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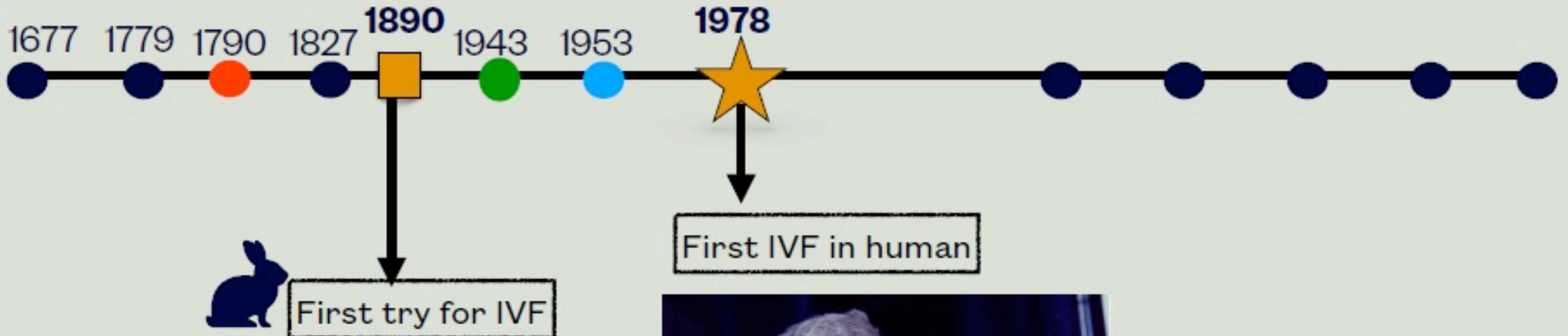
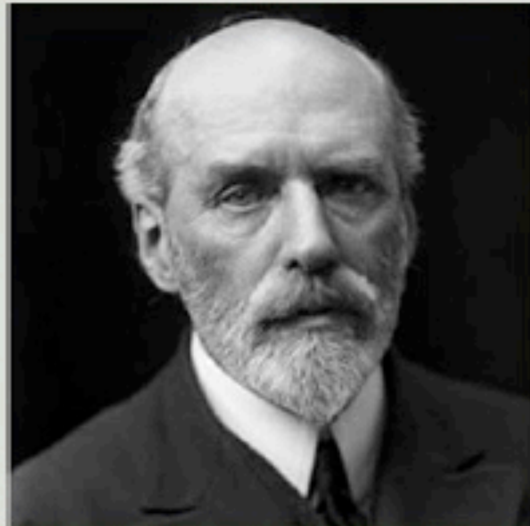
Royan Institute

Cryopreservation of Gametes and Embryos

By: Fatemeh hassani
PhD of Reproductive Biology
Clinical Embryologist in Royan Institute

ART history timeline





Prof. Walter Heape

Steptoe & Edwards

ART history timeline

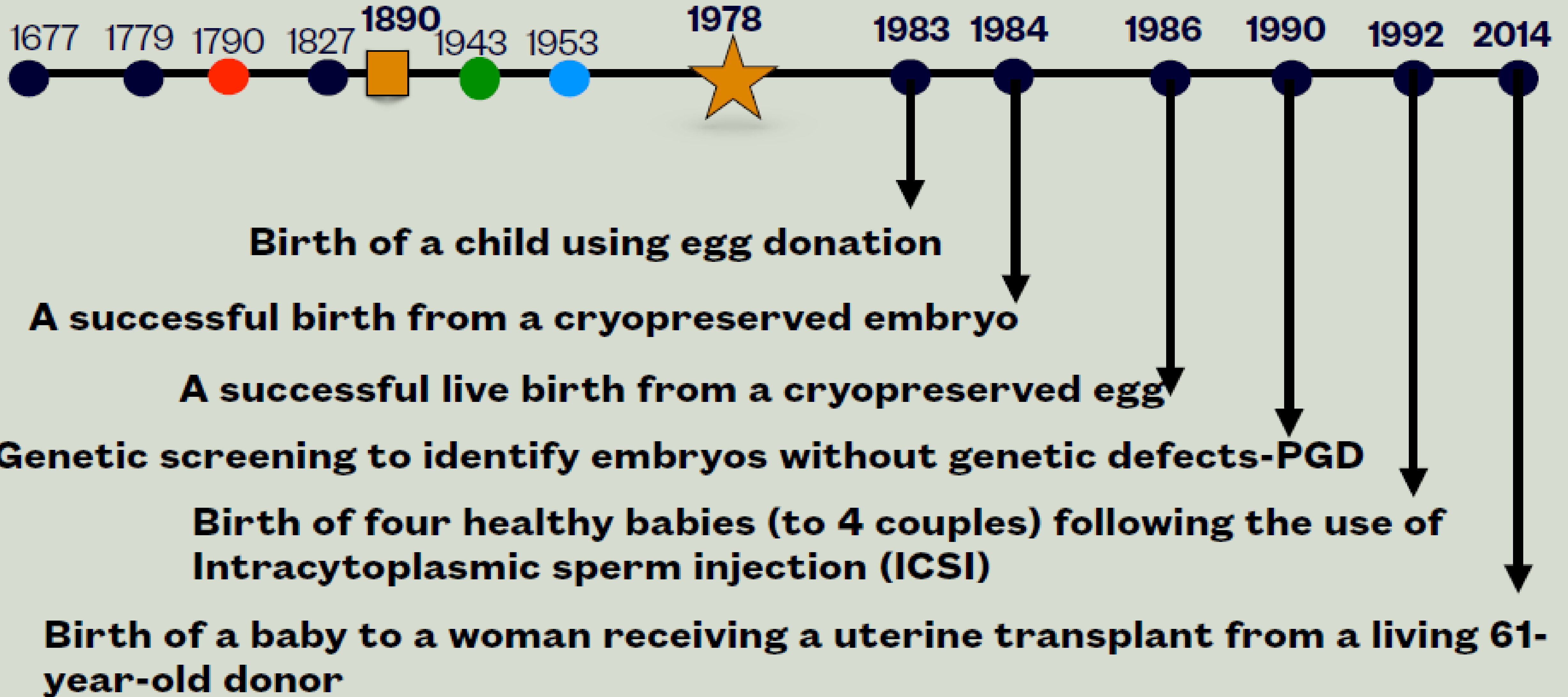
Evening News

LATE SPECIAL

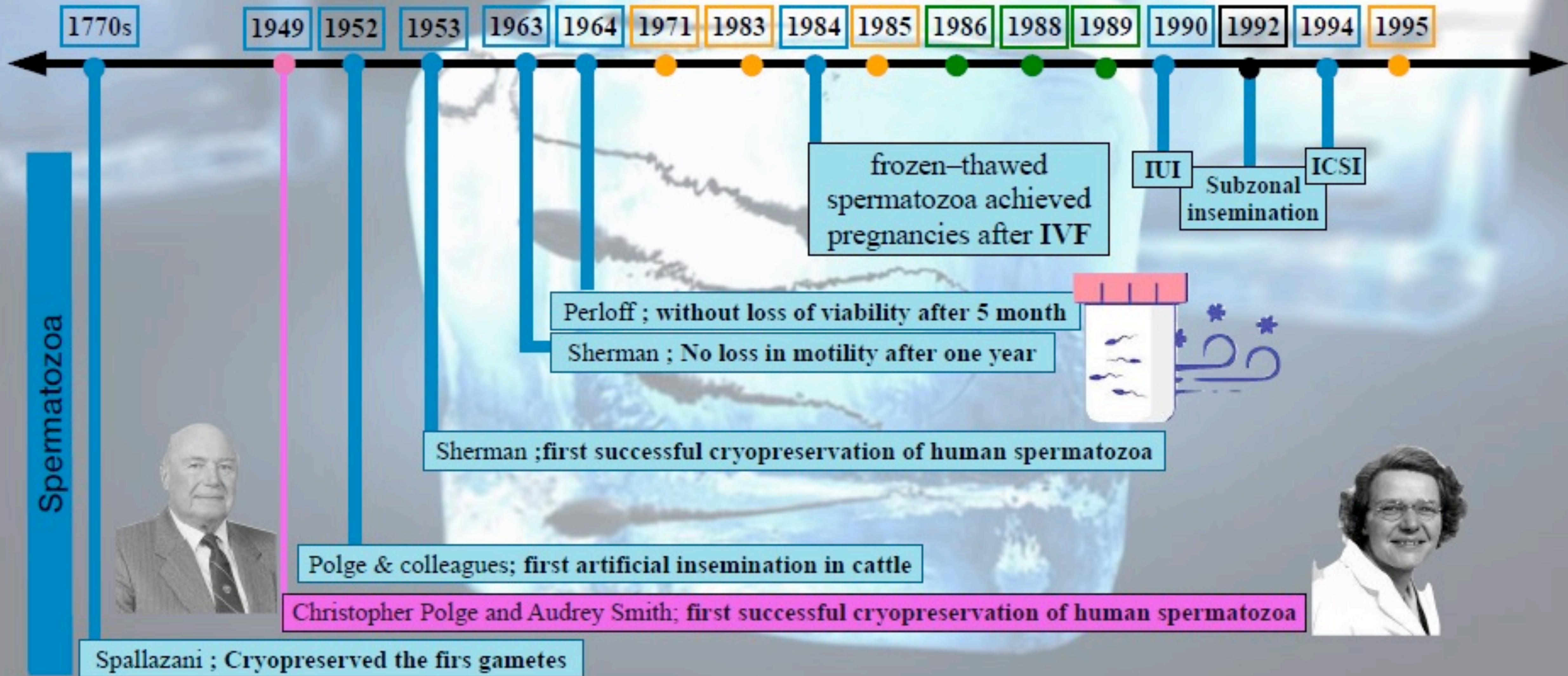
Meet Louise, the world's first test-tube arrival

SUPERBABE

Wide-eyed Louise Brown pictured in hospital 18 hours after she was born. Today she's doing well. See Page Three


History timeline
1677
1779
1790
1827
1890
1943
1953
1978
1983
1984
1986
1990
1992
2014
Birth of a child using egg donation
A successful birth from a cryopreserved embryo
A successful live birth from a cryopreserved egg
Genetic screening to identify embryos without genetic defects-PGD
Birth of four healthy babies (to 4 couples) following the use of Intracytoplasmic sperm injection (ICSI)
Birth of a baby to a woman receiving a uterine transplant from a living 61-year-old donor

Cryopreservation history timeline



Cryopreservation history timeline

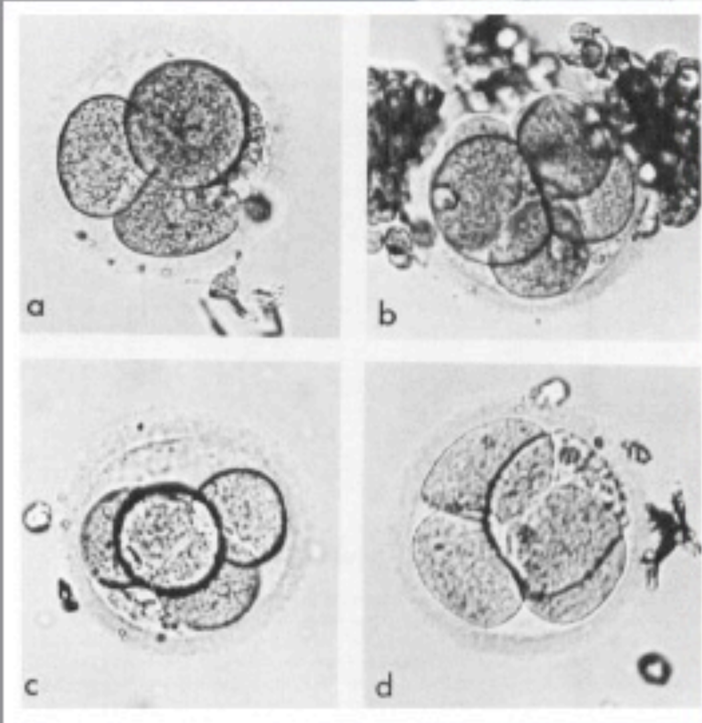
1770s | 1949 | 1952 | 1953 | 1963 | 1964 | 1971 | 1983 | 1984 | 1985 | 1986 | 1988 | 1989 | 1990 | 1992 | 1994 | 1995 | 1997 | 1999

day-2 human embryos were vitrified

Rall; cryopreservation by vitrification of mouse embryos

Trounsone and Mohr; first human pregnancy from embryo cryopreservation

Embryo



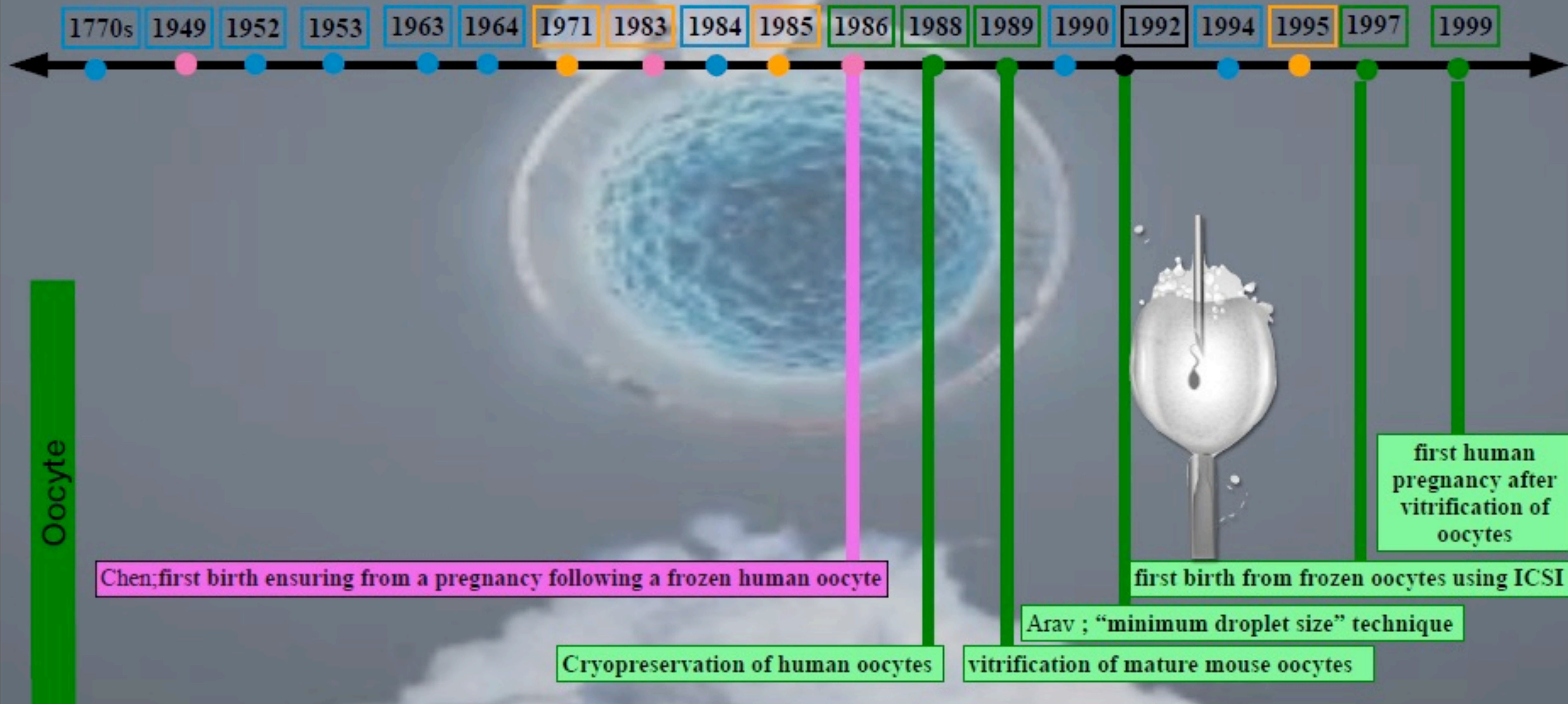
PART XIII. NEW DEVELOPMENTS, TECHNICAL ASPECTS, AND CLINICAL MANAGEMENT

Cryopreservation of Human Embryos

LINDA R. MOHR AND ALAN O. TROUNSON

Department of Obstetrics and Gynaecology
Monash University
Queen Victoria Medical Centre
Melbourne, Victoria 3000, Australia

whittingham ; first Cryopreservation in mouse embryos

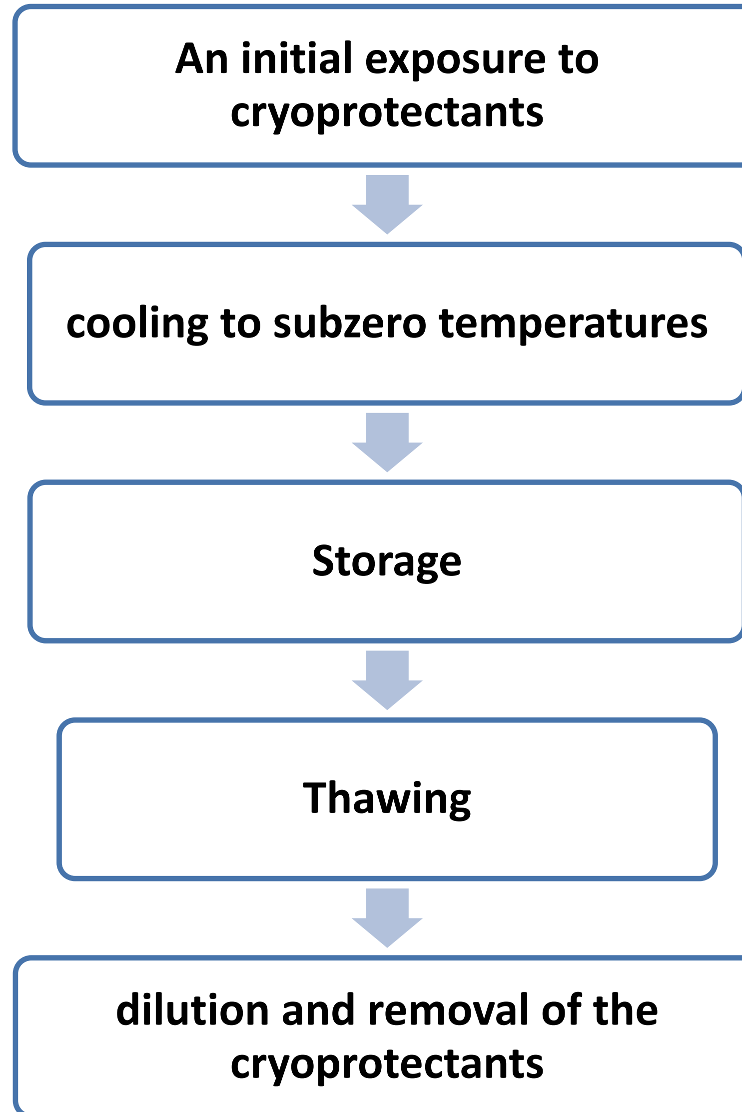


Cryopreservation history timeline
1770s
1949
1952
1953
1963
1964
1971
1983
1984
1985
1986
1988
1989
1990
1992
1994
1995
1997
1999
Oocyte
Chen;first birth ensuring from a pregnancy following a frozen human oocyte
Cryopreservation of human oocytes
vitrification of mature mouse oocytes
Arav ; "minimum droplet size" technique
first birth from frozen oocytes using ICSI
first human pregnancy after vitrification of oocytes

Basic Definition

- **Cryobiology** is the branch of biology that studies the effects of low temperatures on living things.
- **Cryopreservation** is the process of preserving and storing the living systems in a viable condition at low temperatures for future use.

Steps of Cryopreservation



Storage temperature

- Cryopreservation involves cooling and storage of cells in liquid nitrogen at a temperature of - 196o C where all metabolic processes are arrested.
- It reversibly arrests normal physiological processes

Principles of cryopreservation

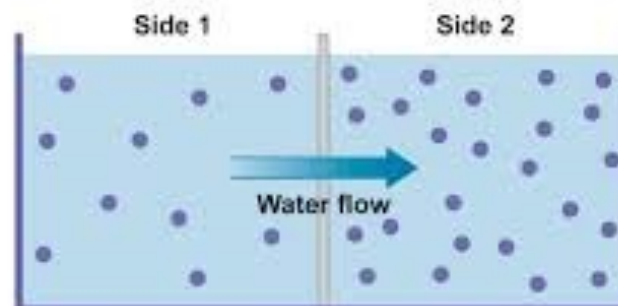
- Water in cell: Around 90% of water is free (water) while the remaining 10 % bounds to other molecular components of the cell (proteins, lipids, nucleic acids and other solutes). This water does not freeze and called hydrated water
  - Removal of water is necessary during freezing to avoid ice crystal formation, dehydration is limited to the free water
  - Removal of hydrated water could have adverse effect on the cell viability and the molecular function (freezing injuries)

PHYSICS

1. Water moves from a region of low osmolarity to a region of high osmolarity.

2. Nucleation temperature is the temperature at which water forms ice crystals.

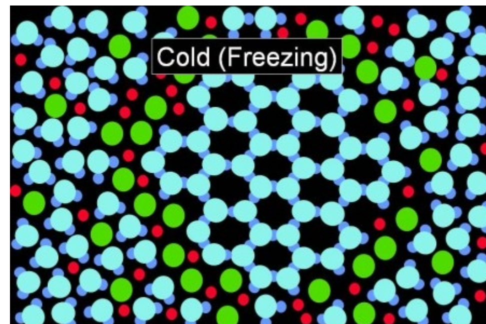
2. Solutes dissolved in the solution decrease the nucleation temperature of the solution.

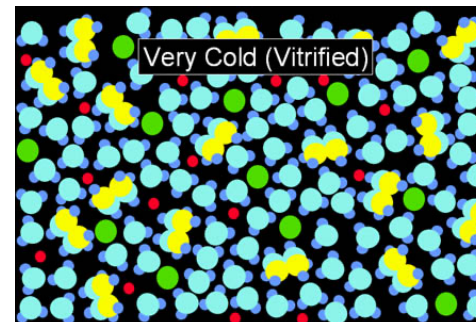


Talwar P and Sareen S,2010

Cryoprotectants additives (CPA)

- The first successful use of a cryoprotectant was in 1949, (glycerol)
- All cryoprotectants are completely miscible with water
- All are hyperosmotic
- They cause the water to form a glass structure rather than ice crystals.
- combination of CPA reduce toxicity
- Decrease the velocity of crystal formation and modify their shape into a smoother pattern

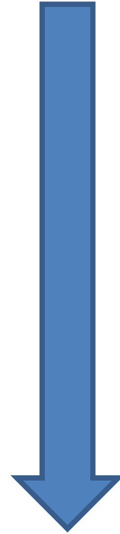




David Whaley et al, Cell Transplantation, 2021

Types of cryoprotectants

- **Permeating agents and Non permeating agents**
- ***Permeating agents***



- Ethylene glycol
- Glycerol
- propandiol
- Dimethyl sulfoxide (DMSO)
- Propylene glycol
- Acetamide

- more slowly than water Inside the cell
- stabilize intracellular proteins
- minimize osmotic damage due to electrolyte concentration effects

Ethylene Glycol is an ideal candidate for human embryo vitrification, high permeation ability and low toxicity compared with other CPAs

David Whaley et al, Cell Transplantation, 2021

Types of cryoprotectants

- Non permeating agents such as high molecular weight
- ➢ Saccharides (Sucrose,Trehalose, Glucose, and Galactose)
- ➢ Macromolecules (Ficoll, Polyvinyl pyrrolidone, Polyethylene glycol)
- Macromolecules are less toxic to the cells compared to permeating agents
- Egg yolk has been added to cryopreservation medium to preserve animal sperm, this is no longer recommended
- ➢ Bacterial contamination
- ➢ Batch-to-batch variation

David Whaley et al, Cell Transplantation, 2021

Mechanism of Action

- **Permeating Agents**

1. Reduction in electrolyte concentration
2. These agents diffuse out of cells more rapidly during warming and prevent osmotic swelling

Nonpermeating Agents

1. Osmotic dehydration during cooling
2. Restrict water influx by acting as an osmotic counter force

Functions as an osmotic buffer

methods of cryopreservation

- There are two methods of cryopreservation

❖ **Slow freezing**

❖ **Vitrification**

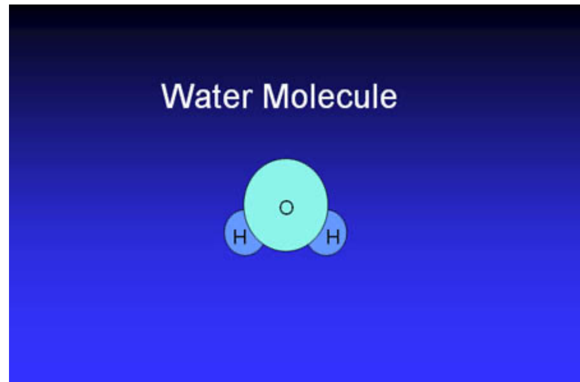
David Whaley et al, Cell Transplantation, 2021

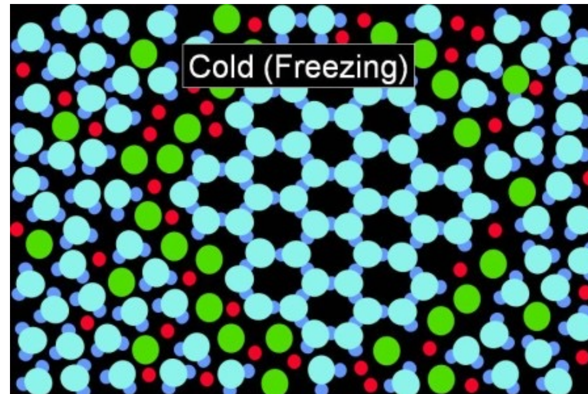
Slow Freezing

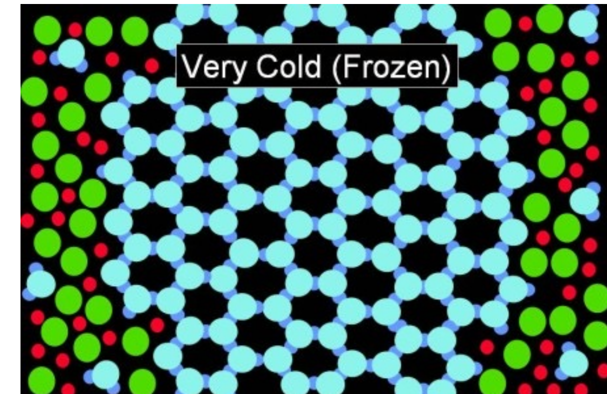
- As the temperature is reduced, less and less energy exists in the system to drive these kinds of molecular motions.

FREEZING

pure water → **organized solid lattice ;crystal.**

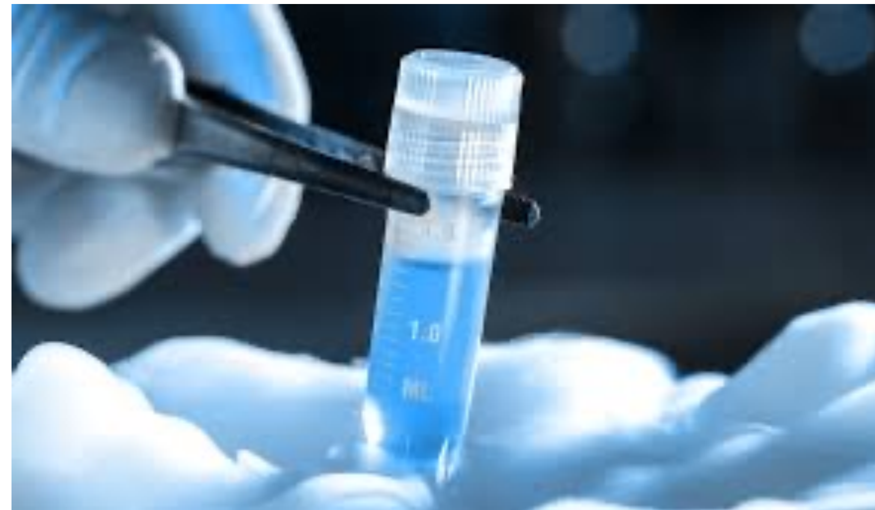






Slow freezing

- The procedures used for oocytes, embryos and ovarian cortex are generally quite similar.
- The cells or tissues exposure to CPA and frozen in straws or ampules
- Following CPA exposure the temperature is slowly lowered, and **ice crystal growth is initiated** in the solution ("seeding"). The ampules or straws are seeded at –6.5 to –7°C, cooled slowly at 0.3–0.5°C /min to approximately –40°C, then quickly cooled to –150°C before final transfer into liquid nitrogen for storage.



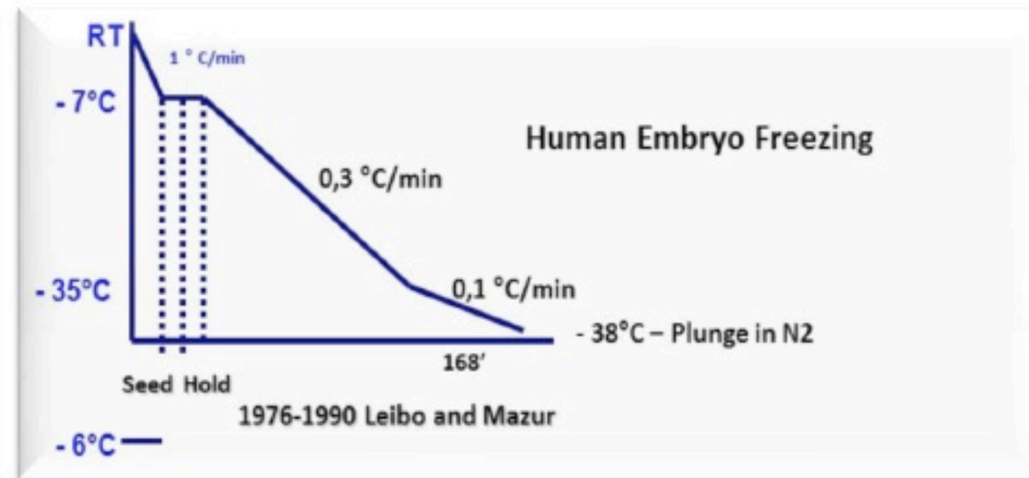
David Whaley et al, Cell Transplantation, 2021

Slow freezing

- A brief pre-equilibration of cells in cryoprotectant solutions: slow, gradual, controlled cooling at rates optimized for the type of cells being cryopreserved
- Special programmable cell freezing equipment
- requires 3-6 hours to complete

David Whaley et al, Cell Transplantation, 2021

Slow Freezing



Vitrification

- In other systems temperature reduction causes

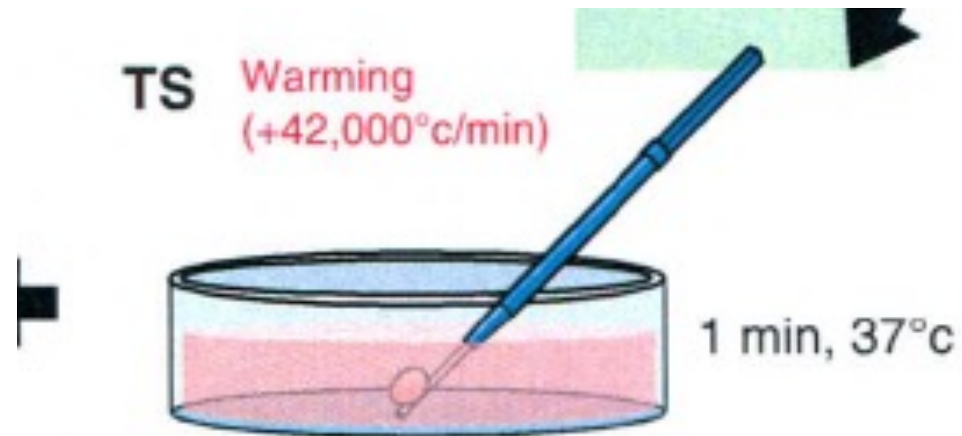
- **rapid cooling of liquid water** —vitrified→ **glass** (Amorphous ice)

- Molecules do not have enough time to form a crystal lattice

Vitrification Protocol

- Hold the Cryotop under a microscope and bring in focus by moving it up and down. If it is difficult to focus on, fix the Cryotop sheet on the bottom of an empty dish and put the embryo on it

Vitrification Protocol

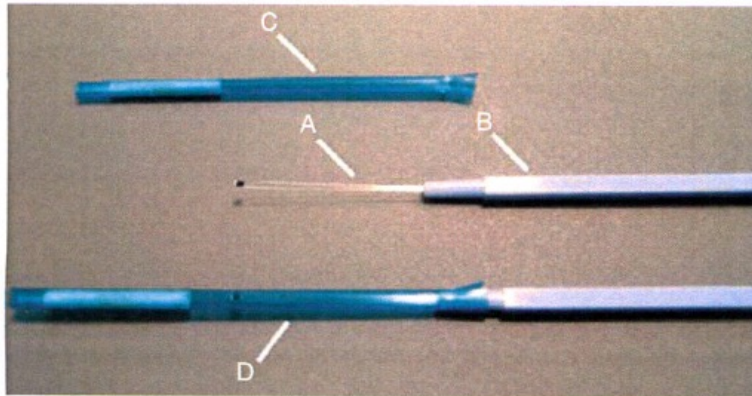


Vitrification Protocol

- Transfer the oocyte into the culture medium. Culture time for recovery is 2 hours.

Vitrification Devices

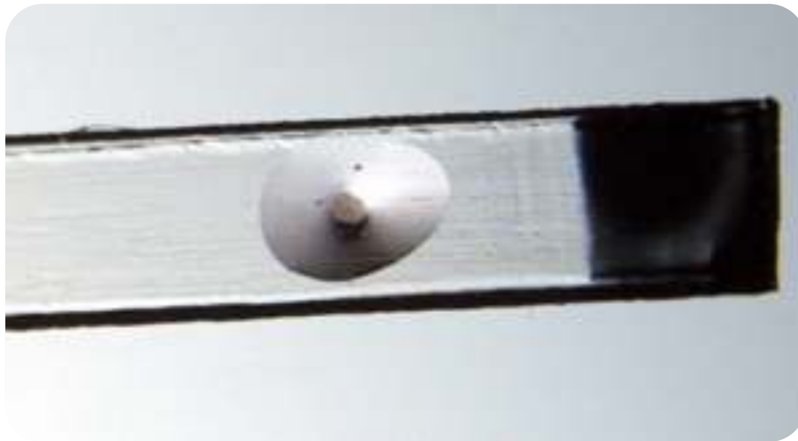
- Write the necessary information on the bar of Cryotop



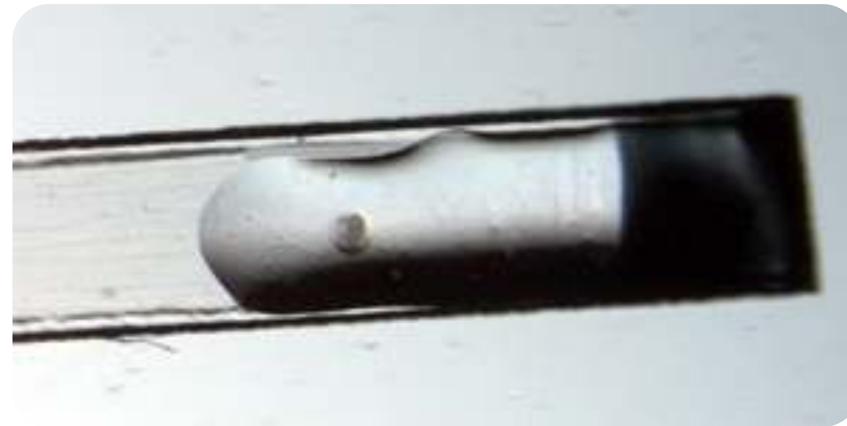
Vitrification Devices

- Put the embryo on the distal end of Cryotop sheet with minimum volume of VS solution (0.1µl or less) under the microscope.

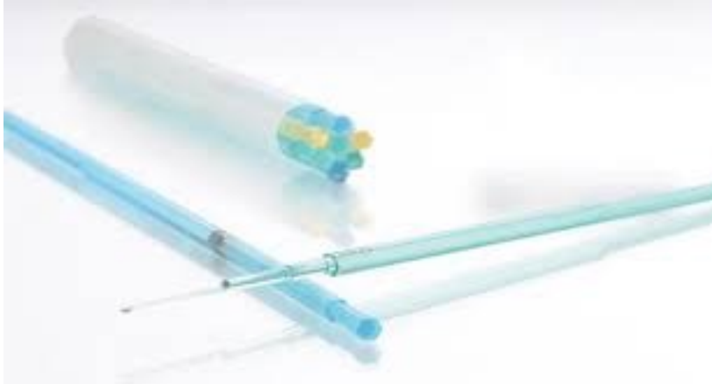
Good example



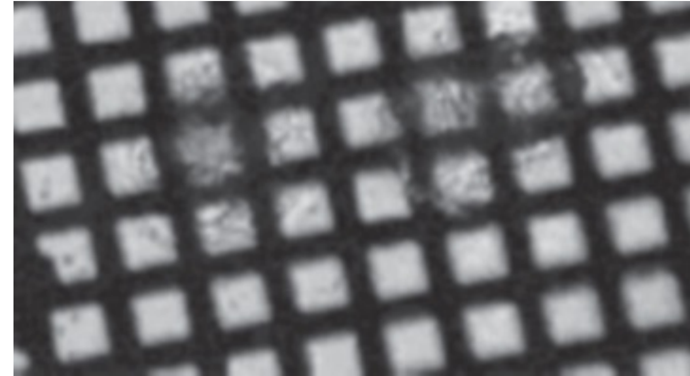
Bad example



Carrier systems



Cryoleaf



Electron microscopic grids

Cryolock

Cryo BioSystem HSV straw

Vitrification Devices – Open or Closed

Open Devices (e.g. Cryotop)

- Faster Cooling/Warming rates
- Direct exposure to LN2
- Contamination

Closed Devices (e.g. HSV)

- Slower Cooling and/or Warming rates
- No direct contact with LN2

Vitrification VS Conventional slow cooling

Table 1 – Comparison between the slow-freezing and vitrification methods

Characteristic	Procedure	
	Slow freezing	Vitrification
Working time	More than 3 h	Fast, less than 10 min
Cost	Expensive, freezing machine needed	Inexpensive, no special machine needed
Sample volume (μL)	100–250	1–2
Concentration of CPA	Low	High
Risk of freeze injury, including ice crystal formation	High	Low
Post-thaw viability	High	High
Risk of toxicity of CPA	Low	High
Status of system	Closed system only	Opened or closed system
Potential contamination with pathogenic agents	Low	High
Manipulation skill	Easy	Difficult

CPA, cryoprotective agent.

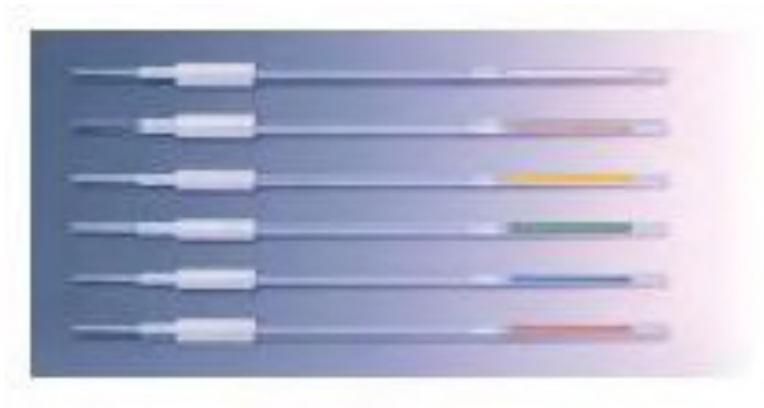
Tae Hoon Jang et ***al*, *2017*,** Integrative Medicine Research

Cryostore Management

- ✓ The capacity of a cryostore depends on the freezing strategy of the clinic and its activity, i.e. the number of cycles with potential freezing performed per year.
- ✓ The number of dewars/ vapor stores can then be predicted, always allowing for a spare dewar for emergency use.

Cryostore Management

- All patients should be screened for major viral markers in advance to minimize the risk of potentially infective material.



Cryostore Management

- **Use different storage tank:**
- ✓ Contagious viral disease sperm tank
- ✓ Emergency or clean tank
- ✓ Sterilization of liquid nitrogen with ultraviolet irradiation (Parmegiani et al., 2010)

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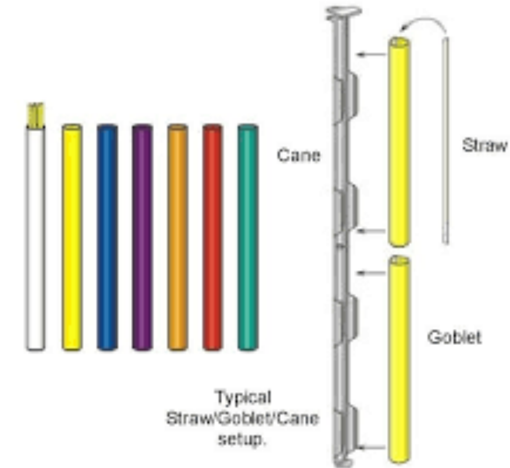
CE
0086

Cryostore Management

- Colored goblets help in keeping track of samples
- They can be arranged in alphabetical order, as shown here: aqua, black, blue, brown, green, gray, orange, pink, purple, tangerine, yellow, or in the order of the rainbow/white light spectrum.
- A canister that has just been removed from liquid nitrogen,

containing straws that are distributed between different colored goblets.



Cryostore Management

Patients who have the same name: all frozen material must have a unique identifiable code, usually with **three identifying features (full name, date of birth, unique clinic accession number);** appropriate checks/witnessing will decrease the chance that cryomaterial for the wrong patients is ever removed from storage.

Cryostore Management

All dewars must be inspected regularly for damage, making sure that the vacuum is functional. Visual inspection of the outside can sometimes reveal “cold spots,” which may indicate a fault with the vacuum.

Cryostore Management

- ✓ Failure to check dewars can have disastrous consequences for cryopreserved material.
- ✓ Dewars should be equipped with an alarm that is triggered by low LN2 levels, either via a platform base that registers weight change, or with a system that monitors temperature inside the dewar

An Ohio fertility clinic said that the remote alarm system on its storage tank was turned off, so it didn't know that the temperature had fluctuated, and that the consequences were worse than it initially thought — all 4,000 eggs and embryos in the cryofreezer are likely nonviable.

Cryostore audit

- ✓ It is important to maintain contact with patients who have their gametes/embryos in storage, and confirm that storage is in accordance with their wishes. The regulatory bodies of many countries produce their own consent forms to cover a defined period of storage.
- ✓ In-house consent forms can act as legal contracts between the patients and the clinic, clearly defining the action to be taken if the patients fail to maintain contact with the clinic or cannot be contacted.

HFEA (00)6 FORM FOR CONSENT TO STORAGE AND USE OF SPERM AND EMBRYOS

N.B. Do not sign this form unless you have received information about these matters and have been offered counselling. You may vary the terms of this consent or withdraw this consent at any time except in relation to sperm or embryos which have already been used. Please insert numbers or tick boxes as appropriate.

Full name (block capitals):

Any other name by which you have been known:

I. USE

a. I hereby consent to the use of my sperm for the following purposes:

i. in treating a named partner YES ☐ NO ☐

Full name of partner:

ii. in treating others YES ☐ NO ☐

iii. in any project of research YES ☐ NO ☐

Please state any particular conditions as to use:

b. I hereby consent to the use of my sperm to fertilise egg(s) in vitro and to the use of embryo(s) developed from these egg(s) for the following purposes:

i. in the treatment of myself together with a named partner YES ☐ NO ☐

Full name of partner:

ii. in treating others YES ☐ NO ☐

iii. in any project of research YES ☐ NO ☐

Please state any other conditions as to use (eg on the use of particular embryos):

Signature: ______________________ Date: DAY |_|_| MONTH |_|_| YEAR |_|_|

II. STORAGE

a. I hereby consent to the storage of my sperm:

Storage period in years   Maximum (10 years)*   YES ☐ NO ☐   If less please state YEARS ☐

*Centres are allowed to store sperm for longer periods for limited uses only.

b. I hereby consent to the storage of embryo(s) developed in vitro from egg(s) fertilised with my sperm:

Storage period in years:

Five years YES ☐ NO ☐   Ten years YES ☐ NO ☐   More than ten years YES ☐ NO ☐

If less than five years or some other period please state the number of years: YEARS ☐

I understand that consent to storage of more than five years must be accompanied by a completed HFEA(96)8 form which has been signed by a registered medical practitioner.**

**This does not apply to donors.

c. If I die or become mentally incapacitated my sperm or the embryo(s) developed in vitro from egg(s) fertilised with my sperm should:

		SPERM		EMBRYOS	
i.	be allowed to perish	YES ☐	NO ☐	YES ☐	NO ☐
ii.	continue in storage for the purpose given in 1a. (for sperm) and 1b. (for embryos) above	YES ☐	NO ☐	YES ☐	NO ☐
iii.	continue in storage for other purposes (please specify below)	YES ☐	NO ☐	YES ☐	NO ☐

____

____

d. Any other conditions of storage (eg for particular embryos). Please state: ______________________________

e. I understand that unless they are used beforehand embryo(s) developed in vitro from egg(s) fertilised with my sperm will have to be allowed to perish at the end of the storage period specified at llb.

Signature: ______________________ Date: DAY |_|_| MONTH |_|_| YEAR |_|_|

Cryostore audit

A specific email group within the clinic for patients with frozen material can be helpful in making sure that communications from patients always receive the appropriate attention.

Cryostore audit

A checklist to cover all eventualities is helpful in conducting a cryostore audit, including the question “Have the patients responded to any communication or sent any new instructions regarding consents?” If any part of the checklist for disposal cannot be completed, further investigation is required before removing any cryopreserved material from the cryostore.

Cryostore audit

- A change in consent to provided extended storage
- A change in consent for removal of gametes/embryos from storage and allowing them to perish
- A couple separate, one partner requests continued storage and the other requests termination of storage. Individual gametes may be stored accordingly, but problems arise in the case of embryos. In the UK, a "cooling off" period of one year is recommended to see if the couple can agree on a united decision; if they cannot agree, storage of cryopreserved embryos must be terminated.

Cryostore audit

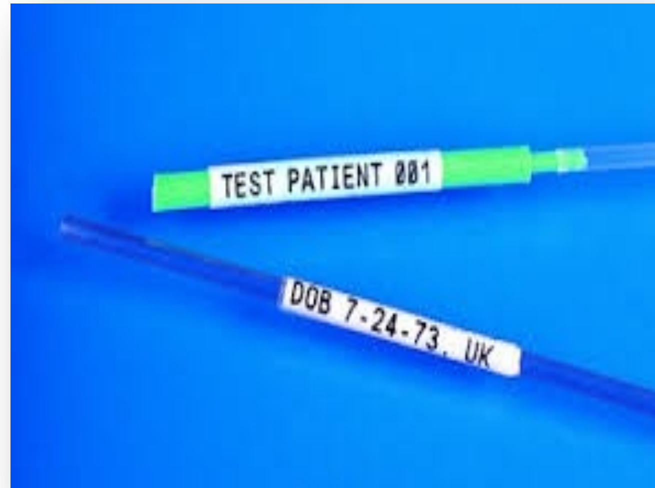
Couples approaching the end of their defined storage period should be contacted in advance, so that they have sufficient time to consider the options (usually to remove the gametes/embryos from storage, extend storage, or donate the material to a licensed research project).

Cryostore audit

Couples may have several gametes/embryos in storage generated in different treatment cycles. The gametes/embryos from specific dates must be used in accordance with the patients' wishes. Cryostorage from different dates should be highlighted in the patients' notes.

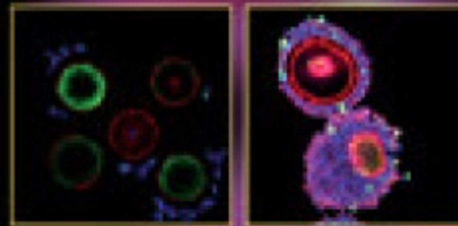
Cryostore audit

Mistakes can be avoided by establishing a system that includes robust checks before any gametes/embryos are removed from storage. Straw labels should have a unique cryonumber for the couple, and labels from thawed straws can be attached to the lab report for the thaw.



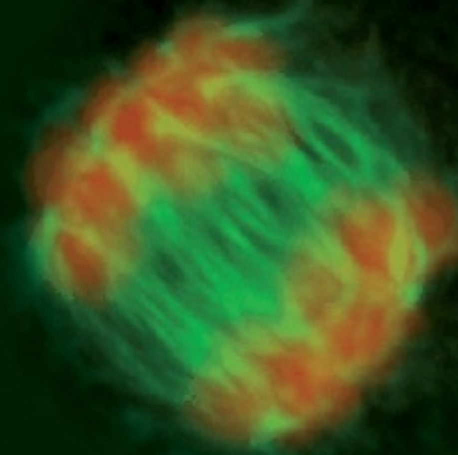
Principles and Practice of

Fertility Preservation

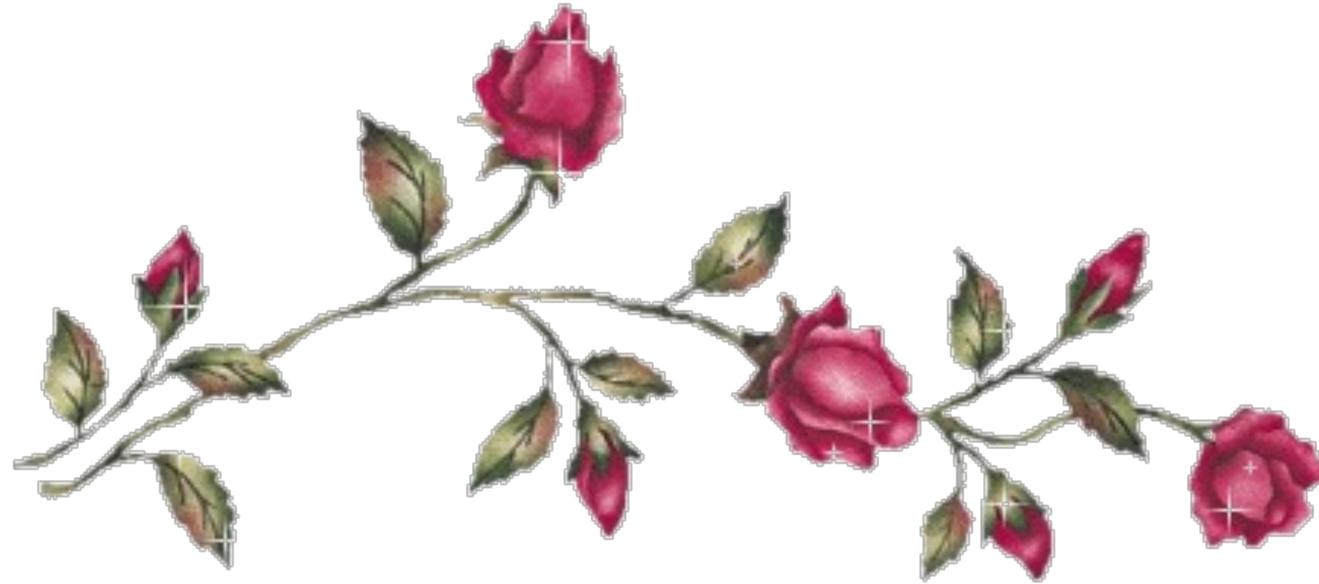


EDITED BY:
JACQUES DONNEZ
S. SAMUEL KIM

CAMBRIDGE Medicine

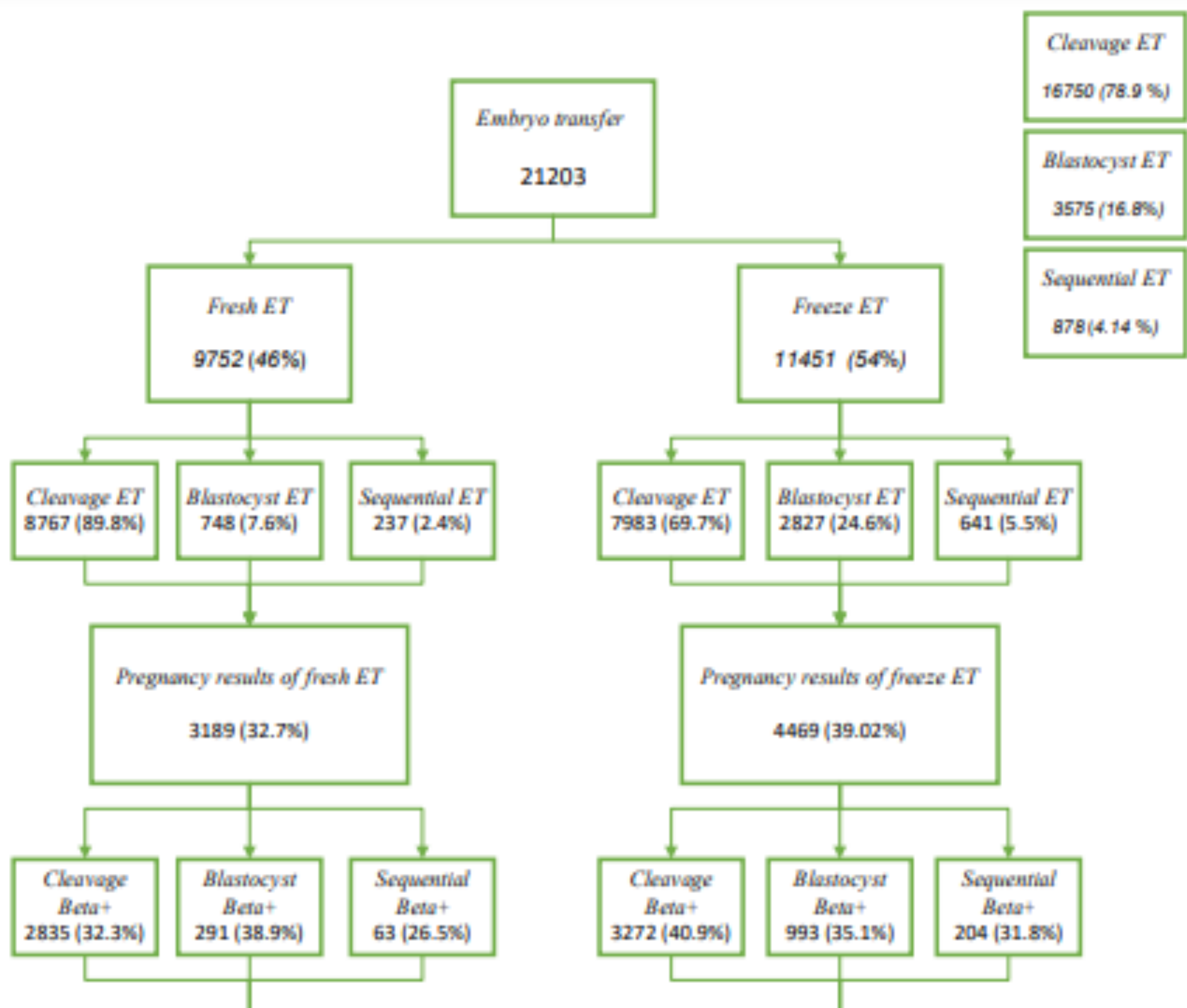

In-Vitro Fertilization
Kay Elder and Brian Dale
THIRD EDITION
CAMBRIDGE
Medicine

Thanks for attention



Practical tips for vitrification

- ✓ Participation in workshops provided by companies that supply vitrification media can be helpful, where experts can demonstrate optimal use of specific devices and media.
- ✓ Visiting a lab that has a good vitrification program can also be helpful.
- ✓ The vitrification technique can be practiced using unfertilized oocytes or abnormally fertilized embryos (both with patient consent) before it is applied in treatment cycles.



Embryo transfer
21203
Cleavage ET
16750 (78.9 %)
Blastocyst ET
3575 (16.8%)
Sequential ET
878 (4.14 %)
Fresh ET
9752 (46%)
Freeze ET
11451 (54%)
Cleavage ET
8767 (89.8%)
Blastocyst ET
748 (7.6%)
Sequential ET
237 (2.4%)
Cleavage ET
7983 (69.7%)
Blastocyst ET
2827 (24.6%)
Sequential ET
641 (5.5%)
Pregnancy results of fresh ET
3189 (32.7%)
Pregnancy results of freeze ET
4469 (39.02%)
Cleavage Beta+
2835 (32.3%)
Blastocyst Beta+
291 (38.9%)
Sequential Beta+
63 (26.5%)
Cleavage Beta+
3272 (40.9%)
Blastocyst Beta+
993 (35.1%)
Sequential Beta+
204 (31.8%)