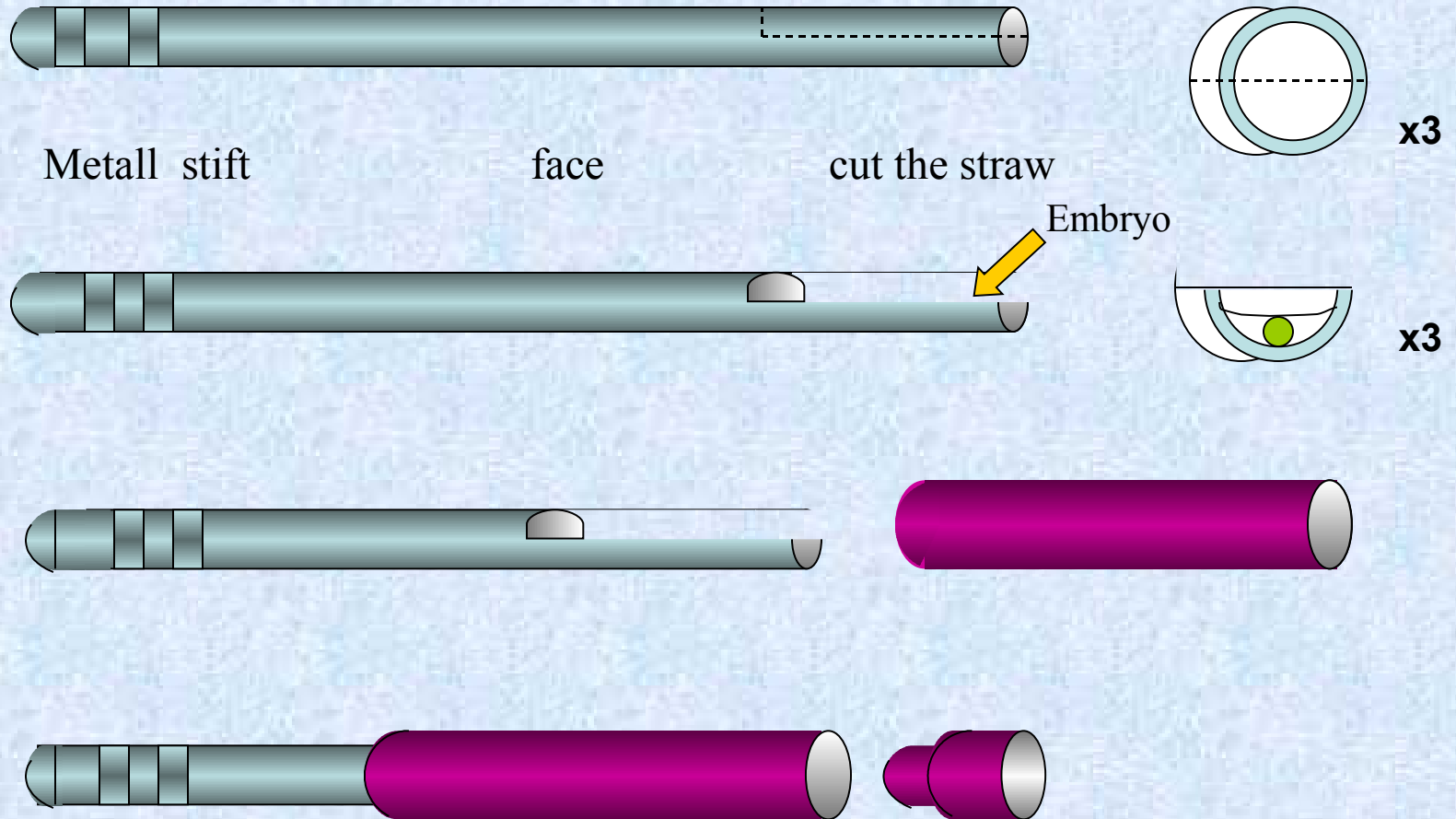
II. 20% DMSO & 20% Ethyleneglycol +
18% Ficoll + 0.3M/l sucrose

Time of vitrification: sol. A - 3 min &
sol. B - 30 sec

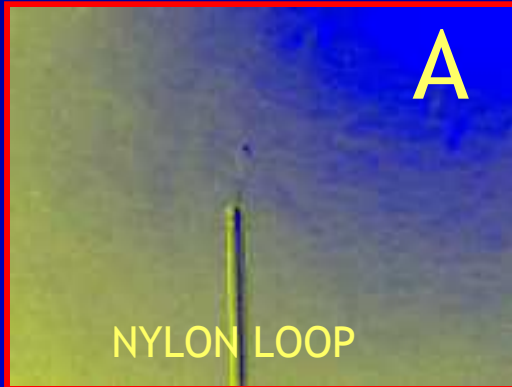
Plunging into LN2

Vitrification

Hemi-Straw System



CRYOLOOP TECHNIQUE

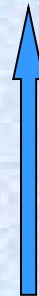


Effect of reduction of the blastocoelic cavity before vitrification

Vanderzwalmer et al., HR, 2002

(Virvoorde, Belgium; Bregenz, Austria; Hiroshima, Japan)

	Control	Artificial shrinkage
Intact Blc after vitrification:	31%	60%
Re-expansion:	19%	40%
Hatching:	6%	25%



Effect of reduction of the blastocoelic cavity before vitrification

Vanderzwalmer et al., HR, 2002

(Virvoorde, Belgium; Bregenz, Austria; Hiroshima, Japan)

	Control	Artificial shrinkage
Pregnancy rate:	4.5%	20.5%
Implantation rate:	1.4%	12.0%



Blastocyst vitrification protocols

- I. Michigan University method (*Smith et al, 2004*)
 - Vanderzwalmer protocol – hemi-straw method
(*Virvoorde, Bregenz, Hiroshima, 2001, 2003*)
- III. Isachenko method (Bonn University)

Model of investigation: mice blastocysts ?

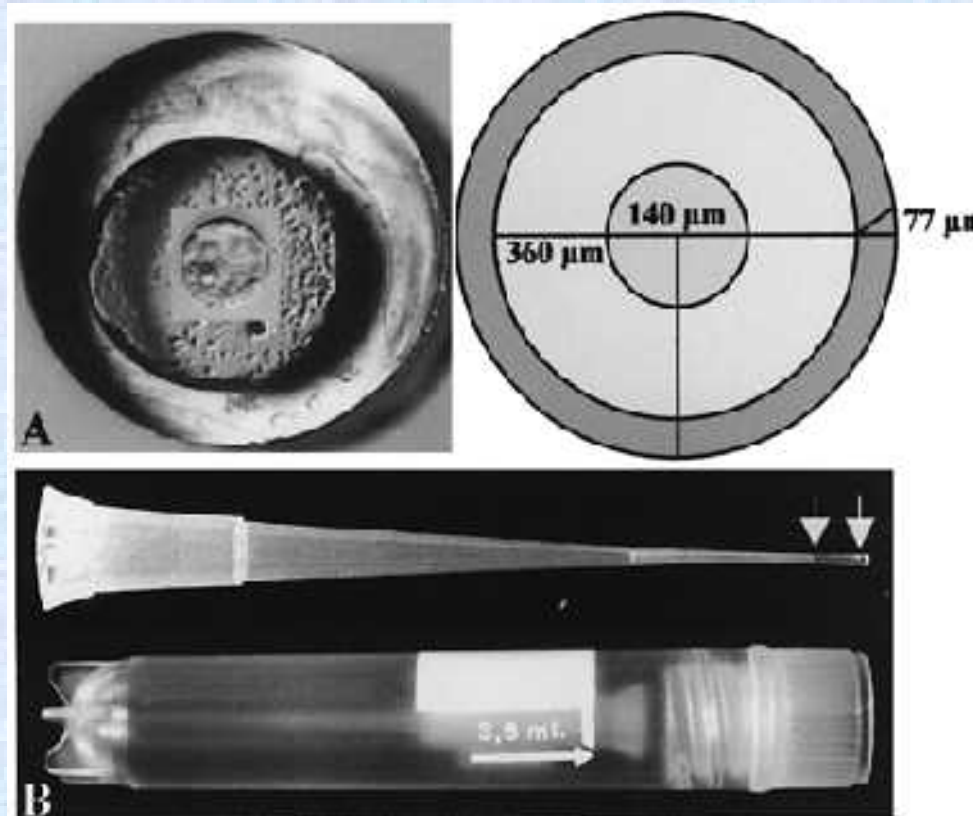
Modifications of Blastocyst cryopreservation protocol

Vitrification

- Cryoprotective solutions (EG; EG/DMSO)
- Safe storage:
 - closed straws (Smith, Michigan University)
 - pipette tips in cryotube (Cremades et al, 2004)
- Effect of blastocoelic cavity reduction (Vanderzwalmer et al., HR, 2002)
- 4. Effect of Assisted hatching (M.Tucker, 1991, Alliot et al, 2003, Kung et al, 2003)

Vitrification in pipette tips

Cremades et al, 2004



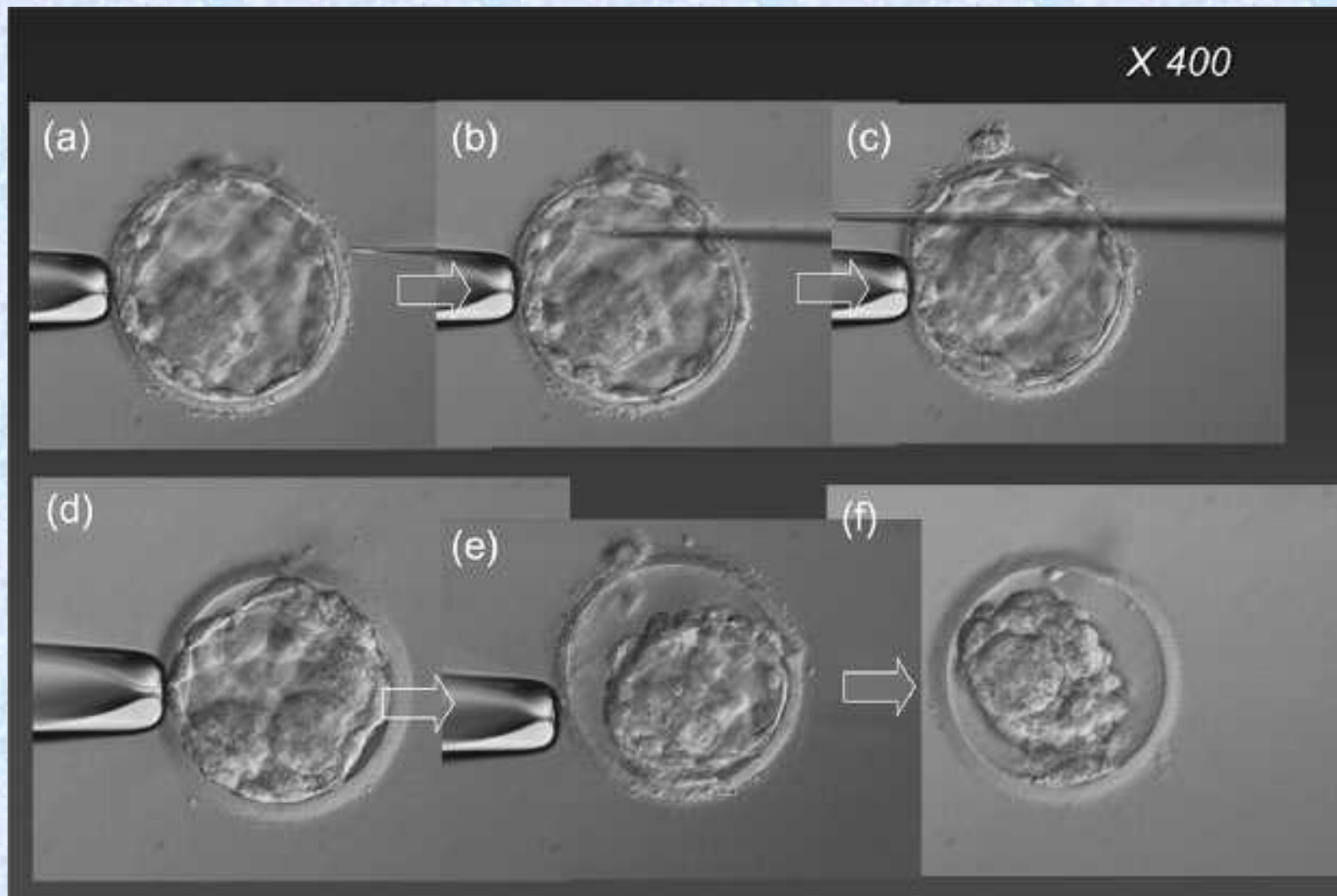
(A) Original and schematic transversal sections, to show the wall and inner diameters of the tip and the size relationship to a blastocyst; (B) tip with 0.5 μl holding medium (between arrows) and inside cryotube.

**Artificial shrinkage of blastocoeles using
either a micro-needle or a laser pulse prior
to the cooling steps of vitrification
improves survival rate and pregnancy
outcome of vitrified human blastocysts**

T. Mukaida, C. Oka, T. Goto and K. Takahashi

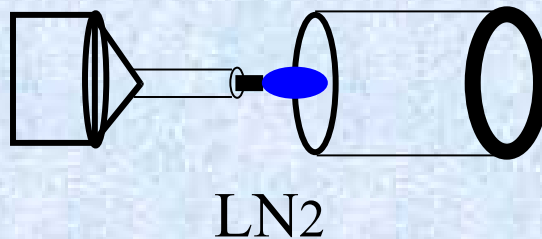
Human Reproduction 2006 21(12):3246-3252

Искусственное уменьшение полости бластоцисты



Проблемы метода витрификации

- Токсичность криопротектантов (DMSO, EG)
- 2. Безопасность для детей (не подтверждена)
- 3. Хранение в жидком азоте (LN₂):
риск контаминации? (Lane et al., 1999; Chung et al., 2000)



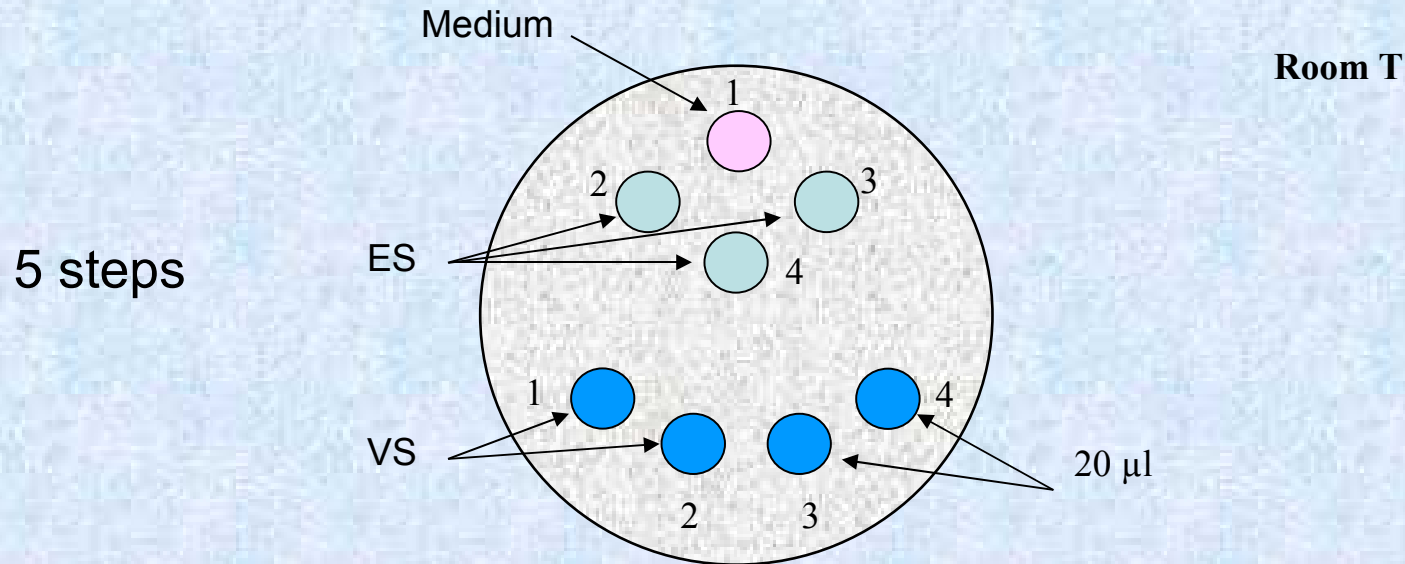
ESHRE Campus Symposium

Brussels, 12-13 March 2004

**Cryobiology & Cryopreservation of
Human Gametes & Embryos**

New method of vitrification

G. Smith et al., University of Michigan, USA



Vitrification: oocytes, cleavage stage embryos & blastocysts

ES - Equilibration solution

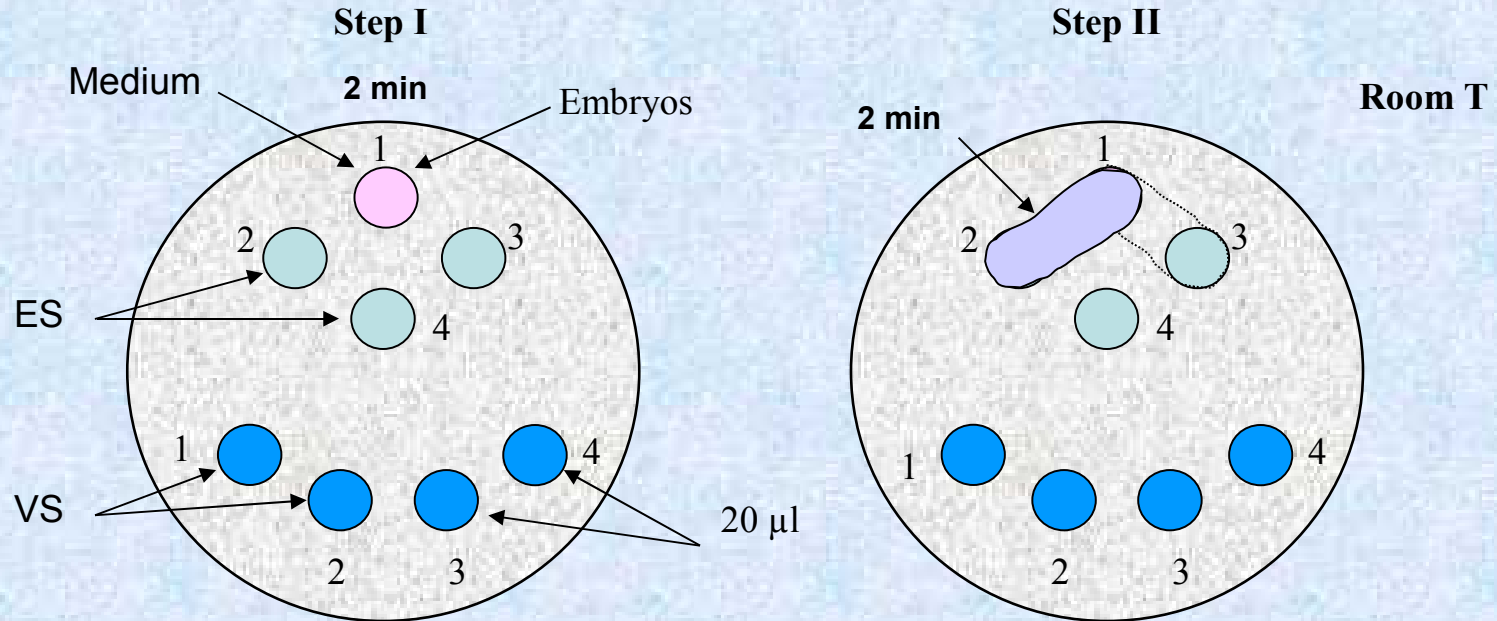
M199 M
7.5% EG
7.5% DMSO
20% SSS

VS - Vitrification solution

M199 M
15% EG
15% DMSO
20% SSS

New method of vitrification

G. Smith et al., University of Michigan, USA



ES - Equilibration solution

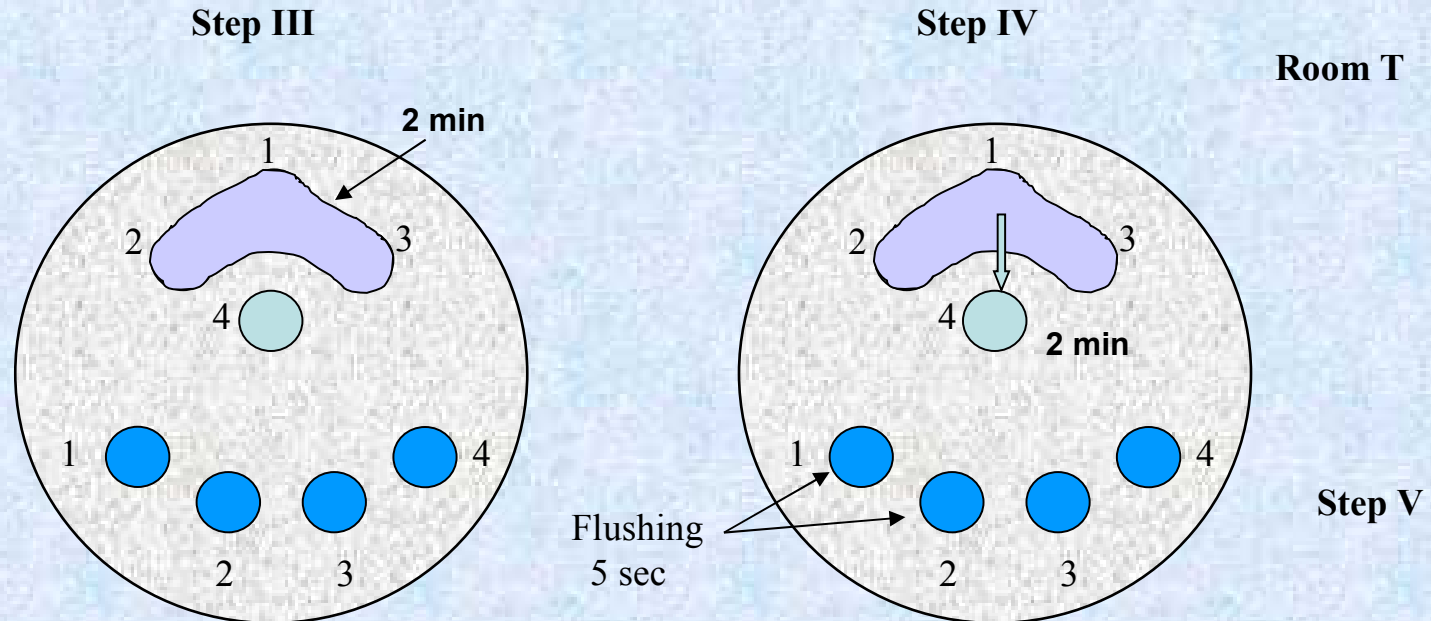
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New method of vitrification

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7.5% EG
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15% DMSO
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Методы витрификации

VitAsep

**Isachenko V et al.
Bonn University, Germany**

I. Vitrification Kit

II. Warming Kit

***Including safe vitrification
straw kit***

Distributor: MTG Medical Technology, Germany

Clinical outcome of cryopreserved by vitrification of **cleavage stage** human embryos

Shafei et al, 2004, Russia

-
- **No of FER cycles:** 118
 - **No of embryos thawed:** 276
 - **Survived / rate:** 244 / **88%**
 - **No. of embryos transferred:** 225 (1.9)
 - **Clinical pregnancies / rate:** 43 / **36%**
 - **Miscarriages / rate:** 7 / 16.3%
 - **Multiple pregnancies / rate:** 8 / 18.6%
 - **Number of life birth:** 7
-

Vitrification in hyaluronan-based medium

Clinical efficiency of vitrification of human embryos

Kuwayama et al, 2004, Japan

No. of vitrified embryos >13,000; 5 years clin. results

	Survival rate	Implantation rate
• 4cell stage:	98%	32%
• Blastocyst stage:	90%	53%

ES - Equilibration solution

M199 M
7.5% EG
7.5% DMSO
20% SSS

VS - Vitrification solution

M199 M
15% EG
15% DMSO
20% SSS

Эксперименты по витрификации в Fertility Unit, Karolinska University Hospital

- Начало проведения тестов: 2005
 - Начало клинического замораживания: 2006
 - Модель для исследования: бластоцисты и
неоплодотворенные ооциты
 - Среды для тестирования: MediCult, Vitrolife
 - Носители: CBS high security straws
 - Выживаемость ооцитов: до 75%
 - Выживаемость бластоцист: до 60-70%
 - Применение витрификации при замораживании
овариальной ткани 2006
-

Human embryos after vitrification



11

Human embryos after vitrification





Пути повышения эффективности криоконсервации бластоцист

- ✓ Переход к новым методам замораживания бластоцист: **витрификация**
- ✓ Рекомендуется проводить редукцию полости бластоцисты перед витрификацией
- ✓ Размораживание бластоцист за 18-20 часов до переноса: **селекция культивированием**



Спасибо за внимание!



Effect of stage of development on vitrification results of blastocysts

Vanderzwalmer et al., HR, 2002
(Virvoorde, Belgium; Bregenz, Austria; Hiroshima, Japan)

Vitrification: 40% EG + 18% Ficoll + 0.3 mol/l sucrose

	Morula	Early Blc	Exp. Blc
Survival rate after vitrification:	54%	58%	20.3%
Pregnancy rate:	27.8%	25%	14.3%
Implantation rate:	20%	16.7%	7.1%

No artificial cavity reduction



Artificial Shrinkage of blastocoeles prior to the vitrification of blastocysts

T. Mukaida^{1,3}, C. Oka², T. Goto² and K. Takahashi¹

- (a) Holding the expanded blastocyst with holding micropipette connected to micromanipulation.
 - (b) Insertion of the micro-needle inside the blastocoele at a point away from the inner cell mass (ICM).
 - (c) Puncture through the blastocoele and removing the micro-needle gradually.
 - (d) Beginning of shrinkage 10s after puncture.
 - (e) Partial shrinkage 30s after puncture.
 - (f) Complete shrinkage 1min after puncture.
-