INDIAN FERTILITY SOCIETY & CRYOBIOSYSTEM-IMV INITIATIVE

CRYOBIOLOGY IN ASSISTED REPRODUCTION

The technology of cryopreservation forms the backbone of a successful ART programme today, whether it is the cryopreservation of supernumerary embryos or freeze-all embryos as a part of segmentation in cases of imminent ovarian hyperstimulation syndrome.

Freezing of embryos as a part of biopsy and preimplantation genetic diagnosis or screening, or gamete cryopreservation either electively, or those diagnosed with malignancy and about to start a potentially sterilizing treatment are other common indications.

Optimising of the cryopreservation technique is required to achieve excellent pregnancy and live birth rates comparable, and sometimes superior to transfer in a fresh cycle. This involves a deep understanding of the media used, the importance of the adherence to the protocols formulated by different manufacturers, and an in-depth knowledge of the various carrier devices used for oocyte and embryo loading.

It gives me immense pleasure that IFS is organising this series of meetings & workshops in different parts of the country in the forthcoming months. I would like to sincerely thank “Cryobiosystem” for supporting us in this academic endeavour.

Dr M Gouri Devi
President - IFS

Cryobiology has been very dear to my heart since years. Things were not very simple in our era of learning and we did not have good teachers . You Tube and Google were not very helpful either and learning was with one to one interaction with a kind teacher if we could find one. It took me a long time to understand nuances of vitrification which I started 2005 onwards with multiple learning failures in the beginning.

Our present endeavour is focussed on vitrification process which is a rapidly emerging technique of cryopreservation. Embryos were routinely cryopreserved by slow freezing methods a decade back. However, there are some constraints with these protocols which includes - longer time required, intracellular ice crystal formation leading to cellular damage and need for expensive programmable freezing equipment.

Vitrification leads to ultra-rapid cooling of a solution containing high concentration of cryoprotectant, inducing a glass like state, as a result avoiding ice crystal formation and cellular damage. It also favours short equilibration time, fast cooling rates and no expensive equipment. Vitrification has come a long way and till date the results obtained with this process are equal or better than conventional slow freezing techniques.

In our meetings, the intricacies of the vitrification technique will be discussed and we sincerely hope that these workshops would benefit senior, junior & trainee embryologists and clinicians with a keen interest in the nuances
of gamete cryopreservation.

I am personally obliged to all local workshop coordinators who have worked hard to bring the program to fruition.

Last but not the least, sincere thanks to Cryobiosystem team for supporting this scientific and educational initiative. I also would like to thank Mr. Shisodia, Mr. Jitender and Mr. Chaman for enabling the same.

Prof (Dr) Pankaj Talwar
Secretary General -IFS

Dear Colleagues

We are delighted to host a series of meetings and workshops all over India focussed on Cryobiology.

Having reached a bottleneck in ART in terms of success rates, the endeavour to improve the pregnancy and livebirth rates has intensified. Whether it is the transfer of a single genetically normal embryo deemed disease free and euploid by preimplantation genetic diagnosis and screening, the transfer of embryo/s in a more physiological uterine environment as a part of "freeze-all" and "segmentation" strategy or simply achieving a higher cumulative success rate by freeze-thaw-transfer of supernumerary embryos produced by a single stimulation, the cornerstone of all these modalities is vitrification.

It gives me immense delight to present a comprehensive handbook on Cryobiology in ART, which we have put together by contributions from the various eminent experts in the field. We have strived hard to include all aspects of cryopreservation pertinent to the field of ART, covering topics from setup of a cryopreservation unit to the safety and risk management issues. This handbook should provide a brief compendium regarding cryobiology in ART to the user.

Moreover, hands-on session focussed on open and closed systems of vitrification have been included to help the delegates refine their skill of vitrification and clear any doubts with the experts.

We hope that you enjoy these knowledge filled capsules of extensive meetings and workshops over the forthcoming months.

I would especially like to thank the Cryobiosystem team for their constant support to help us organize these meetings and bring the program to fruition. Last but not the least, a heartfelt thanks to all the contributors and the local organising chairpersons for their valuable contributions, without which this initiative would not have been possible.

Dr. Pranay Ghosh
National Co-ordinator, IFS Cryobiology initiative

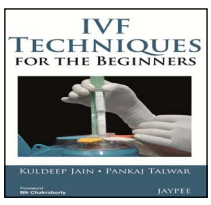
Dr M Gouri Devi
M.D

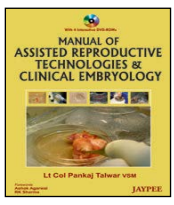
- Director, Gouri Hospitals Ltd.
- Director, Ridge IVF Group.(Runs a chain of IVF centres)
- President, Indian fertility society
- Ex-Secretary General, Indian Fertility Society
- Executive, AOGD governing council
- Member, Executive Board, NARCHI, DGES, FPSI
- Ex Vice President, NARCHI
- Chairperson, Advocacy &Ethics Committee, IFS.
- State Quality Assurance Committee (SQAC)Govt of NCT of Delhi.
- Member: MTP advisory committee, Govt Of NCT of Delhi
- Member Advisory committee on ethical practices in the field of obstetrics, Govt of NCT, Delhi
- Recipient of Kanak Goel Award 1995-1996 from IMA.
- Chairman's Appreciation Award by IMA AMS – 2002
- Dr. APJ Abdul Kalam Excellence Award – 2017
- Economic Times Award one of the Most Inspiring Gynecologists of India

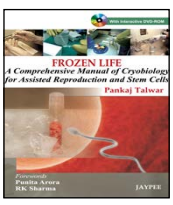
She is a keen academician, has organized many conferences, has been a speaker in many national and international conferences. Has many publications to her credit

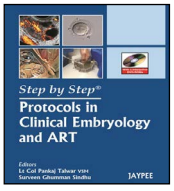
Col Pankaj Talwar
VSM, Professor and HOD
ART Centre, Army Hospital, New Delhi

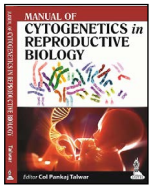
- Sec IFS.
- Secretary Fertility preservation society of India.
- Editorial board of multiple Infertility journals.
- Member Advisory committee ICMR
- Member Infertility committee FOGSI
- Editor nexus – E bulletin of IFS
- Awarded Vishisht seva medal by the President of India for working in field of infertility
- Associate Editor FSR
- Set up two centres for Armed forces .
- Experience of 10,000 and ET cycles .
- Member International society of fertility preservation.
- Trained Human Embryonic Stem Cell Derivation – Israel
- Trained in ovarian cortex freezing (fertility preservation) - Paris
- Trained in PGD – Germany, Spain
- Trained in QA/QC- Spain
- Edited 6 books

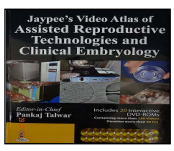












Dr Pranay Ghosh
Director, Elixr Fertility Centre
Consultant, Double Helix Clinical Cytogenetics
and Reproductive Immunology Centre

- MBBS (MAMC)
- MS (MAMC)
- M.Med.Sci ART (University of Nottingham, UK)
- Diploma Reproductive Medicine (University of Kiel, Germany)
- Specialist Training in Reproductive Medicine (NUS, Singapore)
- Fellowship in Minimal Access Surgery
- ESHRE certified Clinical Embryologist

Special areas of interest: Reproductive Immounology, Oocyte and Embryo biology and optimizing strategies for eSET

Organising Chairpersons

Dr Shilpi Sud
LOC: Nagpur

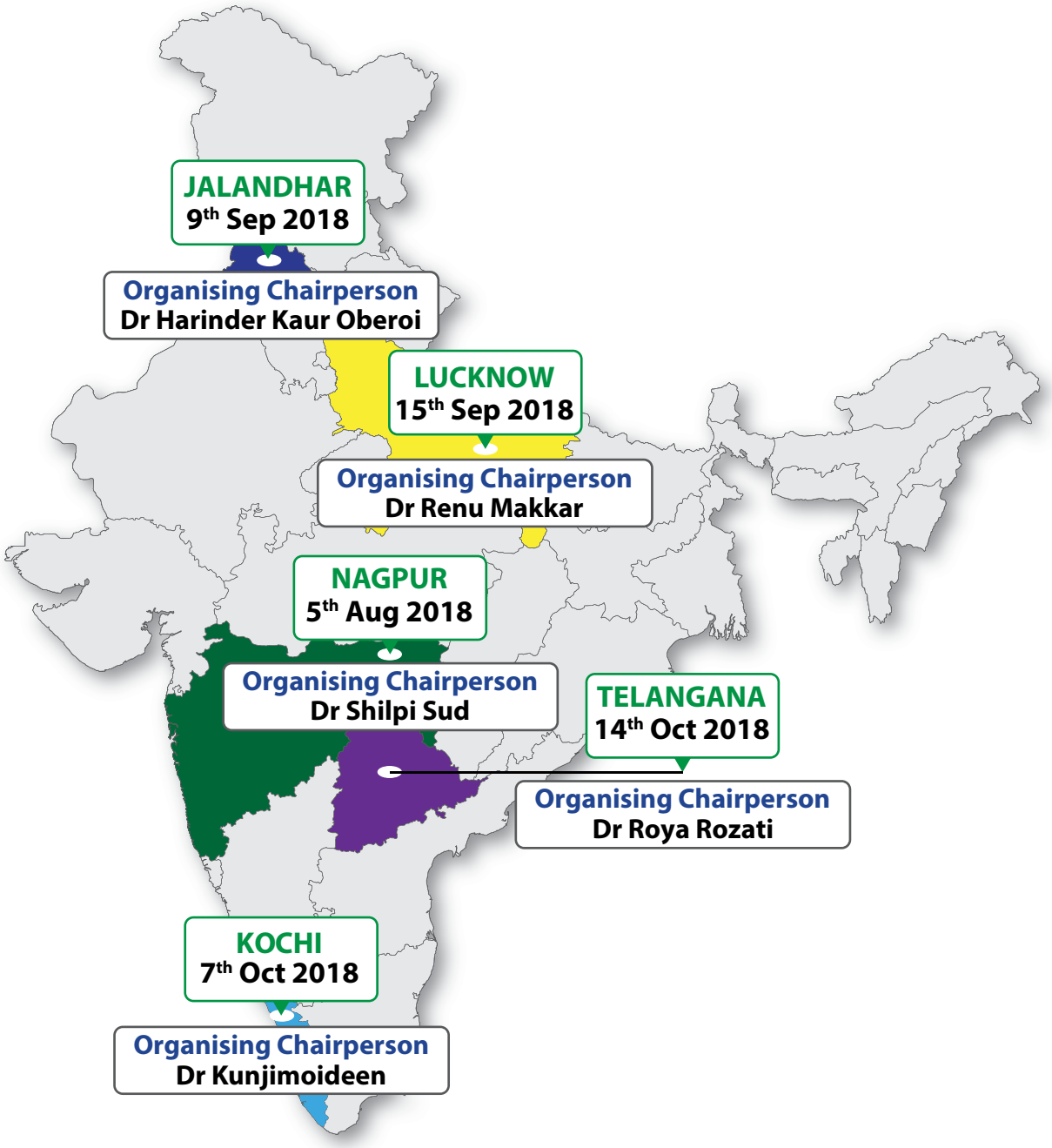
Dr Harinder Oberoi
LOC: Jalandhar

Dr Renu Makker
LOC: Lucknow

Dr Kunjimoideen
LOC: Kochi

Dr Roya Rozati
LOC: Telangana

Venue & Dates


JALANDHAR
9th Sep 2018
Organising Chairperson
Dr Harinder Kaur Oberoi
LUCKNOW
15th Sep 2018
Organising Chairperson
Dr Renu Makkar
NAGPUR
5th Aug 2018
Organising Chairperson
Dr Shilpi Sud
TELANGANA
14th Oct 2018
Organising Chairperson
Dr Roya Rozati
KOCHI
7th Oct 2018
Organising Chairperson
Dr Kunjimoideen

Programme for the day

CRYOBIOLOGY IN ASSISTED REPRODUCTION

Time	Topic
08:30 - 09:00	Registration
09:00 - 09:15	Welcome address
09:15 - 09:35	How to set up a Cryobiology unit
09:35 - 09:55	Advances in Cryobiology
10:00 - 10:20	Is it time to move towards “freeze-all” strategy?
10:20 - 10:40	Oocyte vitrification
10:40 - 11:00	Tea & discussion
11:00 - 11:20	How to have 100% cryosurvival in a vitrification program
11:20 - 11:40	Risk management in cryopreservation in ART
11:45 - 01:30	Hands on demonstration of vitrification and warming
01:30 Onwards	Lunch

List of Contributors

Topic	Contributed by
How to set up a Cryobiology unit	Nishad Chimote
Advances in Cryobiology	Dr Pranay Ghosh
Is it time to move towards "freeze-all" strategy	Dr Renu Makker
Oocyte vitrification	Dr Pranay Ghosh
How to have 100% cryosurvival in a vitrification program	Nancy Sharma
Risk management in cryopreservation in ART	Dr Feseena Kunjimoideen

Nishad Chimote
Lab Director and Senior Embryologist,
Vasundhara Clinic and
Assisted Conception Centre, Nagpur

Dr. Pranay Ghosh
MBBS, MS, M. Med. Sci (ART) (Nottingham, UK),
DRM (University of Kiel, Germany), F.MAS,
ESHRE Certified Clinical Embryologist
Director & Chief Embryologist,
Elixir Fertility Centre

Dr Renu Makker
Director, Makker IVF Centre,
Lucknow

Nancy Sharma
Clinical Embryologist
Akanksha IVF Centre

Dr Feseena Kunjimoideen
MCE(UK) Embryologist,
ARMC IVF Fertility Centre, Kozhikode, Kerala

Index

1. How to set up a Cryobiology unit

Introduction

- The success of any Freezing Program depends on its Embryology and Cryolab. The primary function of a Cryolab is to provide an optimal environment for gametes and embryos to be stored properly in LN2 for long durations and high survival rate upon thawing.

Crux of the matter

Your Cryopreservation program is only as good as the Lab which supports it.

Salient Features

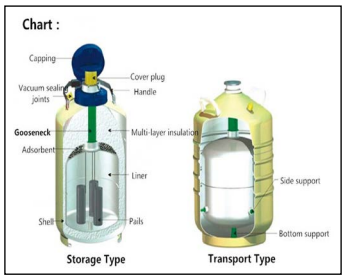
- **Lab Design**
- **Equipments**
- **Consumables**
- **Air Quality**
- **Quality Control**

ART Lab Layout

- **Airlock**
- **Operation Theatre**
- **Embryology lab**
- **Cryopreservation Lab / Room**
- **Andrology Lab**
- **Stock Room**
- **Cylinder room**
- **Changing Room**
- **HVAC Room**
- **Power back up**

Laminar Hood and work station

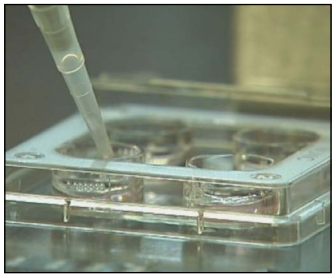
Equipments

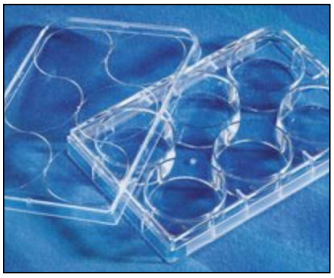


LN2 Dewar's for storage and transportation

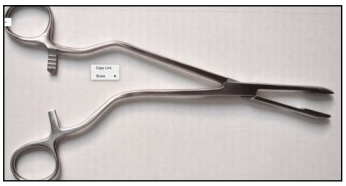
Vitrostash Dewars and Cannistars

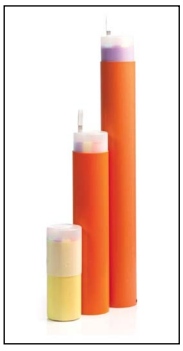
Dishes for Freezing and thawing

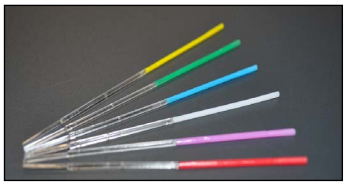




Essentials and Consumables







Stocking and Storage

Acrylic boxes (NO VOC)

Consumable stock and storage

Fridge for Media Storage

Vitrostash Dewars and Cannistars

Label printers and stickers

BMP21 BMP51

Using our LN2 resistant labels, it is strongly recommended to overlap the label on the straw.

Do's and Don't's

- Wear protective Gear and gloves while handling LN2
- Make sure there are at least 2 personnel in the lab
- Keep the doors open while transferring LN2
- Make sure that the O2 monitoring system is working
- Maintain the lab Temp below 24 deg C & Humidity below 40%
- No VOC should be used especially rubber
- Avoid Direct sunlight and High temp near the LN2 Dewars

CRYOBIOLOGY LAB

ESSENTIALS

- Quiet place allowing focused work
- **Preferred LN2 resistant flooring**
- Elevated ambient room temperature around or slightly above 25 C
- Safe and efficient ventilation of the room is an absolute requirement
- Separate storage room, with restricted entry to ensure safety of gametes and embryos

CRYOBIOLOGY LAB

ESSENTIALS

- Plan for the future
- Cryocontainers:
  - always stored on the floor & never on shelves
  - should not hamper free passage
  - Easily accessible without disturbing the other tanks, and
  - roll base is most advisable for each single tank

QUALITY CONTROL

- LABORATORY ENVIRONMENTAL CONTROL
  - Ambient temperature, ventilation and humidity should be maintained
  - Rapid circulation of air in the freezing & cryostorage room
  - Monitor oxygen levels
  - No power fluctuations

- CONTAINERS : maintain at -196C ,avoid fluctuations temperature
- daily checking of LN2 levels, tempertaure
- Warning signs:
  - Not maintaining appropriate level or temperature
  - Fast rate of evaporation
  - condensation or ice appears on the external surface
  - At no point of time transport dewars should go outside the facility for refilling

SOPs & RECORD MAINTAINENCE

- Written quality control protocol for each equipment
- Separate ledgers for
  - Procedure & its frequency
  - Filling of LN2
  - Engineer / maintenance visits

DISASTER MANAGEMENT

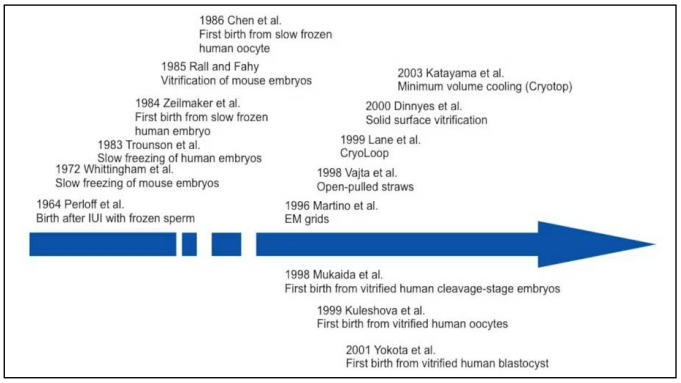
- Emergency plan in place for disaster management
- Easy access and escape route covered with LN2 resistant material to and from the cryostorage room is essential not only for LN2 supply but also to rapid removal of the most valuable biological material in case of emergency

Conclusion

Cryo lab can be setup and maintenance is very essential in order to have a successful freezing program in the ART Center and keeping track of all the things with a proper QC and Embryologist is the key this success

2. Advances in Cryobiology

Introduction



This timeline shows the important landmarks associated with cryopreservation in the field of ART.
Whittingham – slow freezing, Trounson – first slow-frozen pregnancy, Zeilmaker – first slow-frozen live birth

Evolution of vitrification

- "Life & death at low temperatures" – Basile Luyet (1940)
- Joseph Louis Gay-Lussac (1804): small vol. vitrificationFound out that water can be cooled to -12°C without freezing

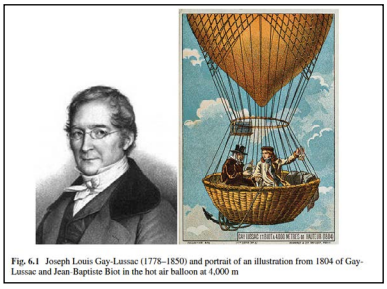


Fig. 6.1 Joseph Louis Gay-Lussac (1778–1850) and portrait of an illustration from 1804 of Gay-Lussac and Jean Baptiste Biot in the hot air balloon at 4,000 m

French chemist & physicist Joseph Louis

When ascending in a hot air balloon, he noticed that the drops in clouds are not frozen despite the subzero temp.

Evolution of vitrification

CRYOPRESERVATION IN HUMAN ART TODAY

Survival rates

	Historically	Today
Gametes		
Spermatozoa	Moderate	Moderate
Oocytes	Poor - Moderate	High
Embryos		
Pronuclear-stage	High	High
Cleavage stages	Moderate	High
Morula	Moderate	High
Blastocysts	Poor	High

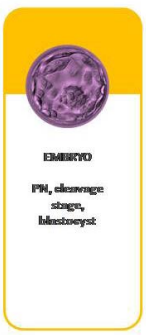
Following the advent of vitrification, high post-thaw survival recovery is possible for all embryonic stages and oocyte now, though sperm cryopreservation post thaw results are still modest.

What all can be frozen?

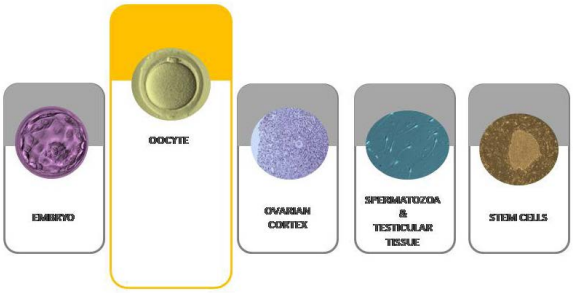
Indications

Indications

1. Avoidance of multiple gestation
2. Unfavorable endometrium/difficult E
3. OHSS (freeze-all)
4. Avoidance of synchronization in oocyte donor cycle
5. Oncofertility
6. Embryo donation programs
7. PGD/PGS



Indications



Indications

1. Better synchronization of donor recipient cycles
2. Oncofertility preservation
3. Male partner unavailable or unable to produce semen sample on day of OPU
4. Pts. at risk of POF
5. OHSS
6. Legal, ethical or moral issues with embryo cryopreservation
7. Women wanting to delay conception
8. Cryobanking oocytes for egg donation
9. Cryoaccumulation (Accu-vit)

Indications

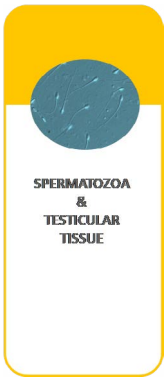
Indications

1. Oncofertility preservation (adv. over oocyte cryopreservation)
- restoration of endocrine function
- in some cases, obviates the need to delay treatment for a stimulation cycle
- resumption of menses
2. Pts. At risk of premature menopause
3. Benign diseases requiring chemotherapy (autoimmune diseases)
4. BMT (benign hematological diseases: sickle-cell anaemia, aplastic anaemia)
- inadequate permeation of CPA
- possibility of re-introduction of malignant cells

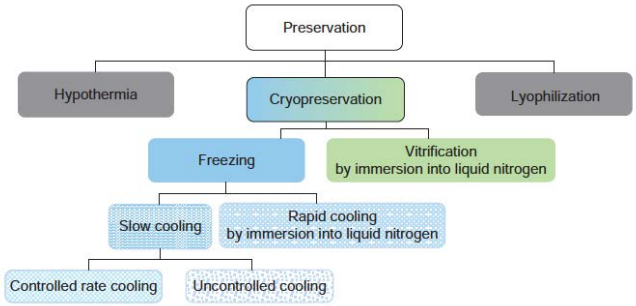
Indications

Indications

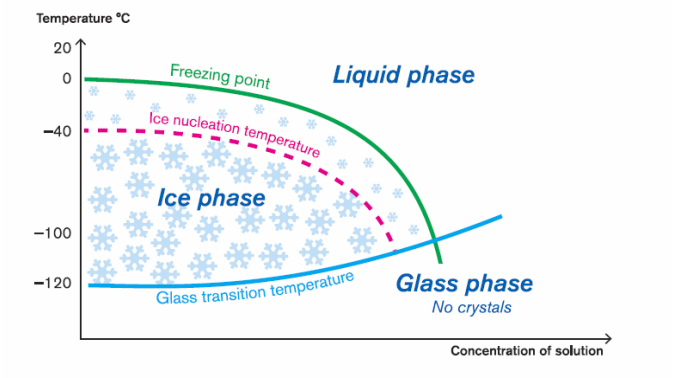
1. Oncofertility preservation
2. Non-malignant diseases like diabetes & autoimmune disorders
3. Donor insemination tand heterologous fertilization (where allowed)
4. Following SSR to avoid repeated biopsies or aspirations
5. Prior to starting ART to avoid "semen collection stress"
6. Males exposed to gonadotoxic agents
7. Sperm Banking



Cryopreservation strategies



Important considerations



Rall & Fahy's phase transition diagram

Important considerations

Arav's pseudo-equation

$$\text{Probablility of vitrification} = \frac{\text{CR X WR X viscosity}}{\text{volume}}$$

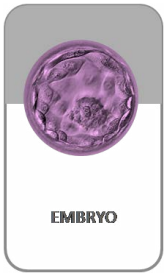
Progress in vitrification

- Move towards surface techniques from tubing techniques
- Minimum volume loading
- Development of devices allowing direct contact and minimal volumes
- Importance of warming rates
- Accelerating cooling rate by direct submersion in LN2
- High viscosity CPA

Advances in cryopreservation

Advances in cryopreservation

1. Move towards surface techniques (cryotop, cryoleaf etc.) from tubing (straw)
2. Replacement of serum substitute supplement by hydroxypropyl cellulose (HPC)
3. Trehalose instead of sucrose
4. LASER blastocoel collapse prior to blastocyst vitrification
5. Preference to open carrier devices

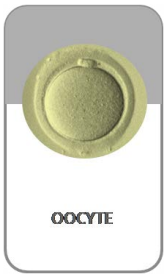


Advances in cryopreservation

- Largest cell with largest volume

Cell	Surface Area	Volume	SA:Vol
Oocyte	4.5 x 10^4 µm^2	9 x 10^5 µm^3	0.05

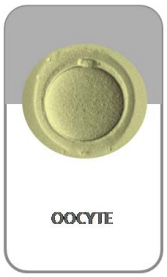
- Spherical shape: permeation gradient, inadequate permeation
- Meiotic spindle
- Actin scaffolding
- Low cell number



Advances in cryopreservation

CRYODAMAGE

- Nucleus & nuclear membrane
- Cytoplasm
  - Microtubules: MS
  - Microfilaments: Cortical scaffolding
  - Mitochondria: Swelling & abnormal distribution
- Zona pellucida
  - Premature corticle granule exocy tosis
  - Zona hardening



Results with oocyte vitrification

ASRM PAGES

Mature oocyte cryopreservation: a guideline

The Practice Committees of the American Society for Reproductive Medicine and the Society for Assiste Reproductive Technology

Society for Reproductive Medicine and Society for Assisted Reproductive Technology, Birmingham, Alabama

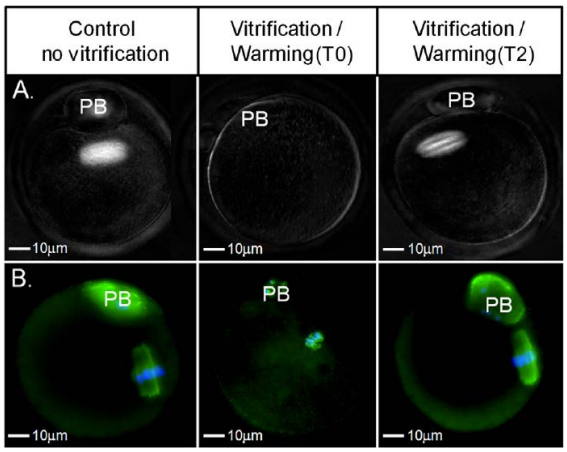
There is good evidence that fertilization and pregnancy rates are similar to IVF/ICSI with fresh oocytes when vitrifie are used as part of IVF/ICSI for young women. Although data are limited, no increase in chromosomal abnormalities, developmental deficits has been reported in the offspring born from cryopreserved oocytes when compared to pregr ventional IVF/ICSI and the general population. Evidence indicates that oocyte vitrification and warming should no lor experimental. This document replaces the document last published in 2008 titled, "Ovarian Tis-

Efficiency of vitrified oocyte (Safety)

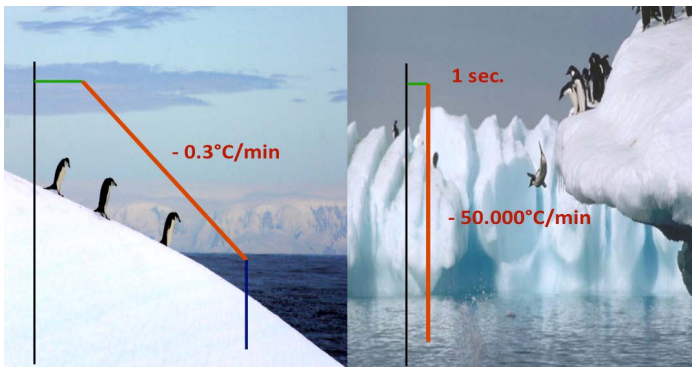
1. Survival & pregnancy rates
   Cobo et al, 2008, 2009, 2010
2. Gene expression
   Di Pietro et al, 2010
3. Aneuploidy
   Garcia et al, 2011
4. Congenital anomalies
   Noyes et al, 2009
5. Perinatal outcomes
   Chian et al, 2008; Cobo et al, 2010

} No Difference

Meiotic spindle integrity & repolymerization (Safety)



Slow-freezing vs. vitrification



Slow-freezing vs. vitrification

- Fadini et al (2009): Vitrification provided higher survival (79% vs. 58%), PR (18.7% vs. 7.6%) & IR (9.3% vs. 4.3%)
- Cao et al (2009): **Higher survival** (91.8% vs. 61%), **high quality embryos** (42 % vs. 24%) and **top quality blastocyst** (33% vs. 12%) with vitrification
- Cytoplasmic effect of OCP: **Spindle recovery** is accelerated in vitrified embryos (Chen & Yang, 2009; Ciotti et al., 2009); **cytoplasmic vacuolization** more pronounced after slow freezing (Nottola et al., 2009)

Open vs. closed systems

REVIEW

Open versus closed systems for vitrification of human oocytes and embryos

Gábor Vajta [a,b,*], Laura Rienzi [c], Filippo Maria Ubaldi [c]

In summary, evidence supporting the suitability of closed systems for oocyte vitrification is scarce compared with the vast amount of published and unpublished excellent results with open systems. Studies of closed systems have been published by isolated research groups, resulting in only 13 reported births and 20 ongoing pregnancies in total (Papatheodorou et al., 2013; Stoop et al., 2012), whereas tens of thousands of healthy babies have been born worldwide during the past 8 years with the open systems. Right now, closed systems should be regarded as experiemental, and extensive work including multicentre prospective randomized trials, are still required to explain why these procedures are successful when applied in one laboratory and inefficient in others, and to prove that the suggested methods are really competitive alternatives to open systems.

Open vs. closed systems

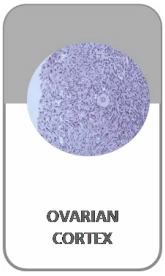
- Cooling rates are higher in open systems (>20,000°C/min)
- Closed systems are safer
- Open systems are more popular (Potdar et al., 2014; Vajta, 2015)

Cumulus surrounded vs. denuded oocyte

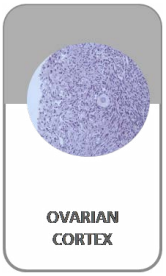
- **Majority experience has been with denuded oocytes**
- **Probably retard the already compromised permeation of CPA**
- **Hinder the assessment of oocyte to be selected for vitrification**
- **Hinder the assessment of re-expansion in ES**
- **Zhou et al. 2010: Bovine model, survival, cleavage and blastocyst formation rates not significantly different**

Advances in cryopreservation

1. Key to success in ovarian cortical transplantation: consider it as a skin graft i.e. avoid microhaematoma formation, micropressure stitches of 9-0 nylon.
2. Transplant is best if performed orthotopic.
3. OTC works both with slow-freezing & vitrification, functional for >5 years, spontaneous pregnancy without ART treatment.



Slow-freezing vs. vitrification



Issue of revascularization

Advances in cryopreservation

1. Cryoprotectant-free vitrification
- Sperm vitrification on carrier device
- Sperm vitrification by direct sperm immersion in LN2
2. Routine concentrations of cryoprotectants can't be used
3. Lyophilization (freeze-drying) of spermatozoa
4. Use of non-permeable CPA mixtures (HSA & sucrose) enhance mitochondrial integrity & prevents initiation of capacitation & AR.
5. Vitrification of immature germ cells (SSC) or tissue (ITT)

However, no e/o restoration of complete functionality in humans

3. Is it time to move towards “freeze-all” strategy

IS IT TIME TO MOVE TOWARDS FREEZE ALL STRATEGY ?

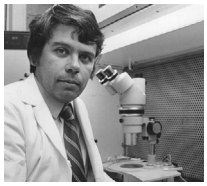
Early history of freezing human embryos or gametes

- 1949 – First human gamete cryopreservation (sperm vitrification)
- 1984 - First live birth with FET
- 1985 – First pregnancies with thawed blastocysts
- 1986 - First live birth with thawed oocytes

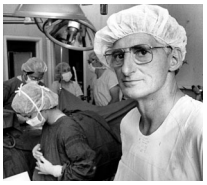
Polge et al 1949, Zeilmaker et al 1984, Cohen et al 1985, Chen 1986

Human Embryo Cryopreservation

First IVF Baby from frozen embryos "Zoe Leyland" was born in Melbourne, in March, 28th, 1984.
National data of USA inn 1986 reported, seven clinical pregnancies had resulted from FET, less than 1% of all cycles



Dr. Alan Trounson



Dr. Carl Wood

Since then, the approach has become the integral part of every IVF centre.
Evolution of cryopreservation techniques toward vitrification formed an accelaration.

Vitrified embryos are always second choice for transfer

Motivation for fresh vs FET studies

Birth rates with FET began to exceed those with fresh

In 2004 we noticed the pregnancy rates in our FET cycles were as good as those in our fresh cycles

In 2005-2006, our live birth rates with FET began to exceed those with fresh transfers

Fresh or Frozen: Which is better?

It is essential in case of

- Pre-implantation genetic diagnosis and screening,
- Ovarian hyper-stimulation,
- Or where there is a poor endometrial development.

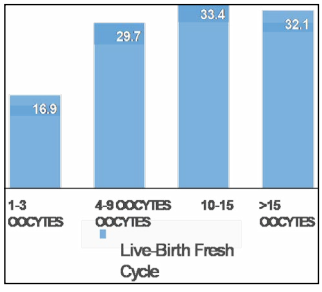
Why Freeze embryos ?

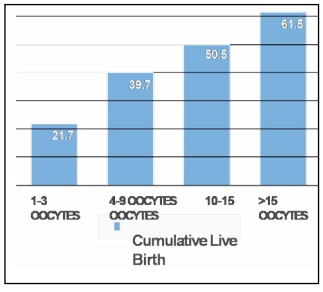
- Superovulation= many eggs=many embryos !
- What do we do with spare embryos ?
- Better to freeze than to discard !
- Reduce risk of OHSS
- Improve pregnancy rates
- Additional option for embryo adoption (regular adoption becoming much harder these days !)

Reason for lower pregnancy rate ?

- The slow freezing and thawing process damaged and killed a lot of cells.
- However , those embryos which were intact after freezing and thawing had the same implantation rate as fresh embryos.

COS vs. Embryo Quality
No. of oocytes vs. Live Birth

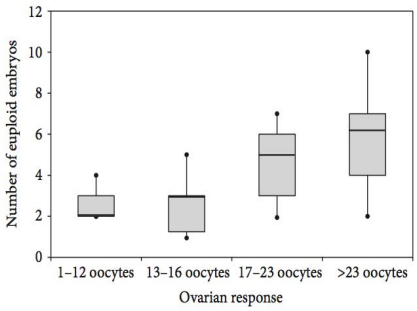




Drakopoulos et al., Hum Reprod 2016

A Higher Ovarian Response after Stimulation for IVF Is Related to a Higher Number of Euploid Embryos

Elena Labarta,[1] Ernesto Bosch,[1] Amparo Mercader,[2] Pilar Alamá,[1] Emilia Mateu,[2] and Antonio Pellicer[1]



Labarta et al., BioMed Research International 2017

Safety: can one go "wild" on stimulation?

When the first case[1] of severe OHSS with elective cryopreservation was reported…
…was this a one-time (and only time only) case?

But now there are yet another six cases[2-4…]
…are these patients genetically prone to OHSS?

[1]Griesinger et al, 2011; [2]Fatemi et al, 2014, [3]Gurbuz et al 2014, [4]Ling et al 2014

Maybe not…

J Assist Reprod Genet (2015) 32:1063–1068
DOI 10.1007/s10815-015-0498-y

CASE REPORT

Ovarian hyperstimulation syndrome after gonadotropin-releasing hormone agonist triggering and "freeze-all": in-depth analysis of genetic predisposition

Samuel Santos-Ribeiro[1,2] · Nikolaos P. Polyzos[1,3] · Katrien Stouffs[4] · Michel De Vos[1] · Sara Seneca[4] · Herman Tournaye[1] · Christophe Blockeel[1]

Freeze-all policy: fresh vs. frozen-thawed embryo transfer

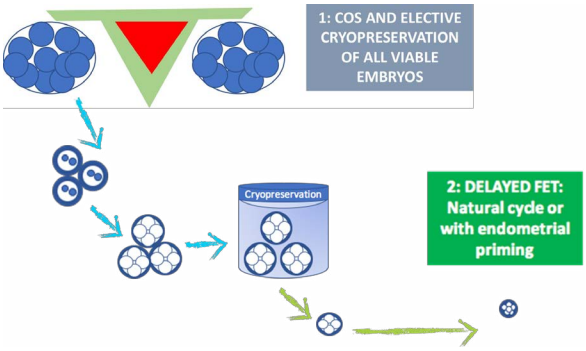
Matheus Roque, M.D.,[a] Marcello Valle, M.D.,[a] Fernando Guimarães, B.S.,[a] Marcos Sampaio, M.D., Ph.D.,[b] and Selmo Geber, M.D., Ph.D.[b,c]

[a] ORIGEN, Center for Reproductive Medicine, Rio de Janeiro; [b] ORIGEN, Center for Reproductive Medicine, Belo Horizonte and [c] Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

- Recently with better results, the policy for "freezing all" has been introduced in some clinics and needs evaluation.
- The "freeze all" policy, that is, all embryos being frozen and transferred in the next cycle, was introduced to avoid ovarian hyperstimulation syndrome (OHSS).
- It allowed the ovulation trigger to be a GnRH agonist to avoid hyperstimulation, which the human chorionic gonadotropin (hCG) trigger would have caused. As implantation and pregnancy rates are lower with GnRH agonist trigger, due to its luteolytic action leading to poor endometrial receptivity, transfer is done in the next cycle.
- Besides, freezing also avoids appearance of OHSS due to increased β hCG levels if pregnancy occurs in the same cycle.

Objectives

The rationale of Freeze-all



Frozen cycle

- Much easier for the patient – and for the clinic
- Much less expensive

2 OPTIONS

1. Natural cycle protocol (for patients with regular ovulatory cycles)
2. Endometrial preparation protocol

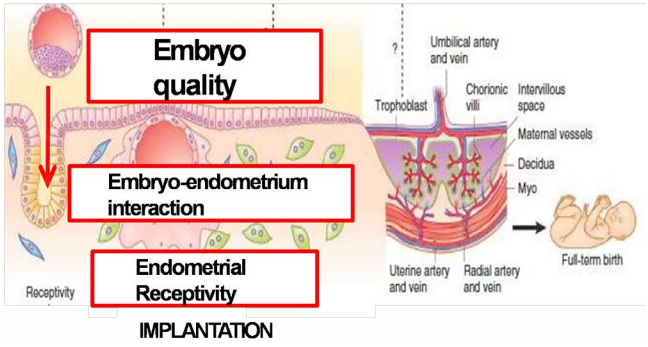
Natural cycle

- No medication
- Monitor ovulation (scan and LH urine strips)
- Embryo transfer 48 hours after ovulation , once the uterine lining is thick and trilaminar
- Progesterone supplementation after ET

Higher success rate

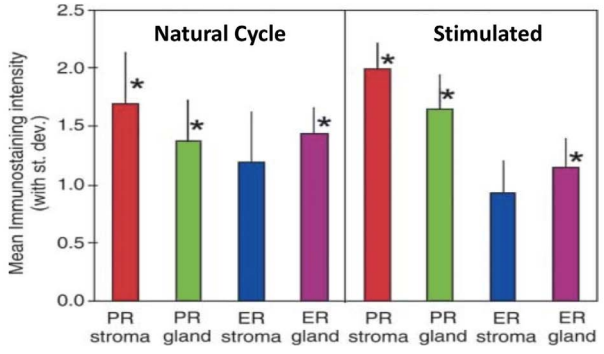
- Paradoxical – but true
- Reason ? The endometrium is more receptive in a frozen thaw cycle
- Not exposed to the supraphysiological levels of estrogen and progesterone induced by superovulation .

COS vs. Endometrial Receptivity



Ovarian Stimulation vs. Endometrium

- Expression of steroidal endometrial receptors
  Natural cycle (LH peak) vs. Stimulated (hCG day)



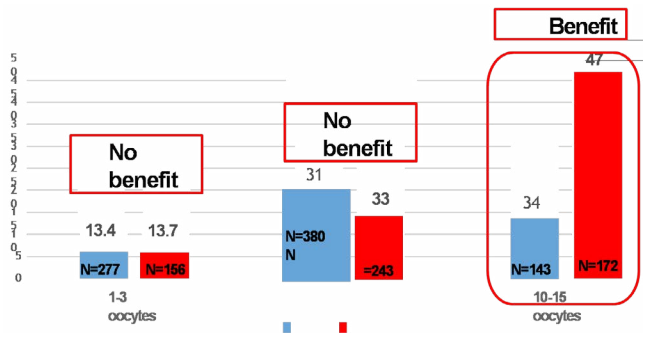
Papanikolaou et al. Hum Reprod 2005;20:3198-203

Freeze-all for all?

- FREEZE - ALL FOR WHOM?

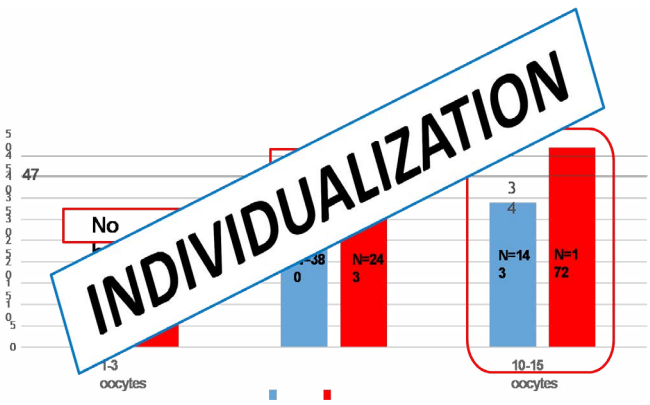
- Hyper responders?
- Normal responders?
- Poor responders?

Freeze-all for all?
Freeze all- stratification- Ongoing Pregnancy



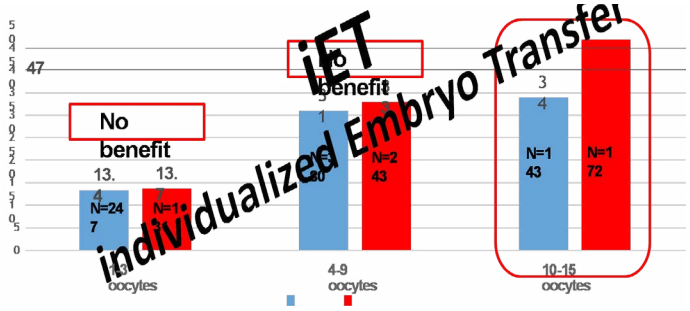
Roque et al., ESHRE 2017; Roque et al., JARG 2017

Freeze-all for all?
Freeze all- stratification- Ongoing Pregnancy



Roque et al., ESHRE 2017; Roque et al., JARG 2017

Freeze-all for all?
Freeze all- stratification- Ongoing Pregnancy



Roque et al., ASRM 2016; Roque et al., JARG 2017

Ovarian Stimulation vs. Endometrium

Ovarian Stimulation -> modification in the pattern of steroidal receptors expression during the late follicular phase

Ovarian Stimulation vs. Endometrium

Group I P ≤ 0.09 ng/mL			Group II P ≥ 1.1 ng/mL		
Case	Endometrial dating	Clinical pregnancy	Case	Endometrial dating	Clinical pregnancy
	d			*d*	
1	2 to 3	—	21	2	Yes
2	3	—	22	3 to 4	—
3	2	Yes	23	4*	—
4	3	Yes	24	3	Yes
5	2	Yes	25	3	—
6	2	—	26	4	—
7	3	Yes	27	2	—
8	2	—	28	2*	—
9	2	Yes	29	2	—
10	3	—	30	2 to 3	No ET
11	2	—	31	3	—
12	2	—	32	3	—
13	2	—	33	2 to 3	—
14	2 to 3	—	34	3	Yes
15	1	—	35	4	—
16	2 to 3	Yes	36	4 to 5	—
17	1 to 2	Yes	37	3 to 4	—
18	2	—	38	3	—
19	2 to 3	—	39	3 to 4	—
20	1 to 2	—	40	2 to 3	—

Ubaldi et al. Fertil Steril 1997; 67:521-6

Ovarian Stimulation vs. Endometrium

Authors	Year	Stimulation type	Endometrial advancement ≤3 days	Endometrial advancement >3 days	Odds ratio	95% CI
			Biochemical pregnancy			
Ubaldi et al.	1997	HMG/agonist	13/32	0/7	0.17	0.03–0.93
Current study	2002	Rec-FSH/antagonist	14/49	0/6	0.23	0.03–1.57
Total			27/81	0/13	0.19	0.05–0.69
			Detection of a gestational sac			
Ubaldi et al.	1997	HMG/agonist	10/32	0/7	0.20	0.03–1.29
Current study	2002	Rec-FSH/antagonist	11/49	0/6	0.25	0.03–2.06
Total			21/81	0/13	0.22	0.06–0.89
			Ongoing pregnancy			
Ubaldi et al.	1997	HMG/agonist	10/32	0/7	0.20	0.03–1.29
Current study	2002	Rec-FSH/antagonist	8/49	0/6	0.28	0.03–2.98
Total			18/81	0/13	0.23	0.05–0.98

Kolibianakis et al. Fertil Steril 2002;78:1025-9

Ovarian Stimulation vs. Endometrium

- COS -> endometrial gene expression
- hCG+7 vs LH+7 – paired samples
- >200 genes – different expression

OVARIAN STIMULATION AFFECTS ENDOMETRIAL RECEPTIVITY

Labarta et al. Hum Reprod 2011;26:1813-25

Ovarian Stimulation vs. Endometrium

- Oocyte donors
  - -P > 1.5 ng/mL (Study group)
  - -P < 1.5 ng/mL (Control group)
- Endometrium biopsy -> genic expression
- 140 genes ”up or down-regulated” – STUDY GROUP

HORMONAL LEVELS AFFECT ENDOMETRIAL RECEPTIVITY

Endometrial Preparation

- Down-regulation (with Lupride) optional
- Check scan on day 2/3
- Prepare uterine lining with estradiol valerate , 2 mg
- One option – gradually increasing doses every 3 days
- Some use 6 mg/ day from day 2 and scan after 7 days
- Once the lining is trilaminar and more than 8 mm, start progesterone and do the transfer

Endometrial Preparation

- May need to increase the Progynova dose
- Patients with poor uterine lining can be difficult to manage !
- Benefit of frozen cycle – we do not have to worry about the eggs – can focus purely on improving the endometrium !

Strategies for patients with poor lining

- Hysteroscopy
- Endometrial injury (to improve uterine blood flow)
- Bromelain (enzyme) , 200 mg daily
- Intrauterine perfusion of recombinant G-CSF(Granulocyte colony stimulating factor, Filgrastim) , active ingredient of Neukine , 300 ug.
- Successful treatment of unresponsive thin endometrium. *Gleicher ,et al. Fertil Steril, Feb 2011*

Kato Protocol

- No fresh transfers at all !
- Electively freeze all embryos
- Transfer only in the next cycle
- Only blastocysts
- Only vitrification

Advanced endometrial histology

- Advanced endometrial histology has been correlated with premature progesterone elevation and implantation failure.

Nikas et al , 1999 . Kolibianakis et al , 2002

Is The Freeze All Strategy for All?

Potential Advantages	Potential Disadvantages
↓ OHSS	↑ Macrosomia
↑ Endometrial receptivity	↑ Hypertensive disorders
↑ Implantation rates	↑ Placenta accreta
↓ Ectopic pregnancy	↑ Posts
↓ Placenta previa	Few RCTs
↓ Pre-term birth	No data on poor responders
↓ Small for gestational age	
↓ Perinatal mortality	

Freezing

- Can freeze embryos at any time –from Day 0- Day -6
- Its useful to freeze only good quality embryos i.e. Grade A embryos
- Poor quality embryos do not survive the process well- just provide false hopes !
- We will freeze even if there is just 1 good quality blastocyst
- Can freeze for as long as you want
- Need to counsel patients re: what they want to do with their supernumerary embryos
- Take informed consent

When to do freeze-all?

- Risk of OHSS
- For all?
- Based on ovarian response?
- P4 levels on the trigger day?
- Implantation failure?
- PGT (blastocyst)?
- Slower developing blastocyst?
- To improve obstetric / perinatal outcomes?

Freeze all- when OHSS risk? Prediction of Moderate to Severe OHSS

2,433 women who received hCG for trigger	≥19 follicles ≥11 mm on the day of hCG	E_2 ≥6,000 pmol/L on the day og hCG	Follicle number ≥19 AND E_2≥ 6,000 pmol/L on the day of hCG
Sensitivity, %	62.3	62.3	72.5
Specificity, %	75.6	63.6	65.1
Positive predictive value, %	6.9	4.8	5.7
Negative predictive value, %	98.6	98.3	98.8

*6,000 pmol/L = 1634.5 pg/mL

Griesinger et al. PLoS One 2016;11:e0149615

Freeze all- for all?

Cochrane Library
Cochrane Database of Systematic Reviews

Fresh versus frozen embryo transfers in assisted reproduction (Review)

Wong KM, van Wely M, Mol F, Repping S, Mastenbroek S

Freeze all- when OHSS risk?

Human Reproduction, Vol.26, No.10 pp. 2593–2597, 2011
Advanced Access publication on August 9, 2011 doi:10.1093/humrep/der251

human reproduction OPINION

An OHSS-Free Clinic by segmentation of IVF treatment

Paul Devroey*, Nikolaos P. Polyzos, and Christophe Blockeel

FREEZE-ALL -> if risk of OHSS
Antagonist protocol + GnRH agonist trigger

Overall effect of ovarian stimulation on the endometrium

- Following COS , the endometrium is " histologically advanced, biochemically different, and genomically dysregulated."

Horcajadas et al , 2007 .

J Assist Reprod Genet (2017) 34:179–185
DOI 10.1007/s10815-016-0834-x

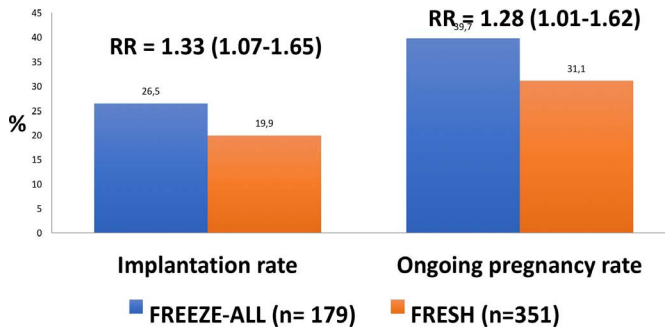
ASSISTED REPRODUCTION TECHNOLOGIES

Freeze-all cycle for all normal responders?

Matheus Roque[1,2] · Marcello Valle[1] · Fernando Guimarães[1] · Marcos Sampaio[3] · Selmo Geber[2,3]

Freeze-all based on ovarian response?

- D+3 embryo transfers/ Fresh ET only if P<1.5 ng/mL
- Observational Study



Roque et al. Fertil Steril 2015; 103:1190-3

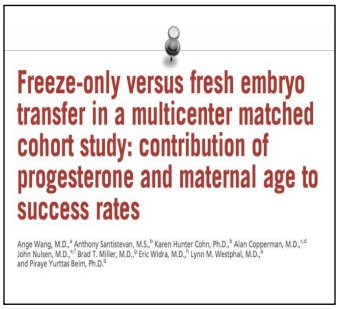
Freeze-all based on ovarian response?

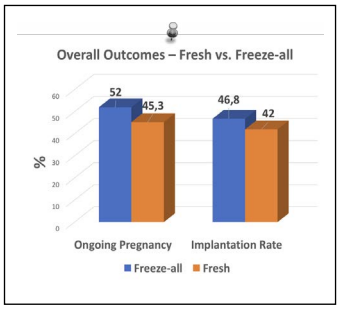
Fresh vs. FREEZE-ALL PCOS Patients

Outcome	Frozen-Embryo Transfer (N=746)	Fresh-Embryo Transfer (N=762)	Absolute Difference between Groups (95% CI)	Rate Ratio in Frozen-Embryo Group (95% CI)	P Value
Primary outcome: live birth — no. (%)†	368 (49.3)	320 (42.0)	7.3 (2.3 to 12.4)	1.17 (1.05 to 1.31)	0.004
Pregnancy loss — no./total no. (%)					
Among biochemical pregnancies	108/492 (22.0)	161/492 (32.7)	−10.8 (−16.3 to −5.2)	0.67 (0.54 to 0.83)	<0.001
Among clinical pregnancies	64/438 (14.6)	107/428 (25.0)	−10.4 (−15.7 to −5.1)	0.58 (0.44 to 0.77)	<0.001
First trimester	42/438 (9.6)	56/428 (13.1)	−3.5 (−7.7 to 0.7)	0.73 (0.50 to 1.07)	0.10
Second trimester	22/438 (5.0)	51/428 (11.9)	−6.9 (−10.6 to −3.2)	0.42 (0.26 to 0.68)	<0.001

Roque et al. Fertil Steril 2015; 103:1190-3

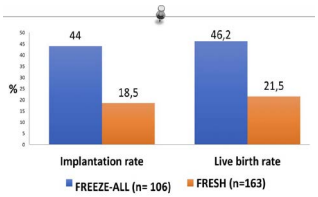
Freeze-all and P4 levels on the trigger day?

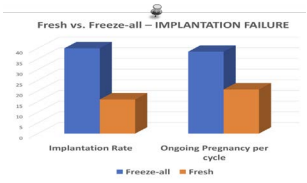




Wang et al. Fertil Steril; 2017; 108:254-261

Freeze-all for Implantation Failure ?





Shapiro et al. Reprod Biomed Online,2014; 29:286-90

Magdi et al. Fertil Steril 2017; 108:72-77.

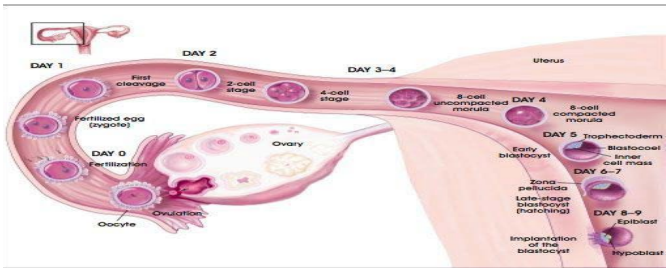
LUTEAL PHASE SUPPORT-FET CYCLES

OUTCOME	Surface First Menstrual Cycle After OPU	Afterward	P
Pregnancy Rate (PR)	49,8%	43,8%	0,171
Clinical PR	44,1%	36,1%	0,066
Live Birth Rate	37,6%	27,3%	0,013

Lattes et al. Hum Reprod 2017; 32:368-374

Blastocyst

- *A blastocyst* is a highly differentiated, highly developed embryo that has grown to the point where it is ready to attach to the uterine wall (implantation).
- *Blastocyst transfer* is claimed to be more physiological than pronucleate or cleaved-embryo since it mimics nature more closely..



What might be the disadvantages of a blastocyst

- Patient may not have an embryo suitable for transfer.
- Increase rate of monozygotic twins

What might be the disadvantage of using Blastocysts for transfer?

Blastocyst freezing

- With the refinement of extended culture systems, it is becoming more reliable to obtain blastocysts in vitro .
- Due their high implantation rates, it is becoming a common practice to limit transfer to one or two blastocysts at a time. Therefore, surplus blastocysts require an efficient cryopreservation method .
- Slow freezing was the main method of cryopreservation , but vitrification is now on the rise.
- Blastocysts represent a unique challenge in cryostorage due to their size, multicellular structure and presence of blastocoele.

Blastocyst freezing

- Vitrification is the glass-like solidification of a solution at a low temperature without ice crystal formation, which is made possible by extreme elevation in viscosity during freezing. This can be achieved by increasing the freezing and warming rates and/or increasing the concentration of the cryoprotectants .
- Unlike slow freezing, vitrification results in the total elimination of ice crystal formation, both within the cells being vitrified and outside the cells in the surrounding solution .

Obstetric and perinatal outcomes

- Multiple pregnancy is the main source of obstetric and perinatal morbidity associated with ART.
- Blastocyst transfer allowed one or two blastocysts to be transferred with high implantation potential, while minimizing the risks of multiple pregnancies
- There are still concerns regarding the overall safety of vitrification and whether it can cause or lead to chromosomal abnormalities, congenital malformation, and/or developmental abnormalities in the offspring.

Bogliolo L, Ariu F, Fois S, Rosati I, Zedda MT, Leoni G, Succu S, Pau S, Ledda S: Morphological and biochemical analysis of immature ovine oocytes vitrified with or without cumulus cells. Theriogenology 2007, 68:1138-1149

Obstetric and perinatal outcomes

- Noyes et al reviewed a total of 58 reports (1986-2008) on 609 live born babies from cryopreserved oocytes (308 from slow-freezing, 289 from vitrification and 12 from both methods). Twelve newborns (1.3%) had birth anomalies, which is comparable to the number of congenital anomalies that occur in naturally conceived infants.

Noyes N, Porcu E, Borini A: Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. Reprod Biomed Online 2009

Obstetric and perinatal outcomes

- Takahashi et al reported congenital birth defects of 1.4% using vitrified blastocysts which was similar to fresh blastocysts.

Takahashi K, Mukaida T, Goto T, Oka C: Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. Fertil Steril 2005, 84:88-92.

- Mukaida et al analyzed 560 deliveries of 691 healthy babies following the transfer of vitrified blastocysts. The congenital and neonatal complication rate was 3%, which was comparable to that in their fresh blastocysts transfer group (2.3%).

Mukaida T, Takahashi, K., Goto, T., Oka, C.: Perinatal outcome of vitrified human blastocysts in 7 year experience (2670 attempted cycles). Human Reproduction 2008, 23:i48.

Obstetric and perinatal outcomes

- No perinatal abnormalities were reported in Liebermann's report on 348 deliveries following transfer of vitrified blastocysts .

Liebermann J: Vitrification of human blastocysts: An update Reprod Biomed Online 2009, 19 Suppl 2.

- These findings may provide preliminary reassurance on the safety of blastocyst vitrification. A final verdict on the actual effect of blastocyst vitrification on congenital and perinatal outcomes may not be possible until large-scale trials or further meta-analysis of rapidly accumulating reports can be performed.

Factors that can affect the outcome

- Pre-vitrification blastocyst selection
- Post-thaw blastocyst selection
- Assisted hatching
- Blastocoele collapse (assisted shrinkage)
- Media protocols
- Freezing rate
- Warming rate
- Operator-dependent factors
- Hydrostatic pressure

Embryo developmental pace

- There is biological variation in embryonic develop developmental pace
- Some embryos from expanded blastocysts on day 5 of development, others on day 6
- Day 5 blastocysts implant more readily than day 6 blastocysts in fresh IVF cycles following ovarian stimulation .

Shapiro et al 2001.


ORIGINAL ARTICLES: ASSISTED REPRODUCTION

Abnormal implantation after fresh and frozen in vitro fertilization cycles

Erica T. Wang, M.D., M.A.S.,[a] Anupama S. Q. Kathiresan, M.D.,[a] Catherine Bresee, M.S.,[b] Naomi Greene, M.P.H., Ph.D.,[a] Carolyn Alexander, M.D.,[a] and Margareta D. Pisarska, M.D.[a]

[a] Department of Obstetrics and Gynecology; and [b]Cedars Sinai Biostatistics & Bioinformatics Core, Cedars Sinai Medical Center, Los Angeles, California

Many benefits of Vitrification

- Reduce the risk of high order multiple births
- Reduce OHSS- we can freeze all embryos , rather than transfer
- In the fresh cycle , we need to transfer 1-2 embryos (since the rest are being frozen , the patient is not worried that her embryos are being wasted)
- Cumulative success rate is very high.
- We are now doing more frozen transfers in oue clinic than fresh transfers !

When to thaw the Embryos

- Can thaw on the day of transfer itself
- Easy to make out if the blastomeres are viable or not. Dead blastomeres are dark
- Some clinic will thaw 24 hours in advance
- Will allow the thawed embryos to cleave in vitro-this confirms the embryos are viable and help them to select the best embryos !

Why we worry ?

- Cost of liquid nitrogen
- Outside city patient cost?
- Patient will come or not
- Burden on Lab
- Record keeping
- Legal Problems in cases of separation
- How to handle embryos in case of divorce.

Need to work towards this

- Gradual process
- Need to master the technical skills
- Develop confidence in your LAB
- Training
- Monitoring
- Quality Control

- The IVF lab is the heart of the IVF clinic
- You do not need to become an embryologist but you need to be very knowledgeable
- **Each embryo should be treated as patient .**

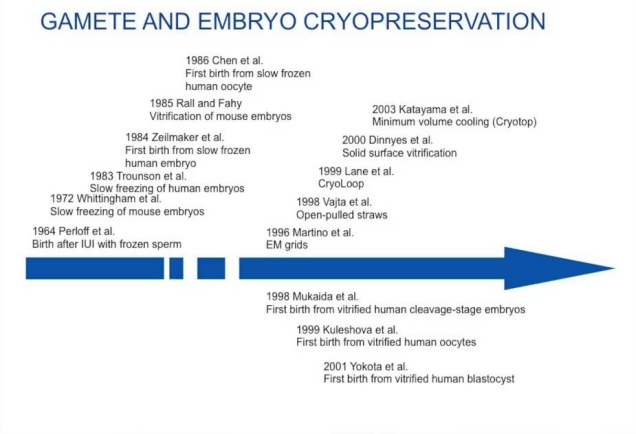
When nothing is sure, everything is possible.

Margeret Atwood

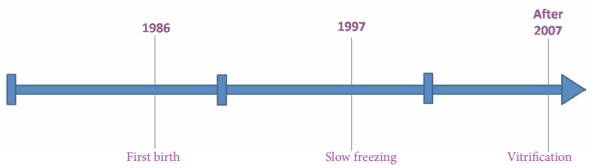
4. Oocyte vitrification

Oocyte cryopreservation

Progress in slow freezing & vitrification



Progress in slow freezing & vitrification



Survival	20-30%	40-50%	80-90%
Pregnancy Rate (PR)	<40%	40-50%	70-80%
Clinical PR	?	?	40-50%
Live Birth Rate	?	?	5-8%

Peculiarities of oocyte

- Largest cell with largest volume

Cell	Surface area	Volume	SA:Vol
Oocyte	4.5 x 104 µm2	9 x 105 µm3	0.05
Sperm	120 µm2	28 µm	4.3

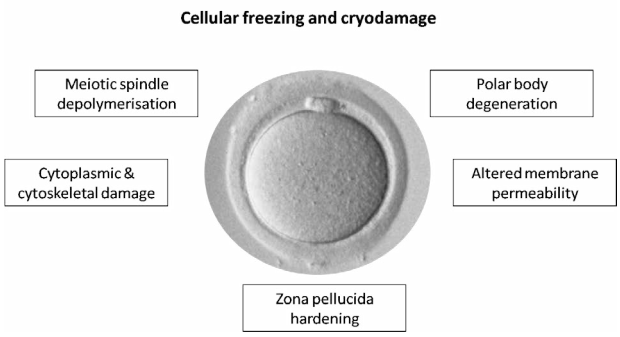
- Spherical shape: permeation gradient, inadequate permeation
- Meiotic spindle
- Actin scaffolding
- Low cell number – all or nothing

Peculiarities of oocyte

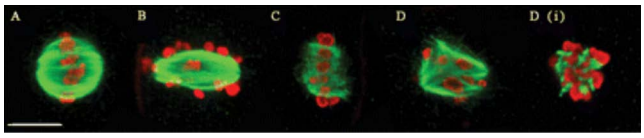
CRYODAMAGE

- Nucleus & nuclear membrane
- Cytoplasm
  - Microtubules: MS – risk of aneuplodies
  - Microfilaments: Cortical scaffolding
  - Mitochondria: Swelling & abnormal distribution
- Zona pellucida
  - Premature corticle granule exocytosis
  - Zona hardening

Cryoinjuries

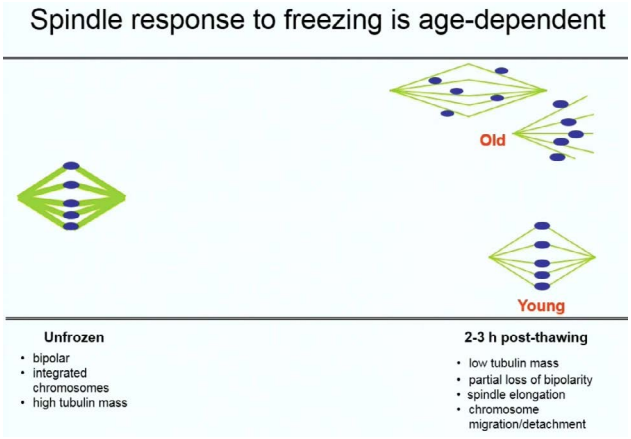


Cryoinjuries



From: Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation
Hum Reprod. 2009;24(9):2114-2123. doi:10.1093/humrep/dep182

Cryoinjuries



Indications

1. Better synchronization of donor recipient cycles
2. Oncofertility preservation
3. Male partner unavailable or unable to produce semen sample on day of OPU
4. Pts. At risk of POF
5. OHSS
6. Legal, ethical or moral issues with embryo cryopreservation
7. Women wanting to delay conception
8. Cryobanking oocytes for egg donation
9. Cryoaccumulation (Accu-vit

Oocyte donation

- Oocyte cryopreservation offers greater options in terms of flexibility

- Not necessary to commit all oocytes to single sperm source

- Simplified donor/recipient synchronization

- Allows quarantine pending testing for infectious diseases

Legal/ethical issues

Issues related to:

- Fate of unused/excess embryos
- Relationship breakups
- Death of a partner
- Legal restrictions on embryo cryopreservation

Results with oocyte vitrification

ASRM PAGES

Mature oocyte cryopreservation: a guideline

The Practice Committees of the American Society for Reproductive Medicine and the Society for Assiste Reproductive Technology

Society for Reproductive Medicine and Society for Assisted Reproductive Technology, Birmingham, Alabama

There is good evidence that fertilization and pregnancy rates are similar to IVF/ICSI with fresh oocytes when vitrified are used as part of IVF/ICSI for young women. Although data are limited, no increase in chromosomal abnormalities, developmental deficits has been reported in the offspring born from cryopreserved oocytes when compared to pregn ventional IVF/ICSI and the general population. Evidence indicates that oocyte vitrification and warming should no lon experimental. This document replaces the document last published in 2008 titled, "Ovarian Tis-

Results with oocyte vitrification

REVIEW

Oocyte vitrification in the 21st century and post-warming fertility outcomes: a systematic review and meta-analysis

Neelam Potdar [a,*], Tarek A Gelbaya [a], Luciano G Nardo [b]

Abstract Oocyte cryopreservation is a rapidly developing technology, which is increasingly being used for various medical, legal and social reasons. There are inconsistencies in information regarding survival rate and fertility outcomes. This systematic review and meta-analysis provides evidence-based information about oocyte survival and fertility outcomes post warming to help women to make informed choices. All randomized and non-randomized, controlled and prospective cohort studies using oocyte vitrification were included. The primary outcome measure was ongoing pregnancy rate/warmed oocyte. Sensitivity analysis for donor and non-donor oocyte studies was performed. Proportional meta-analysis of 17 studies, using a random-effects model, showed pooled ongoing pregnancy and clinical pregnancy rates per warmed oocyte of 7%. Oocyte survival, fertilization, cleavage, clinical pregnancy and ongoing pregnancy rates per warmed oocyte were higher in donor versus non-donor studies. Comparing vitrified with fresh oocytes, no statistically significant difference was observed in fertilization, cleavage and clinical pregnancy rates, but ongoing pregnancy rate was reduced in the vitrified group (odds ratio 0.74), with heterogeneity between studies. Considering the age of women and the reason for cryopreservation, reasonable information can be given to help women to make informed choices. Future studies with outcomes from oocytes cryopreserved for gonadotoxic treatment may provide more insight.

Results with oocyte vitrification

Summary of randomized controlled trials comparing fresh versus vitrified oocytes.

	Cobo 2008 (24)	Cobo 2010 (26)	Rienzi 2010 (25)	Parmegiani 2011 (19)
Patient population	Oocyte donors	Oocyte donors	Infertile patients <43 years of age requiring ICSI with >6 mature oocytes	Infertile patients <42 years of age requiring ICSI with >5 mature oocytes
No. patients	30 vitrification 30 fresh	295 vitrification 289 fresh	40 vitrification 40 fresh	31 vitrification 31 fresh
Mean age at retrieval	26	26	35	35
No. oocytes	231 vitrification 219 fresh	3286 vitrification 3185 fresh	124 vitrification 120 fresh	168 vitrification NA fresh
No. oocytes per retrieval	18.2	11	13	NA
Survival	96.9%	92.5%	96.8%	89.9%
Fertilization rate	76.3 vitrification 82.2 fresh	74% vitrification 73% fresh	79.2% vitrification 83.3% fresh	71% vitrification 72.6% fresh
No. transferred vitrification vs. fresh	3.8 vitrification 3.9 fresh	1.7 vitrification 1.7 fresh	2.3 vitrification 2.5 fresh	2.5 vitrification 2.6 fresh
Day of transfer	3	3	2	2–3
Implantation rate	40.8% vitrification 100% fresh	39.9% vitrification 40.9% fresh	20.4% vitrification 21.7% fresh	17.1% vitrification NA fresh
CPR/transfer vitrification vs. fresh	60.8% (23 vitrification transfers) 100% (1 fresh transfer)	55.4% vitrification 55.6% fresh	38.5% vitrification 43.5% fresh	35.5% vitrification 13.3% fresh
CPR/oocyte thawed	6.1%	4.5%	12%	6.5%

Note: All used vitrification with Cryotop, 15% EG + 15% DMSO + 0.5M sucrose. CPR = clinical pregnancy rate.

Practice Committee. Oocyte cryopreservation. Fertil Steril 2013.

Results with oocyte vitrification

Oocyte Vitrification: Oocyte donors (mean age 27)
(Cobo et al, 2010)

	Fresh (n=3185)	Vitrified (n=3039)
Survival	_	92.5%
Fertilisation	73.3%	74.2%
Top quality day 3 embryos	60.7%	58.4%
Implantation (day 3)	40.9%	39.9%

Open vitrification using EG/DMSO/Sucrose

Results with oocyte vitrification

Other oocyte vitrification studies using donor oocytes

	Fresh IR	Cryopreserved IR	Survival
Nagy et al, 2009 (Blastocyst transfer)	47.4%	55.3%	89.0%
Garcia et al, 2011 (Blastocyst transfer)	42.9%	43.9%	89.4%
Trokoudes et al, 2011 (Cleavage stage transfer)	25.6%	24.7%	91.4%

Results with oocyte vitrification

Multicentre study: Unselected infertility cycles (mean age 36)
(Rienzi et al, 2012)

	486 warming cycles 2721 warmed oocytes
Survival	84.7%
Fertilisation	75.2%
Top quality day 2/3 embryos	48.1%
Delivery rate per cycle	26.3%
Implantation (newborn/embryo transferred)	15.8%

Open vitrification using EG/DMSO/Sucrose

Decisional making model based on recursive partitioning analysis (per patient basis)

- 0 = delivery not obtained
- 1 = delivery obtained

	%	N
0.000	71.6	322
1.000	28.4	128
Total	100.0	450

Number of vitrified MII

≤ 8 MII

	%	n
0.000	77.4	263
1.000	22.6	77
Total	75.6	340

> 8 MII

	%	n
0.000	53.6	59
1.000	46.4	51
Total	24.4	110

Rienzi et al; ESHRE 2011

Efficiency of vitrified oocyte (Safety)

1. Survival & pregnancy rates
   Cobo et al, 2008, 2009, 2010
2. Gene expression
   Di Pietro et al, 2010
3. Aneuploidy
   Garcia et al, 2011
4. Congenital anomalies
   Noyes et al, 2009
5. Perinatal outcomes
   Chian et al, 2008; Cobo et al, 2010

} No Difference

Perinatal outcome of children born after oocyte cryoreservation

IVI) Perinatal outcome of child born after oocyte vitrification. Paired cohorts study.

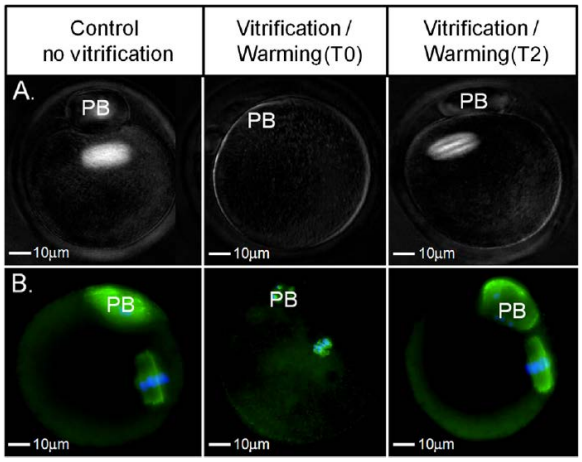
Perinatal outcome

	Vitrified	Fresh
Nº Deliveries	160	262
Nº New born	212	315
Perinatal mortality	3 (1.4)	0
Mean maternal age at delivering	39.2 ± 4.9	39.6 ± 5.3
Mean gestational age (weeks)	37.4 ± 2.5	38.0 ± 2.3
No. of deliveries at <37 weeks (%)	37 (23.1)	47 (17.9)
No. of deliveries at <34 weeks (%)	9 (5.6)	12 (4.6)
Birth weight (mean ± SD)	2,718 ± 0.668	2,896 ± 0.658
No. of LBW (%) <2500gr.	65 (17.4)	75 (23.8)
No. of VLBW (%) <1500 gr.	14 (1.9)	13 (4.1)
Median Apgar score at 1 min.	8.9 ± 0.6	8.9 ± 0.8
Median Apgar score at 5 min.	9.3 ± 0.6	9.6 ± 0.3
Sex of new born. Female.	113 (53.7)	172 (54.6)
Sex of new born. Male.	99 (46.3.)	143 (45.4)
Incidence of major congenital anomalies	4(1.8)	2 (0.6)

NS

Cobo, Serna. Hum Reprod 2011

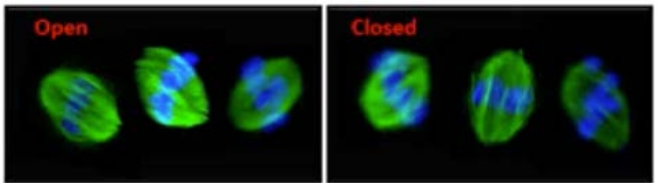
Meiotic spindle integrity & repolymerization (Safety)



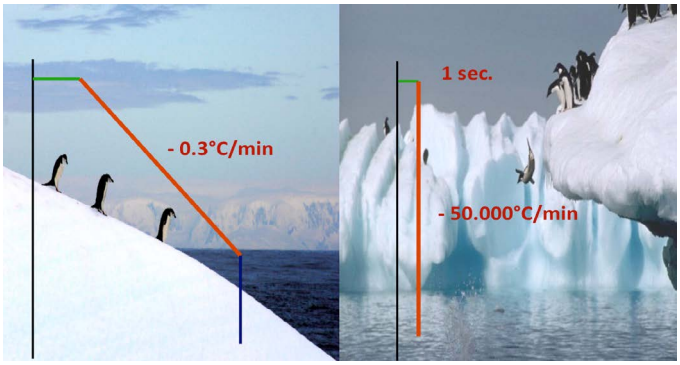
Microtubule & microfilament perturbations

The use of open or closed devices does not affect spindle morphometry

	Control	Open System	Closed System	P value
No. Oocyte	32	24	11	
Major axis (µm)	122±22	123±20	140±22	0.117
Minor axis (µm)	92±14	86±09	93±14	0.185
Area maximum projection (µm²)	929±235	850±200	1001±218	0.235



Slow-freezing vs. vitrification



Slow-freezing vs. vitrification

- Fadini et al (2009): Vitrification provided higher survival (79% vs. 58%), PR (18.7% vs. 7.6%) & IR (9.3% vs. 4.3%)
- Cao et al (2009): Higher survival (91.8% vs. 61%), high quality embryos (42 % vs. 24%) and top quality blastocyst (33% vs. 12%) with vitrification
- Cytoplasmic effect of OCP: Spindle recovery is accelerated in vitrified embryos (Chen & Yang, 2009; Ciotti et al., 2009); cytoplasmic vacuolization more pronounced after slow freezing (Nottola et al., 2009)
- Data suggesting superiority of vitrification based on metabolomic & proteomic considerations (Gardner et al.)

Open vs. closed systems

REVIEW

Open versus closed systems for vitrification of human oocytes and embryos

Gábor Vajta [a,b,*], Laura Rienzi [c], Filippo Maria Ubaldi [c]

In summary, evidence supporting the suitability of closed systems for oocyte vitrification is scarce compared with the vast amount of published and unpublished excellent results with open systems. Studies of closed systems have been published by isolated research groups, resulting in only 13 reported births and 20 ongoing pregnancies in total (Papatheodorou et al., 2013; Stoop et al., 2012), whereas tens of thousands of healthy babies have been born worldwide during the past 8 years with the open systems. Right now, closed systems should be regarded as experiemental, and extensive work including multicentre prospective randomized trials, are still required to explain why these procedures are successful when applied in one laboratory and inefficient in others, and to prove that the suggested methods are really competitive alternatives to open systems.

Open vs. closed systems

- Cooling rates are higher in open systems (>20,000oC/min)
- Closed systems are safer
- Open systems are more popular (Potdar et al., 2014; Vajta, 2015)

Cumulus surrounded vs. denuded oocyte(Safety)

- Majority experience has been with denuded oocytes
- Probably retard the already compromised permeation of CPA
- Hinder the assessment of oocyte to be selected for vitrification
- Hinder the assessment of re-expansion in ES
- Zhou et al. 2010: Bovine model, survival, cleavage and blastocyst formation rates not significantly different

Immature vs mature oocyte

- To date, only 1 live birth reported following GV stage cryopreservation & IVM
- Recently, several pregnancies & live births obtained by immature oocyte retrieval followed by IVM & then vitrification

Conclusions

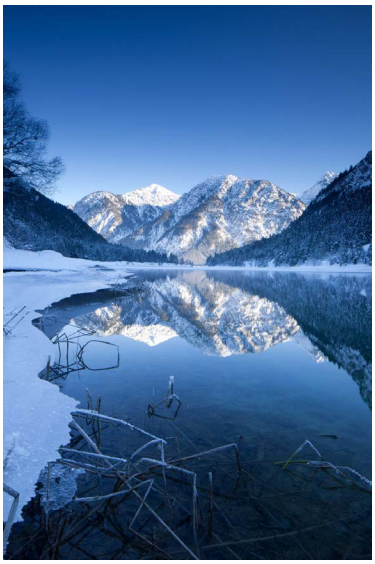
- Oocyte vitrification is no longer experimental and can be offered to patients esp. oncofertility and oocyte cryobanking
- Warming rates are especially crucial for thaw survival of oocytes
- Due regards towards timing of oocyte vitrification and performing ICSI 2-3 hours post-thaw to enable spindle recovery
- CPA exposure causes alteration in intracellular calcium signaling
- More studies regarding safety

5. How to have 100% cryosurvival in a vitrification program

HOW TO HAVE 100% CRYOSURVIVAL IN VITRIFICATION PROGRAM

Cryopreservation is ceasing and resuming cell metabolism

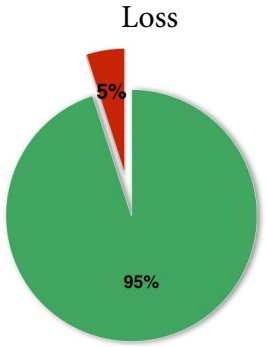
DEFINITION



Vitrification (from Latin vitreum, "glass" via French vitrifier) is the transformation of a substance into a glass - Wikipedia

It is a cryopreservation method that allows solidification of the cells and extracellular milieu into a glass like state without formation of ice.

AIM IS TO ACHIEVE 100% CRYO SURVIVAL



- Achieved 90%-95% Survival rates in CRYOPRESERVATION but the AIM is to achieve 100% CRYOSURVIVAL
- 95% Cryosurvival - 5% Repercussion
- 5% is very very precious for patients who need ART.
- EACH CELL MATTERS FOR SUCCESS.

Factors affecting Cryopreservation

FACTORS AFFECTING CRYOPRESERVATION

- INTRINSIC
- EXTRINSIC

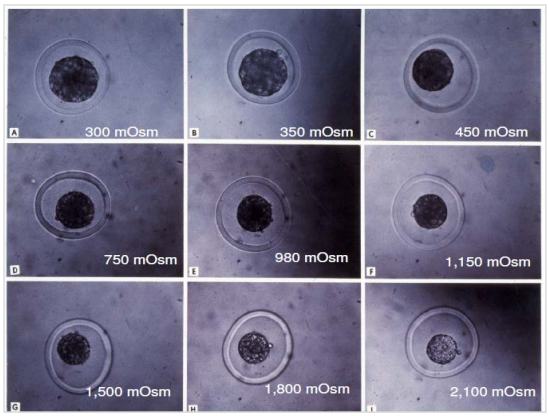
Both intrinsic and extrinsic factors influence the outcome of oocyte and embryo cryopreservation and both types of factors must be considered when deriving cryopreservation protocols.

INTRINSIC FACTORS

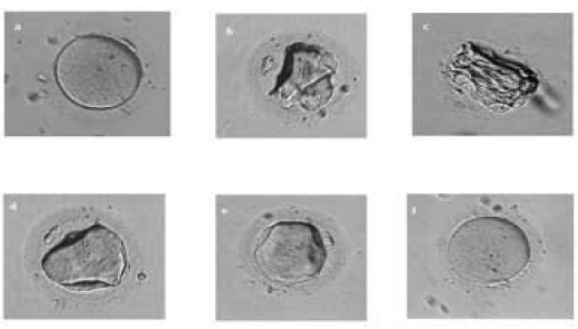
- Intrinsic factors are the inherent cryobiological properties of the cell membrane at different stages of development,such as its ability to cope with osmotic stress during vitrification.

1. Exposure to CPA solutions
2. Osmotic response.
3. Permeability of the cell membrane
4. Pathways for the movements of water and CPAs across the cell membrane.

1. Exposure to CPAs



2. OSMOTIC RESPONSE TO CPA SOLUTION



3. OSMOTIC RESPONSE

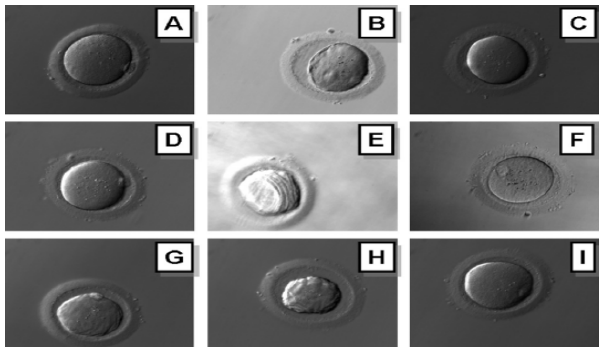


Figure 3 Metaphase-II human oocyte features during vitrification/warming. (**A**–**E**) Vitrification: (**A**) MII oocyte before equilibration; (**B**) oocyte shrinking at equilibration; (**C**) oocyte recovery at equilibration; (**D**) full-size recovery at equilibration; (**E**) shrunken oocyte in vitrification solution before loading on the carrier device. (**F**–**I**) Warming: (**F**) oocyte at first step of warming (1 M sucrose solution) on release from the carrier device; (**G**–**H**) oocyte shrinking during the second step of warming (0.5 M sucrose solution); (**I**) oocyte recovery during final washing.

4. PERMEABILITY - STAGE OF DEVELOPMENT

(i) Ratio of membrane surface area to cell water volume

(ii) The stage-specific membrane permeability for water

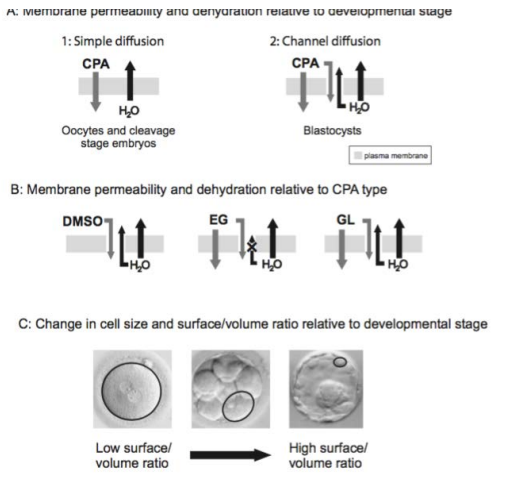
(iii) The stage-specific membrane permeability for CPAs

PERMEABILITY - STAGE OF DEVELOPMENT

1) Low permeability to CPA is typical for metaphase-II (MII) oocytes and early stages of development whereas higher permeability is representative for morulae and blastocysts (Pedro et al., 2005).

Longer exposure to CPAs - OOCYTE & ZYGOTE

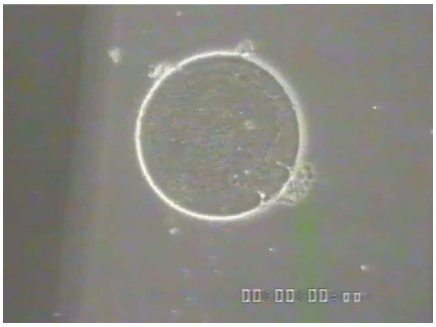
In Human morulae and blastocysts, glycerol, EG and DMSO move rapidly through the cell membrane by facilitated diffusion through channels whereas PrOH enters only slowly by simple diffusion (Kasai and Edashige, 2010)



PHYSICAL STRESSES DURING CRYOPRESERVATION

1. Direct effects of reduced temperature

2. Physical changes associated with ice formation .

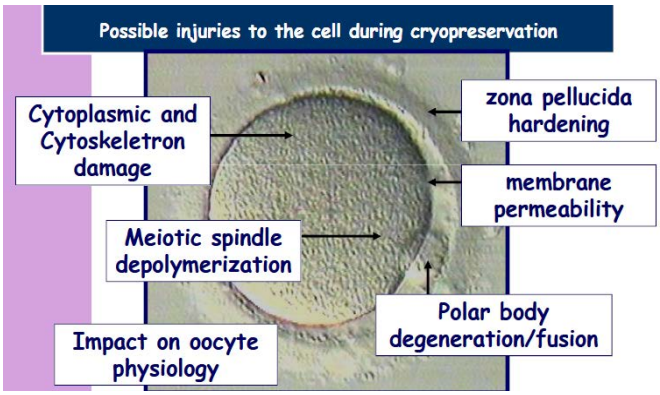


CRYO DAMAGES

- +15 c to -5 c = Chilling injuries
- -5 c to -50 c = Osmotic damage
- -50 c to -150 c = Fracture damage

Factors Associated with Cooling and Cryopreservation that Contribute to Cellular Injury and Death in Biological Systems (Shaw and Troson 2000)

System	Type/cause of damage
All	Intracellular ice formations, extracellular ice formation, apoptosis, toxicity, calcium imbalance, free radical, ATP levels, general metabolism, fertilization failure, cleavage failure, pHi, parthenogenetic activation,
Membrane	Rupture, leakage, fusion, microvilli, phase transition
Chromosomes	Loss/gain, polyspermy, polygyny (failure to extrude polar body), tertrapliody
DNA	Apoptosis, fusion, rearrangements
Cytoskeleton	Microtubules dissolve, actin
Proteins/enzymes	Dehydration, loss of function
Ultrastructure	Microvilli mitochondria, vesicles, cortical granules, zona pellucida
Zona Pellucida	Hardening, fracture
Lipids	Free radical?



Human Reproduction Vol.23, No.8 pp. 1778–1785, 2008 doi:10.1093/humrep/den127
Advance Access publication on May 12, 2008

Severe cytoplasmic abnormalities of the oocyte decrease cryosurvival and subsequent embryonic development of cryopreserved embryos

B. Balaban, B. Ata, A. Isiklar, K. Yakin and B. Urman[1]

Assisted Reproduction Unit, American Hospital of Istanbul, Guzelbahce Sokak No 20, Nisantasi, Istanbul 34365, Turkey

[1]Correspondence address. Tel: +90-212-3112000; Fax: +90-212-3112339. E-mail: burman@superonline.com

BACKGROUND: Abnormalities of oocyte morphology affect embryo quality and viability. Whether morphological abnormalities of the oocyte influence cryosurvival and further development of derived embryos is not known. The aim of this study was to compare cryosurvival and progression to the blastocyst stage of frozen–thawed embryos derived from normal and abnormal oocytes. **METHODS:** A total of 5292 Grade 1 and 2 embryos from 964 women were frozen, thawed and subsequently cultured up to the blastocyst stage. The study was performed on excess embryos from patients who did not opt for cryopreservation. Cryosurvival, progression to the blastocyst stage and hatching were correlated with morphological characteristics of the oocytes that embryos were derived from. **RESULTS:** Presence of a cytoplasmic abnormality of the oocyte significantly decreased cryosurvival. This detrimental effect was more pronounced in embryos derived from oocytes with vacuolar cytoplasm or with central granulation. Furthermore, these embryos did not have the potential to develop into good quality blastocysts or reach the hatching stage. On the other hand, presence of a single extracytoplasmic abnormality of the oocyte did not affect cryosurvival and the potential to develop into good quality blastocysts. Grade 2 embryos derived from oocytes with irregular shape or a large perivitelline space had decreased cryosurvival. However when these embryos survived cryopreservation, their potential to develop good quality blastocysts or to reach hatching stage was unaffected. **CONCLUSIONS:** Embryos derived from oocytes with vacuolar cytoplasm or central granulation do not seem to bear the potential to develop good quality blastocysts or to reach hatching stage after cryopreservation. The presence of extracytoplasmic abnormalities alone does not affect blastocyst development despite decreasing cryosurvival.

EXTRINSIC FACTORS

- Extrinsic factors are related to technical practice such as the type of carrier device or adaptation of the protocol to the stage of embryo development . (Vanderzwalmen et al., 2002).

1. Extrinsic factors:

- Reduced cooling rate/higher warming rates
- Modification of the vitrification protocol for aseptic carriers.
- User

TO OVERCOME CRYO -DAMAGECRYOPROTECTIVE AGENTS

CRYOPROTECTIVE AGENTS (CPA)

- Goal: minimize damage during the freezing/ thawing or the cooling/ warming period & reduce the

- Damage from temperature
- Damage from CPA themselves

CPAs should always be used in combination with non-permeating osmolytes such as sucrose or mannitol. These act as osmotic buffers to protect against cell swelling.

GOAL IS TO ACHIEVE 100% CRYO SURVIVAL

Table 1 Oocyte key performance indicator values.

KPI			Competence	Benchmark
O1	Morphological survival	Freezing	≥50%	75%
		Vitrification	70%	85% (95% for donors <30 years)

Table 3 Embryo key performance indicator values.

KPI		Competence		Benchmark
E1	Morphological survival: fully intact	Freezing	40%	55%
		Vitrification	70%	85%
E2	Morphological survival: ≥50% intact	Freezing	60%	85%
		Vitrification	85%	95%

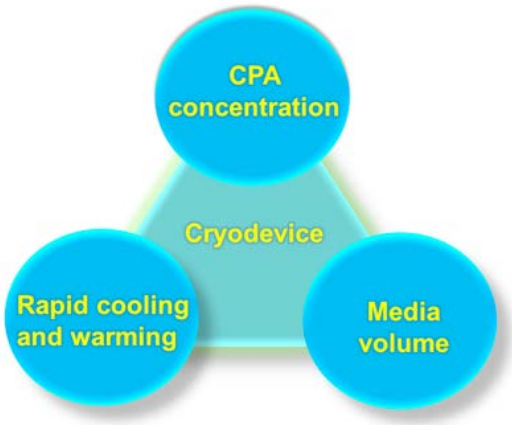
Table 4 – Key performance indicators.

Key performance indicator	Calculation	Competency value	Benchmark value
Blastocyst cryosurvival rate	no. blastocysts appearing intact × 100 / no. blastocysts warmed	≥90%	≥99%

SUCCESSFUL VITRIFICATION GRADE

- Successful cryopreservation of human embryos depends predominantly upon the selection of high quality embryos.
- For oocytes, the timing of vitrification is important, and oocytes must be vitrifed after 2-4 hours of incubation after retrieval, and immediately after denudation
- **Different quality = Different Survival**

SUCCESSFUL VITRIFICATION



1. CPA

- Permeating Toxicity : EG < DMSO/ PrOH
- Non Permeating :
- Trehalose
- Sucrose

- Hydroxy Propyl Cellulose
- SSS (Serum Substitute Supplement)
- DSS (Dextran Substitute Supplement)
- HAS (Human Albumin Solution)

Cooling media

Brand	Equilibration Solution (ES)	Vitrifcation Solution (VS)	Basic Composition
KETAZATO	EG / DMSO / HPC	EG / DMSO / HPC TREHALOSE	HEPES with Basic Culture Media
SAGE (ORIGIO)	7.5% (v/v) DMSO 7.5% (v/v)EG Human Albumin (12mg/ml	15% (v/v) DMSO15% (v/v) EG Human Albumin (12mg/ml) 0.6 M Sucrose	It is a MOPS buffered solution of modified HTF containing : Essential amino acids Non-essential amino acids Gentamicin Sulfate (0.01g/L)
CRYOTECH	EG / DMSO / HPC	EG / DMSO / HPC TREHALOSE	Modified HEPES Buffered MEM
IRVINE	7.5% (v/v) DMSO 7.5% (v/v)EG	15 % DMSO15 % Ethylene Glycol 20% DSS 0.5 M Sucrose	M – 199 HEPES Buffered Medium Gentamicin
VITROLIFE Omni	Ethylene Glycol Propanediol	Ethylene Glycol Propanediol Sucrose	MOPS Buffered Solution Human Albumin Serum Amino Acids Hyaluronan Gentamicin Energy substrates
Medicult	7.5% (v/v) PrOH 7.5% (v/v)EG HAS	15 % PrOH 15 % Ethylene Glycol HAS 0.5 M Sucrose	M – 199 HEPES Buffered Medium Gentamicin

Cooling media

Brand	Vitrification solution 1	Vitrification solution 2	Vitrification solution 3	Vitrification solution 4	Basic Compostion
COOK BLAST	Cryobase buffer	Cryobase buffer 8 % Dimethyl Sulphoxide 8% Ethylene Glycol	Cryobase buffer 16 % Dimethyl Sulphoxide 16 % Ethylene Glycol 0.68 M Trehalose	Dimethyl Sulphoxide (DMSO)	Cryobase buffer 10 mm HEPES Buffered Solution 20.0 mg/ml Human Serum Albumin 0.01 mg/ml Gentamicin
GLOBAL BLAST	Dimethyl sulphoxide (7.5 % v/v) Ethylene Glycol (7.5 % v/v) Human Serum Albumin (10 mg/ml)	Dimethyl sulphoxide (15 % v/v) Ethylene Glycol (15 % v/v) Sucrose (0.5 M) Human Serum Albumin (10 mg/ml)			Sodium chloride , Potassium chloride , Calcium chloride , Potassium phosphate , Magnesium sulfate , Sodium Bicarbonate , Glucose , Lactate Na Salt , Sodium Pyruvate , Glycine , L-Alanine , L-Arginine HCL , L-Asparagine , L-Aspartic Acid L-Cystine, L-Glutamic Acid, Glycyl-
VITROLIFE Blastocyst	No cryoprtectant	Ethylene Glycol Propanediol	Ethylene Glycol Propanediol Ficoll		MOPS Buffered Solution Amino Acids Hyaluronan

Warming media

Brand	Thawing Solution (TS)	Diluent Solution (DS)	Washing Solution (WS)
KETAZATO	Trehalose	Trehalose	HEPES with Basic Culture Media
SAGE (ORIGIO)	Human Albumin (12mg/ml) 1.0 M Sucrose	Human Albumin (12mg/ml) 0.5 M Sucrose	Human Albumin (12mg/ml)
CRYOTECH	Trehalose Hydroxy Propyl Cellulose	Trehalose Hydroxy Propyl Cellulose	Modified HEPES Buffered Hydroxy Propyl Cellulose
IRVINE	1.0 M Sucrose 20 % Dextran Substitute Supplement	0.5 M Sucrose 20 % Dextran Substitute Supplement	20 % Dextran Substitute Supplement
VITROLIFE Omni	Sucrose	Sucrose	Sucrose
Medicult	1M Sucrose	0.5M Sucrose	0.25M Sucrose

Warming Media

Brand	Warm solution 1	Warm solution 2	Warm solution 3	Warm solution 4	Basic Compostion
COOK BLAST	Cryobase buffer with 0.33 M Trehalose	Cryobase buffer with 0.2 M Trehalose	Cryobase buffer		Cryobase buffer 10 mm HEPES Buffered Solution 20.0 mg/ml Human Serum Albumin 0.01 mg/ml Gentamicin
GLOBAL BLAST	Sucrose (1.0 M) Human Se- rum Albumin (10 mg/ml)	Sucrose (0.5 M) Human Serum Albumin (10 mg/ml)	Human Serum Albumin		Glutamine , L-Histidine, L-Isoleucine , L-Leucine , L-Lysine HCL, L-Methi- onine , L-Phenylalanine , L-Proline , L-Serine , L- reonine , L-Trypto- phan , L-Tyrosine , L-Valine , EDTA , Phenol Red , HEPES, Gentamicin Sul- fate (10µg/ml)
VITROLIFE Blastocyst	Sucrose	Sucrose	No cryo- protectant		MOPS Buffered Solution Amino Acids Hyaluronan
VITROLIFE Omni	Sucrose	Sucrose	Sucrose	No sucrose	MOPS Buffered Solution Human Albu- min Serum Amino Acids Hyaluronan Gentamicin Energy substrates

2. COOLING & WARMING RATES

- The high cooling and warming rates applied at vitrification provide an unique benefit compared to the traditional freezing:
- The possible partial and sometimes total elimination of chilling injury, as the sample passes through the dangerous temperature zone quickly enough to disallow sufficient time for damage to develop..

The effects of cooling rates

Equilibrium vitrification (Rall and Fahy, 1985)

$$\frac{\text{Cooling rate (L) X [CPA] (H)}}{\text{Sample Volume (M)}}$$

Ultra rapid freezing (Trounson et al 1987)

$$\frac{\text{Cooling rate (H) X [CPA] (M)}}{\text{Sample Volume (M)}}$$

Minimum Drop Size (MDS) **Vitrification** (Arav et al, 1987,1992,1997,2002)

Minimal Volume (open pulled straw) **Vitrification** (Vajta et al 1997)

$$\frac{\text{Cooling rates (VH) x [CPA] (M)}}{\text{Sample Volume (VL)}}$$

(L=Low; H = High; VH = Very High; M = Moderate; VL = Very Low)

$$\text{Probability of Vitrification} = \frac{\text{Cooling \& Warming Rates X CPA Conc}}{\text{Volume}}$$

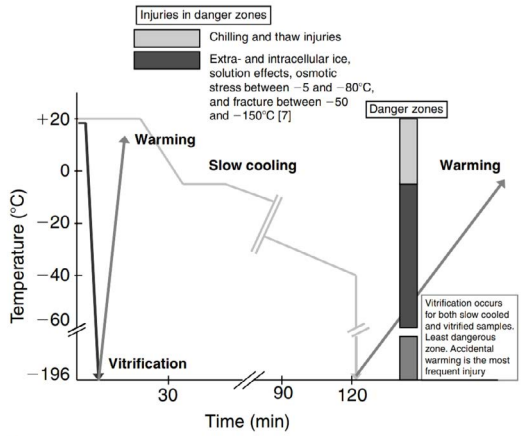
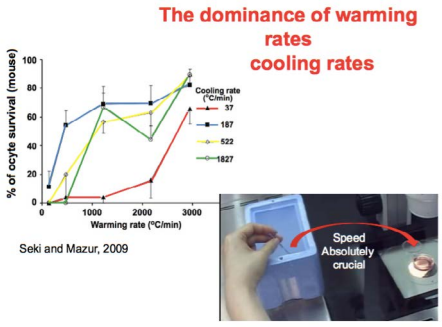


Figure 2.2. Cooling rates and dangerous zones during cryopreservation.



The dominance of warming rate over cooling rate in the surviva of mouse oocytes subjected to a vitrification procedure ☆

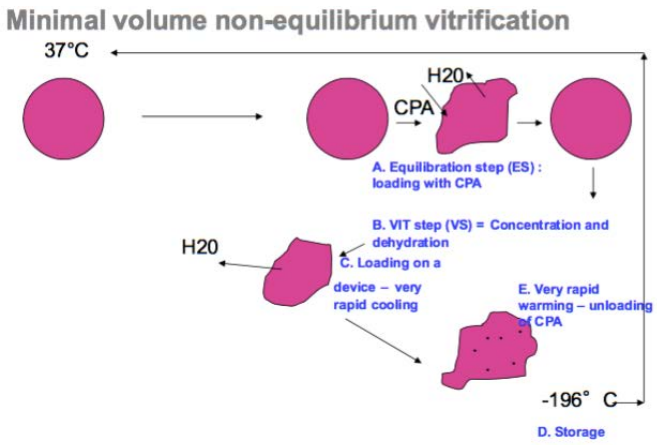
Shinsuke Seki, Peter Mazur

Show more

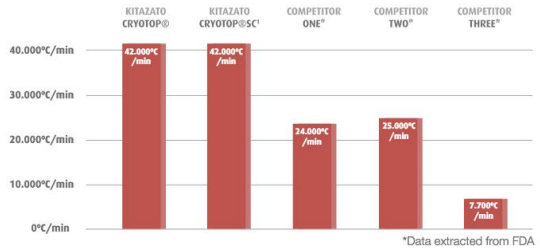
https://doi.org/10.1016/j.cryobiol.2009.04.012 Get rights and cont

3. MINIMUM VOLUME VITRIFICATION

- The first attempt to use ultrarapid cooling in everyday practice was based on the very simple idea of dropping the embryo containing solution directly into the liquid nitrogen.
- Theoretical advantages in maximizing cooling rate by doing so were limited at first by the relatively large volume of the drop as well as the delay before the drop floating on the surface of liquid nitrogen sank.
- Different carrier tools were applied to minimise the volume and to submerge the sample quickly into the liquid nitrogen, including electron microscopic grids , open pulled straws and cryoloops.



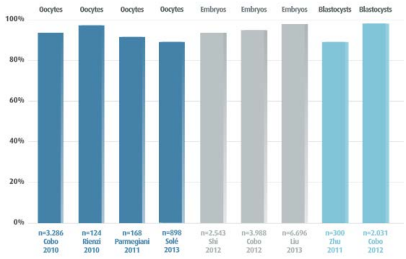
4. CRYODEVICES



*Data extracted from FDA

Warming rates of different vitrification devices

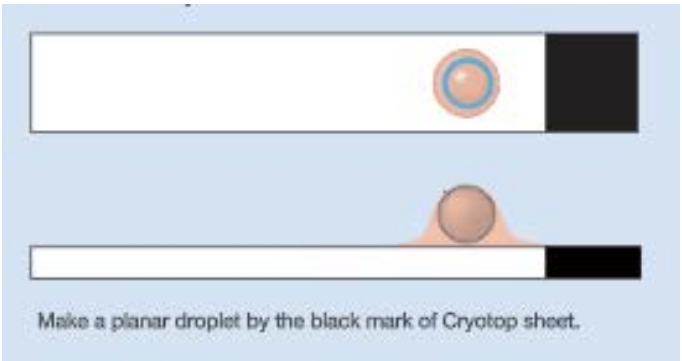
High cooling rate and warming rate are achieved by minimal volume of the solution in the vitrification. All types of Cryotop® allow loading of the specimens with a volume of 0.1µL; this minimal volume allows the reduction of the concentration of "cryoprotectant agents".



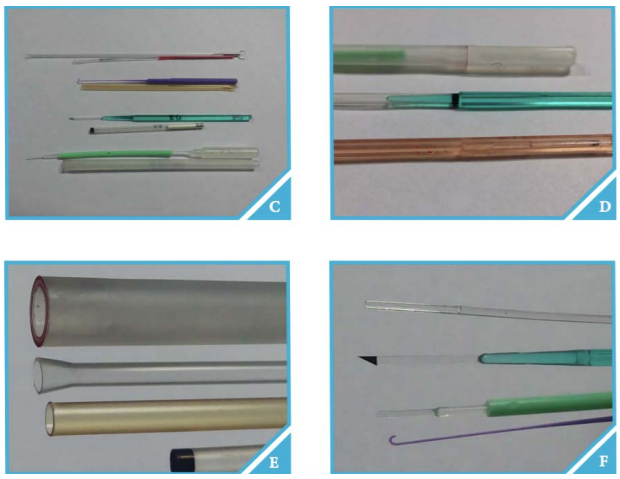
CRYOTOP® SURVIVAL RATES IN HUMAN SPECIMEN

There are high survival rates for oocytes and embryos in all stages of development, reported in numerous clinical publications with the largest study samples in the whole sector.

LOADING



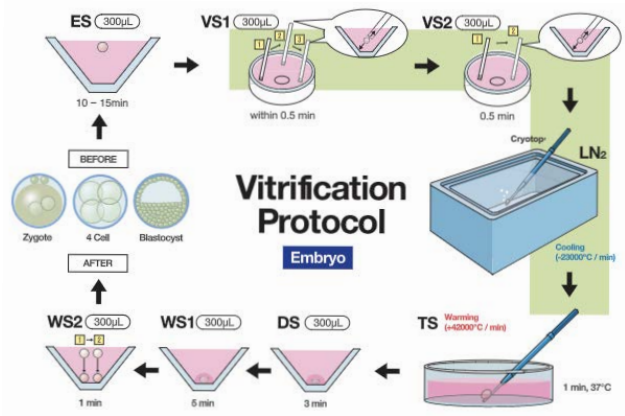
CRYO DEVICES



The device: open versus closed vitrification

	Vitrification	Warming	Storage
Cryo TOP	open	open	open
			closed?
Cryo Loop	open	open	open
Flexipet	open	open	open
Cryologic	open	open	open
.....			
Cryo TIP	closed	closed	closed
Cryopette	closed	closed	closed
.....			
Rapid i	semi-closed	open	closed
CBS HS VIT	closed	open	closed
Vitrisafe	closed	open	closed

FOLLOW THE PROTOCOL



- Equilibration
- Vitrification
- High Cooling Rate >10000/m

The Cryotec Method Perfect Survival And Safety

Cryotec Method is the most trustable method of cryopreservation.This open style method is recognized as easy, simple and repeatable for all.

Cryotec is a special device that allows to minimize the volume that will be cool and warm with a result up to **100% of survival** for oocytes and all the stage of pre-implantation embryos.

CRYOTECH ADVANTAGE; "WHY 100% SURVIVAL?"

Best Vitrification Solution

1. Highest Vitrification Capability by addition of HPC
2. No Serum, No protein contained in solutions
3. Endotoxin free Trehalose used instead of Sucrose

Best Vitrification Container; Cryotec

1. Multiple cooling devise: for Closed or Open cooling
2. Longer and Wider handle and sheet: easy writing and loading samples
3. Safe and clear material

Best exclusive Vitri- and Warm plate

1. On-focus vitrification plate
2. Easy warming plate: no blind well for warming

Table 1. Survival and development rates of human pronuclear (PN) embryos cryopreserved by either slow cooling or vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/cryopreserved rate (%)	1730/1944 (89)[a]	5881/5881 (100)[b]
Cleaved/surviving rate (%)	1557/1730 (90)[a]	5469/5881 (93)[b]
Blastocyst/cleaved rate (%)	796/1557 (51)[a]	3058/5469 (56)[b]
Blastocyst/cryopreserved rate (%)	796/1944 (41)[a]	3058/5881 (52)[b]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.01$).

Table 2. Survival and pregnancy rates with human 4-cell embryos cryopreserved by either slow cooling or vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/cryopreserved rate (%)	857/942 (91)[a]	879/897 (98)[b]
Pregnancy/transfer rate (%)	172/536 (32)[a]	136/504 (27)[a]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.01$).

LOADING

Table 3. Survival and pregnancy rates with human blastocysts cryopreserved by either slow cooling as compared with vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/vitrified rate (%)	131/156 (84)[a]	5695/6328 (90)[b]
Number of blastocysts transferred	127	5659
Pregnancy/transfer rate (%)	50/98 (51)[a]	2516/4745 (53)[a]
Live birth/transfer rate (%)	40/98 (41)[a]	2138/4745 (45)[a]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.05$).

Table 4. Survival, pregnancy and delivery rates after single embryo transfer of human blastocysts vitrified with either the Cryotop or the CryoTip method.

	Cryotop	*CryoTip*
Survived/vitrified rate (%)	221/227 (97)	82/88 (93)
Pregnancy/transfer rate (%)	131/221 (59)	42/82 (51)
Delivery/transfer rate (%)	113/221 (51)	39/82 (48)

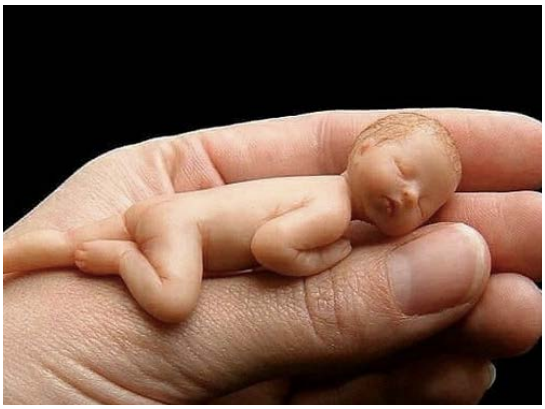
No significant differences between corresponding values were found.

USER

Technical proficiency of the embryologist.

Success of the vitrification method depends on a correct interplay between a
"sufficient"
High cooling rate,
Permeation of a sufficient high concentration of penetrating cryoprotectant,
Dehydration by a non-penetrating cryoprotectant, and a "sufficient" high warming

Everything is for keeping the precious life of human oocyte/ embryo with no damage no risk until the day patients dream will come true……



6. Risk management in cryopreservation in ART

Risk management in Cryopreservation in ART

Flow

- Introduction
- Risk management in cryopreservation
- ESHRE, ASRM guideline
- Summary

INTRODUCTION

- Patients who consent to the frozen storage of sperm or embryos quite rightly expect the storing centre to do everything reasonably possible to keep them in optimum conditions.
- Both the process of cryopreservation and the cryofacility are loaded with risk, from patient/sample processing, through to the eventual utilization or disposal of specimens.

INTRODUCTION

- The cryopreservation of sperm, embryos and reproductive tissues is an essential element of any assisted conception (or indeed cancer) service.
- To those patients who consent to storage, their frozen sperm or embryos are absolutely priceless and quite rightly they expect them to be kept in the best possible condition.

INTRODUCTION

- The process of cryopreservation of cells and tissues either for research or for therapeutic use is loaded with risk from beginning to end.
- Centres must focus on key areas of potential hazard or incident, particularly those associated with injury, loss or damage to stored material, and misidentification of stored material.
- involving any would have more fundamental consequences for the laboratory including financial loss and a threat to continuation of a service or project.
- Control measures to prevent injury are as much about education in the use and handling of liquid nitrogen as they are about protective clothing and more attention should be focused on preventing explosion, asphyxiation, burns and injury from manual handling.

Managing risk in Cryopreservation

- Define risk
- How to identify/quantify risk
- Risk associated with Cryopreservation
- Specific examples and controls

Unavoidable Risk

- Dynamically derived events e.g. infections, epidemics, manmade or natural disasters
- Will occur at some point despite risk avoidance measures
- Outcomes are about coping with the consequences - contingency planning (what if……)

Cryopreservation facilities

- Cryopreservation facilities should be rationally and safely located outside but close to the laboratory and, for safety reasons, with visible access to the interior (e.g. via a window, camera).
- Adequate ventilation and low oxygen alarms should be installed. Personal low oxygen alarms are recommended, as additional security measure.
- Cryostorage units should be continuously monitored and equipped with alarm systems, detecting any out of range temperature and/or levels of liquid nitrogen (LN2).
- Protection devices (e.g. glasses, face shield, cryogloves, apron, footwear) should be used during LN2 handling.
- All staff dealing with LN2 should be trained in safety aspects of its use.

ESHRE Guideline Group on good practice in IVF labs December 2015

key performance indicators (KPIs) for competence

- Regardless of the freezing technique preferred in the IVF laboratory, evidence-based key performance indicators (KPIs) for competence and benchmark values should be used in IVF laboratories for a standardized success in cryopreservation.
- These values should be checked weekly/monthly depending on the number of cryopreservation cycles in the center.

key performance indicators (KPIs) for competence

- KPIs should be objective and relevant, regularly checked and discussed, and communicated to all staff. KPIs can be based on a reference patient group with good prognosis, as well as on the whole patient population.
- Appropriate statistics can be used to account for patient variation and the number of previous treatment cycles patients may have already undertaken.

key performance indicators (KPIs) for competence

- All relevant data concerning laboratory work must be recorded in a database that allows KPI extraction and statistical analysis. Corrections, either written or electronic, should be traceable. Data should include:

- Morphological characteristics of gametes and embryos.
- Detailed information of the procedures, including timing and staff involved.
- All information needed to comply with the requirements of national and international data registries.

Avoidable Risk

- Found by: observation, common sense, past experiences
- RM benefits from an open (no blame) reporting culture
- Learning from mistakes - near miss/incident reporting

Failure to control risk

- Loss of Material (patient, research)
- Errors in ART
- Loss of reputation/referrals/contract
- Loss of license
- Loss of business
- Loss of fingers, toes, eyes, life…..

Major risk and cryopreservation of Reproductive cells and tissues

- 1.Injury to personnel

- 2. Loss of stored material
- 3. Damage to stored material
- 4. Misidentification of material
- 5. Risk to recipient/ART errors

- 5. Financial
- 6. Regulation
- 7. Quality Assurance

} Overarcging

The risk management process

- Should focus on

- minimizing losses, including staff injury, premature warming of cells and tissues, mistaken identity, and transmission of infection.

- Early warning and monitoring systems should be in place for

- quality assurance
- to prevent incidents involving cryovessels turning critical.

Human Reproduction Vol.20, No.7 pp. 1751–1756, 2005

The risk management process

Centres must ensure that every reasonable practical measure that can be put in place is done so, and that resourcing of the service adequately reflects the liability it represents.

The risk management of storage services

- Becoming an increasingly sensitive and important issue, particularly with regard to:

- injury to personnel,
- sample loss,
- premature sample thaw and
- the possibility of transmission of infectious disease between samples (Tedder et al., 1995).

Formal Risk Identification

- Risk Assessment
- Method for early identification of adverse events (hazards)

- Implement controls
- SOPs
- training
- equipment
- facilities
- reduce the chance of those occurring
- reduce insurance premiums

Assessing risk

- Risk Assessment – difficult (prior knowledge)

- Break down the process
- Areas/rooms e.g. cryoroom
- Process/Procedures

- Incidents – i.e actual occurrences
- probability can be estimated
- Controls implemented for future
- Open reporting culture essential

"Learn by your mistakes and those of others"

The risk assessment

- Centres should at least begin the process by carrying out formal risk assessments for all of the various components, which contribute to the cryostorage service.
- A risk assessment is defined as

- A method for the early identification of adverse events (hazards), which precedes a management phase, which would then include the identification and implementation of specific control measures to deal with each potential hazard.

Human Reproduction Vol.20, No.7 pp. 1751–1756, 2005

The risk assessment

- the area of impact may be the cryostorage facility and the nature of the risk could be one of the following:

- financial (risk to the business);
- natural event (flood/fire);
- human resources (training/staffing levels);
- infection control; health and safety;
- compliance with regulation and patient or user satisfaction (quality assurance).

Human Reproduction Vol.20, No.7 pp. 1751–1756, 2005

Australian/New Zealand model

- Each potential risk is quantified with a basic scoring system and ranked in order of priority.
- A commonly used scoring system is one based on an Australian/New Zealand model (AZ/NZ54360:1999) where the risk score is a product of the consequences and the chance of it occurring

Australian/New Zealand model

- A score is given and an indication of the adequacy of current controls.
- Obviously, with adequate controls in place, a low risk score will be obtained.

- Example might be staff injury (or worse) due to the failure of an oxygen depletion monitor, which potentially could have severe consequences possibly even leading to a fatality, so is given a relatively high score.

Risk assessments demonstrating risk scoring system.

Risk register Area of impact: Sperm cryopreservation/cryoroom Page 3 of 3

Location/ management unit: Andrology, ACU Risk assessor: Date: 17/5/04 Date of review: 17/5/05

Risk matrix Ref.	Description of risk (What and how can it happen?)	Adequacy of existing controls A	I	U	Risk assessment Consequences (C)	Likelihood (l)	Risk rating (CxI)	Risk ranking
10/16	Failure of vacuum on storage vessel	√			3	2	6	2
10	Failure of oxygen depletion monitor	√			5	1	5	6
11	Errors in cryopreservation procedure error insemen analysis	√			2	2	4	8
11/15	Error in storage location/inventory loss of stored samples	√			3	2	6	3
12/15	Inappropriate disclosure of information/access to computer files. Loss of computer data	√			3	2	6	7
13/15	Poor labelling of patient files,stored vials or straws. T card	√			3	2	6	4
10/15	Failure of alarms system and monitoring	√			3	2	6	5
14	Injury due to moving nitrogen cylinders		√		2	4	8	1

A=Adequate I=Inadequate U=Uncertain

Multiple fatalities 5 Single fatality 4 Major 3 Serious 2 Minor 1 Negligible 0

Certain 5 Likely 4 Possible 3 Unlikely 2 Rare 1 Impossible 0

Specific attention should focus on

- In terms of cryostorage, specific attention should focus on the following areas:

- physical security of vessels and specimens;
- liquid nitrogen supply and staff safety;
- the relative safety of the containment system (vials or straws);
- the type of nitrogen storage (liquid vs vapour phase);
- the suitability of equipment to do the job;
- witnessing and security of labelling;
- screening of patients for infectious diseases prior to storage;
- sample processing in order to lessen the risk of transmission;
- early warning and monitoring systems;

Physical security of vessels and specimens

- Risk registers should perhaps include reference to less likely occurrences such as fire, theft or even a major accident such as a heavy vehicle impacting on the building.

Liquid nitrogen supply and staff safety

- Storage within any freezer that warms much above 21358C, the so-called 'glassy transformation temperature' (Merryman, 1963) is likely to result in significant damage to the frozen cells or tissues.
- Maintenance of ultralow temperatures and hence a continued supply of liquid nitrogen must therefore be ensured for the safekeeping of specimens.

Supply failure

- Centres must ensure that if the nitrogen supply fails for whatever reason, there are fail-safe mechanisms in place to cope.
- Supply failure can occur for a number of reasons,

- for example, if the pressurized vessel vacuum fails or discharges its contents; the automated refrigerator over-fills (see later); the supply company fails to deliver.

Supply failure

- Such events could be survived, providing contingency measures are put in place, such as

- extra capacity in terms of nitrogen delivery vessels
- appropriate written procedures and training for all relevant staff for dealing with supply problems.

- Centres are often highly dependant on delivery from the nitrogen supply companies for regular filling of pressurized storage vessels.

Supply failure

- Controls should also anticipate

- potential delivery vehicle problems, for example heavy road traffic, accident or breakdown and
- contingency may include having spare capacity,

- either by keeping a surplus in spare pressurized vessels or
- by keeping an external bulk tank e.g. .1000 litres.

Loss , damage to samples

- Damage to samples due to

- inappropriate processing,
- inadvertent thaw or less likely,
- contamination from another sample or microorganism.

- Complete sample losses can result from misidentification, poor inventory control and poor record keeping with regard to the use of samples in therapeutic procedures.

Loss or Damage to stored material

Liquid nitrogen supply

- Delivery failure
  - Natural disaster
  - Breakdown
  - Other ……..
- Delivery vessel failure
- Staff failure
- Autofill system failed

Injury to staff

- Injury to staff, or indeed members of the public, can occur if nitrogen is not treated with respect.
- Supply vessels

- should be regularly checked and serviced
- their movement throughout the building should be carefully controlled.
- Neither delivery/laboratory staff nor members of the public should accompany a vessel being transported to upper floors of a building in an elevator.

Injury to staff

- An efficient nitrogen extraction system and a suitable oxygen monitoring system should be mandatory
- Staff should have some training in the use of cryogenic vessels and all available personal protective equipment (PPE).

Injury to staff

Hazards
A – asphyxiation (Death)
T – temperature (Burns)
P – pressure (Explosion)

Other
- Occupational health (transport of LN2)

- Who is at risk?

1.Delivery/portering/visitors/assessors
2. Laboratory staff

Injury to personnel Other

1. Burns
   PPE, emergency procedures

2. Explosion
- Flasks
- Cryovials
- Ice plugs

Relative safety of the containment system (vials or straws)

There are basically four options:
1. PVC straws;
2. cryovials;
3. ionomeric resin (CBS) straws; and
4. glass ampoules.

- Ionomeric resin (CBS) straws have a more effective sealing method than PVC straws and are less likely to break.
- Glass ampoules are rarely used but represent a considerable hazard if they explode or break
- The ionomeric resin straw would appear to be the inventory option with the lowest associated risk

Human Reproduction Vol.20, No.7 pp. 1751–1756, 2005

Storage in the gaseous phase of nitrogen (vapour storage)

- Large towers with drawers for cryovials also provide suitable conductivity, although they are cumbersome and do not easily allow the storage of different containment systems i.e. they are limited to vials.
- A relatively full freezer, with an appropriately designed racking and inventory will achieve temperatures as low as 21908C just below the freezer lid with a temperature range of between only 21758C to 21908C (Table I).

Using suitable equipment and materials to do the job

- All equipment has a finite lifespan and as nitrogen vessels age their vacuum will slowly diminish until eventually the vessel will hold nitrogen for too short a period for it to be of economic use.
- There is usually warning of this (frost on outside of vessel, shortened fill intervals) and plenty of time to provide a replacement.

Human Reproduction Vol.20, No.7 pp. 1751–1756, 2005

Witnessing and security of labelling

- Witnessing is now an established part of the assisted conception service, especially in the UK where it is mandatory for a second person to verify all processes involving transfer of gametes or embryos, e.g. transfer of sperm/cryoprotectant to straws or vials.

Labels on straws or vials

- Labels on straws or vials must withstand immersion in liquid nitrogen and/or extreme cold.
- Anecdotal reports of poor practice such as paper tags for labelling straws and inaccurate and misaligned markings on straws have led to a number of transcription incidents.

Human Reproduction Vol.20, No.7 pp. 1751–1756, 2005

Labels on straws or vials

- The consequences could be (and no doubt have been) disastrous.
- Labelling must be clear and accurate, using appropriate labelling pens and avoiding poor handwriting, which can also lead to transcription errors.

Screening of patients for infectious diseases prior to storage

- The screening of all patients for HIV and hepatitis B and C.
- Centres should not become complacent and over-reliant on screening, however, particularly as quarantine to cover the 'window of seroconversion' may not be practical and other, as yet undiscovered, pathogens will no doubt cause us a problem in the future.

Methods of sperm cryopreservation/sample processing to reduce transmission risk

- Ideally, laboratories should aim to install a comprehensive system which provides

- early warning in emergency situations
- also provides continuous monitoring of vital equipment,.

- additional features

- such as remote call-in facility for interrogating and diagnosing any fault or incident, often circumventing the need for immediate on-site visit.

Early warning and monitoring

- Early warning and monitoring systems should be in place for quality assurance and to prevent incidents involving cryovessels turning critical
- An efficient nitrogen extraction system and a suitable oxygen monitoring system should be mandatory and staff should have some training in the use of cryogenic vessels and all available personal protective equipment(PPE)

Early warning and monitoring

- Ideally, laboratories should aim to install a comprehensive system which not only provides early warning in emergency situations but also provides continuous monitoring of vital equipment, serving as important quality assurance.

ASRM Guidelines for Human Embryology Laboratories

- A written protocol should include cryoprotectant used (including source and shelf life), media used, type of freezing container (e.g.,straw, vial, or ampule), stage of embryo for freezing, freezing rate including procedure for manual or automatic seeding, and storage conditions.

The Practice Committee of the Society for Assisted Reproductive Technology. Revised guidelines for human embryology and andrology laboratories. Fertil Steril. 2008;90(5 Suppl):S45-S59.

ASRM Guidelines for Human Embryology Laboratories

- All embryo freezing containers (e.g., each straw or vial) must be permanently labeled with at least two unique identifiers.
- A method of ensuring prompt, accurate retrieval of cryopreserved specimens must be employed.
- Duplicate records of all embryos in storage should be kept, in separate locations, exclusive of the patient chart information.

The Practice Committee of the Society for Assisted Reproductive Technology. Revised guidelines for human embryology and andrology laboratories. Fertil Steril. 2008;90(5 Suppl):S45-S59.

ASRM Guidelines for Human Embryology Laboratories

- All embryo freezing containers (e.g., each straw or vial) must be permanently labeled with at least two unique identifiers.
- A method of ensuring prompt, accurate retrieval of cryopreserved specimens must be employed.
- Duplicate records of all embryos in storage should be kept, in separate locations, exclusive of the patient chart information.

The Practice Committee of the Society for Assisted Reproductive Technology. Revised guidelines for human embryology and andrology laboratories. Fertil Steril. 2008;90(5 Suppl):S45-S59.

ASRM Guidelines for Human Embryology Laboratories

- Time limits for embryo storage should be established by each individual laboratory and determined prior to freezing
- If the laboratory performs cryopreservation,there should be a system in place for the detection of low levels of liquid nitrogen.

The Practice Committee of the Society for Assisted Reproductive Technology. Revised guidelines for human embryology and andrology laboratories. Fertil Steril. 2008;90(5 Suppl):S45-S59.

ASRM Guidelines for Human Embryology Laboratories

- Procedures for thawing embryos should include cryoprotectant concentrations and media used, temperature requirements for thawing, criteria for assessing embryo viability,time period for embryo culture prior to transfer, protocol for patient preparation for frozen embryo transfers and conditions under which embryo transfers will take place

The Practice Committee of the Society for Assisted Reproductive Technology. Revised guidelines for human embryology and andrology laboratories. Fertil Steril. 2008;90(5 Suppl):S45-S59.

ESHRE Guideline -Cryopreservation facilities and material

- Cryopreservation facilities should be rationally and safely located outside but close to the laboratory and, for safety reasons, with visible access to the interior (e.g. via a window, camera).
- Adequate ventilation and low oxygen alarms should be installed. Personal low oxygen alarms are recommended, as additional security measure.
- Cryostorage units should be continuously monitored and equipped with alarm systems, detecting any out of range temperature and/or levels of liquid nitrogen (LN2).
- Protection devices (e.g. glasses, face shield, cryogloves, apron, footwear) should be used during LN2 handling.
- All staff dealing with LN2 should be trained in safety aspects of its use.

ESHRE Guideline Group on good practice in IVF labs December 2015

ESHRE Guideline -Different cryopreservation approaches

- For cleavage-stage embryos and blastocysts, high success rates have been reported when using vitrification.
- However, for pronuclear and cleavage-stage embryos, good results can also be obtained using slow-freezing methods.

ESHRE Guideline Group on good practice in IVF labs December 2015

ESHRE Guideline -In order to minimise any risk of transmission of infection via LN2

- Contamination of the external surface of cryo-devices should be avoided when loading them with samples.
- Safety issues have been raised regarding direct contact of the biological material with the LN2; however, at this point closed devices cannot be favoured over open devices. Laboratories should make decisions based upon their results, risk analysis and regulations in place.
- Specimens from sero-positive patients should be stored in high-security closed devices. Dedicated vapour phase tanks are recommended

ESHRE Guideline Group on good practice in IVF labs December 2015

ESHRE Guideline -At cryopreservation, documentation on biological material should include

- Labelling of devices;
- - Cryopreservation method;
- - Date and time of cryopreservation;
- - Operator;
- - Embryo quality and stage of development;
- - Number of oocytes or embryos per device;
- - Number of devices stored per patient;
- - Location of stored samples (tank, canister).

ESHRE Guideline Group on good practice in IVF labs December 2015

ESHRE Guideline -At cryopreservation, documentation

- Cryo-devices must be clearly and permanently labelled with reference to patient details, treatment number and/or a unique identification code.
- periodic inventory of the contents of the cryobank is recommended, including cross-referencing contents with storage records.

ESHRE Guideline Group on good practice in IVF labs December 2015

ESHRE Guideline -At thawing, documentation on biological material should include:

- Thawing method;
- - Date and time of thawing;
- - Operator;
- - Post-thawing sample quality
-
- A double-check of patient identity is recommended in the following steps: transfer of samples into labelled cryo-dish, loading of the labelled device, deposition in the cryobank, removal from the cryobank.
- During storage and handling of cryopreserved material, care should be taken to maintain adequate and safe conditions. Temperatures should never rise above -130°C.

ESHRE Guideline Group on good practice in IVF labs December 2015

Summary

- Centres must look at their resourcing in a risk management context.
- To believe that patients are unaware of such risk is irresponsible; indeed, patient expectations are constantly on the increase and they more or less expect such measures to be in place already.
- Centres should begin the risk management process and identify areas of risk within their own service: priority should obviously be given to the areas of highest risk.
- This has to be affordable, manageable, easy to use and implemented alongside other risk reduction strategies.

Summary

- Identification of such risk relies on a combination of a systematic prospective approach (risk assessment) with a documented and formal recognition of previous mistakes or near misses. Risk potential can be formally scored and ranked to provide services with the means of prioritising and allocating appropriate resources.
- Failure to implement a comprehensive risk management strategy could be translated as 'negligence' should a similar incident subsequently occur.
- The risk management of any services including cryopreservation should therefore be considered fundamental and integrated into business planning, objective setting and departmental budgeting to ensure continued improvement and the delivery of a quality and safe service or indeed research project.

Summary

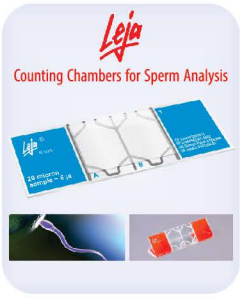
- Identify potential hazard formally – even if low risk
- Hazards with low risk - no controls
- Hazards with high risk - implement controls

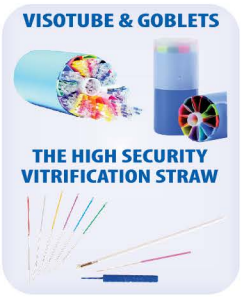
- SOPs
- training
- Equipment/materials
- facilities

- Institute support easier with formal risk assessment

Thank You

Cryo Bio System

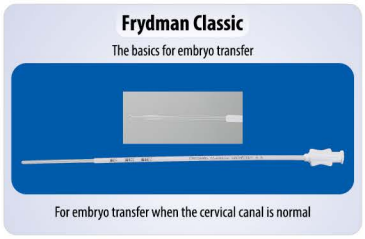


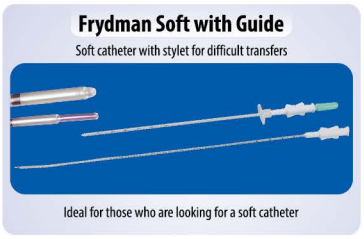


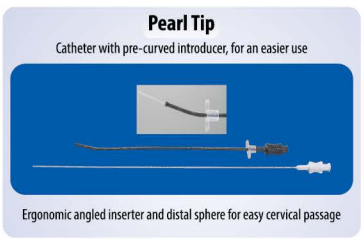
Cryo Bio System

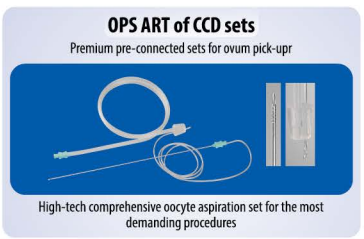
CCD Your partner for Life

Embryo Transfer Catheters & Oocyte Retrieval Needles









Cryo Bio System

IrvineScientific
Grow With Us™

Andrology

ICSI Media

IVF Culture Media

Vitrification Freeze & Thaw

Cryo Bio System India
(A Division of IMV India Pvt. Ltd.)
Plot No. 750, Udyog Vihar Phase- V, Gurugram (HR)-122016
T : +91-124-4770707 E : infocbs@cryobiosystemindia.com

No Conflict of Interest and The Bulletin is being brought out by Education Grant by Cryobio System

Cryo Bio System