Zygote Cryopreservation: Which Zygote & Which Method

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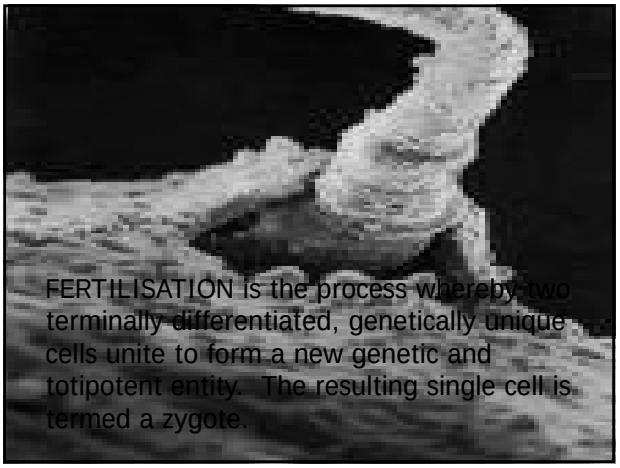
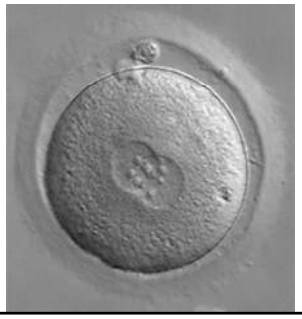
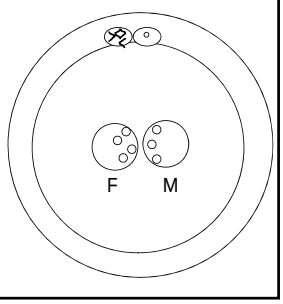


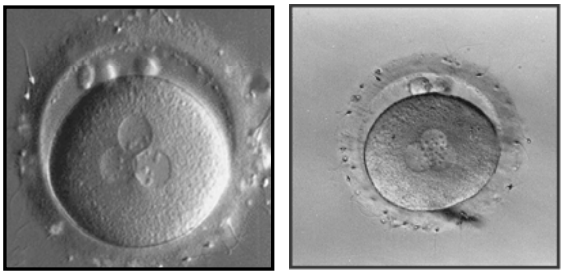
FERTILISATION is the process whereby two terminally differentiated, genetically unique cells unite to form a new genetic and totipotent entity. The resulting single cell is termed a zygote.

Zygote
Normal Fertilization
2 Pronuclei (PN) & 2 Polar Bodies (PB)





Abnormal Fertilization
3 PN = Polyspermic/Dygynic



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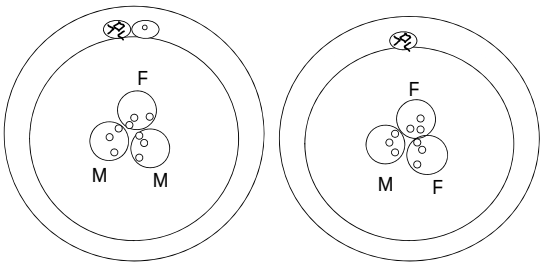
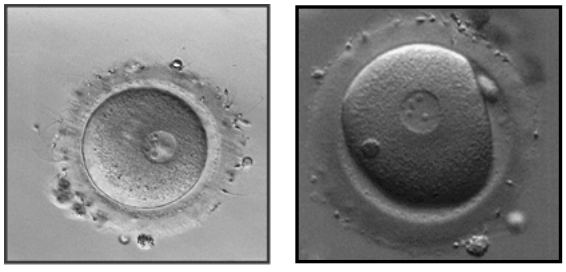


Polyspermic Digynic

Abnormal Fertilization
1 PN = Parthenogenetic/Silent Fertilization



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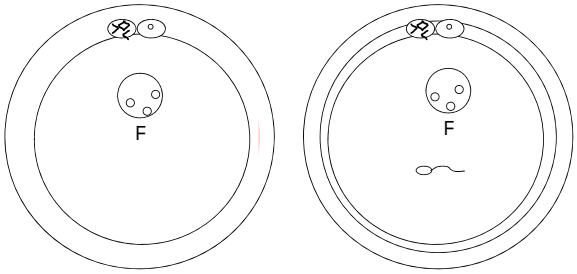
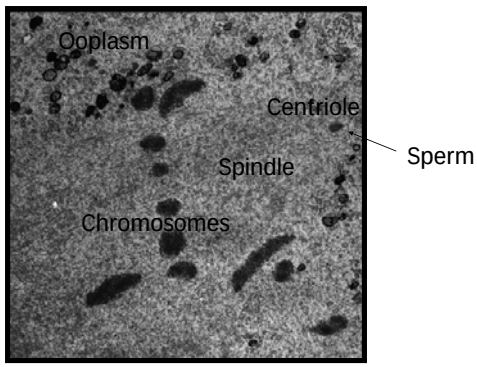


Syngamy

- Union of the male and female pronucleus within the zygote
- Characterized by the breakdown of nuclear membranes and the coming together of the male and female chromosomes on the first mitotic bipolar spindle in preparation for cell division

Syngamy – Human Mitosis I



Fertilization & Syngamy *In Vitro*

- Median time to 2nd PB extrusion 2h 39min, maximum time 8h
- Pronucleus can be visualized as early as 2h 51min post-injection (median time 4h 59min for male and female pronucleus)
- Male pronucleus appears at same time or before female pronucleus (median time difference for asynchronous appearance is 31min and maximal difference 2h 15min)
- Maximum time for female pronucleus observation 11h 26min
- Median time to pronucleus abuttal 7h 9min (maximum 12h 48min)
- Female pronucleus (22 µm) smaller in diameter than male pronucleus (24 µm) at abuttal and located closest to 2nd PB
- Pronuclei begin to break down around 18-20 hours
- Time course following IVF very similar

Payne et al., 1997
Nagy et al., 1994

Why Cryopreserve Zygotes?

Advantages

- Satisfies the legal requirements in several countries i.e. Germany
- Not viewed as an embryo so less moral and ethical conflict associated with discard
- Maximizes the chances of pregnancy from a single stimulation cycle
- Unicellular
- Lack of spindle apparatus (c.f. oocyte)
- Liquid phase transition temperature is lower than for oocytes rendering the membranes more resistant to chilling injury

Ghetler et al., 2005

- High post-thaw survival rates
- Little impact on viability
- Survival is easy to diagnose by passage through syngamy and progression to first cleavage

Comparative Viability of Fresh & Frozen Zygotes

Publication	No. Zygotes[A] Frozen	Survival (%A)	Cleaved (%A)	Frozen Zygote Implantation Rate % (%A)	Fresh Zygote Implantation Rate % Zygotes
Fugger et al., 1988	82	76 (93)	62 (76)	5 (4)	3
Miller et al., 1995	239	208 (87)	197 (82)	11 (9)	13
Damario et al., 2000	398		359 (90)	22 (19)	25

Why Cryopreserve Zygotes?

Disadvantages

- Preservation of many pre-embryos with little developmental potential
  - Can be ameliorated by preserving only those zygotes exhibiting good PN scores
- Little opportunity to select the one or two most viable pre-embryos from a cohort for transfer
- Potentially increases the number of cycles before achievement of pregnancy
- Time-consuming and laborious

When to Cryopreserve Zygotes?

- S Phase in the zygote begins at 9-10 h post-insemination and ceases 3-5 h later
- Duration of G2 Phase is 4-6 h
- Duration of M Phase is 3-3.5 h

Balakier et al., 1993

- Therefore optimal time for cryopreservation of human zygotes is 16-20h post-insemination when majority of zygotes are in the G2 Phase of cell cycle

Cryopreservation

- Exposure to cryoprotectants
- Cooling to subzero temperatures
- Storage
- Thawing
- Dilution and removal of cryoprotectants
- Restoration to physiological environment

Cryopreservation

- Freezing - Ice formation
- Vitrification - Glass formation

Freezing

- Equilibration with cryoprotectant
- Loading into straws, vials or ampoules
- Relatively rapid cooling to seed temperature
- “Seeding” at -7°C to induce ice formation
- Slow cooling → -0.3 /min to temperatures of -30°C to -40°C
- Plunge into liquid nitrogen and store

Freezing Solutions

- Phosphate or Hepes buffered medium
- Supplemented with:
  - Albumin or SSS (5% - 20%)
  - Serum (20%)
  - Sucrose (0.1M)
- Cryoprotectants
  - PROH (1.5M)

Freezing Protocol

- 1.5M PROH for 15min
- 1.5M PROH + 0.1M sucrose 10min
- Load into freezing straws

Freezing Program

- Room temperature
- -2°C/min to -5°C to -7°C
- Hold for 5 - 10min for manual seeding
- -0.3°C to -0.4°C down to -30°C to -40°C
- Plunge into liquid nitrogen

Suggested Improvements to Freezing Protocols

- Extend post-seeding time from 5min to 20min
- Cool to -180°C rather than -30°C before removal to liquid nitrogen storage

Boone et al., 2005

Thawing

- Warming rate critical to the success of slow cooling procedures as ice recrystallises at temperatures above -120°C
- Temperature is raised slowly from -196°C to about -120°C by brief exposure to air
- Temperature rapidly raised by plunging in a waterbath of 30-32°C
- Step-wise removal of cryoprotectant in thaw solutions containing decreasing concentrations of cryoprotectant to eliminate cell swelling as rehydration occurs

Thawing Protocol

- Room temperature – 40sec
- 30°C waterbath – 30sec
- 1.0M PROH + 0.2M sucrose – 5min
- 0.5M PROH + 0.2M sucrose – 5min
- 0.2M sucrose – 5min
- Buffered medium – 5min
- Culture

Cryopreservation

- Freezing - Ice formation
- Vitrification - Glass formation

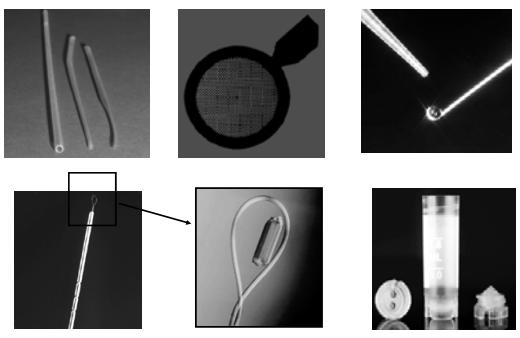
Vitrification

- Utilises a solution of >40% solutes
- Very rapid cooling rates of >15,000 °C
- Very brief exposure to vitrification solution that contains high concentrations of cryoprotectants supplemented with:
  - sucrose
  - Ficoll
  - poly ethylene glycol (PEG)

Vitrification

- Carrier modified to achieve very high cooling rates
  - Open pulled straws (OPS)
  - Flexipet
  - Copper electron-microscope grids
  - Cryoloops
  - Cryotops
  - Cryotips
  - Fibreplugs

Vitrification Carriers



Vitrification Protocol

- Oocyte/Embryo loaded onto carrier in minimal volume of vitrification solution
- Plunged directly into liquid nitrogen
- In the case of fibreplugs, the carrier is touched to a liquid nitrogen cooled stainless steel surface
  - has the advantage that the zygote does not come into direct contact with liquid nitrogen so eliminates the potential for microbial contamination

Warming

- Step-wise removal of cryoprotectant in warming solutions containing decreasing concentrations of sucrose to eliminate cell swelling as rehydration occurs

Vitrification Protocol

Kuwayama et al., 2005

Vitrification in Cryotips/Cryotops

- 7.5% Ethylene Glycol + 7.5% DMSO +20%SSS – 5 to 15 min
- 15% Ethylene Glycol + 15% DMSO + 0.5M sucrose + 20% SSS – 3 x 20 sec

Warming for cryotips at 27°C

- 2 x 1.0M sucrose +20% SSS – 1 min
- 2 x 0.5M sucrose + 20% SSS – 2 min
- 3 x wash in culture medium + 20% SSS – 3 min
- Restore to pre-equilibrated culture medium

Warming for cryotops at 37°C

- 1.0M sucrose +20% SSS – 1 min
- 0.5M sucrose + 20% SSS – 3 min
- 2 x wash in culture medium + 20% SSS – 5 min
- Restore to pre-equilibrated culture medium

Publication	Method	Carrier	No. Zygotes[A]	Survival (%A)	Cleaved (%A)	Blastocyst (%A)	Implantation Rate % (%A)
Hoover et al., 1997	S		761	718 (94)	647 (85)		7 (6)
Damario et al., 1999	S		724	657 (91)	650 (90)		20 (18)
Kattera et al., 1999	S		701	452 (64)	369 (53)		5 (3)
Senn et al., 2000	S		1000	804 (80)	750 (75)		11 (8)
Selman et al., 2002	V	OPS	27	17 (67)	14 (52)		18 (7)
Isachenko et al., 2003	V	OPS	59	42 (71)	35 (59)		15 (8)
Veeck, 2004	S		1441	1101 (76)	997 (69)		17 (12)
Kuwayama et al., 2005	V	Cryotop	5881	5881 (100)	5469 (93)	3058 (52)	
	S		1944	1730 (89)	1557 (90)	796 (41)	
Al-Hasani et al., 2007	V	Cryotop	339	302 (89)	243 (72)		16 (11)

S = slow cooling V = vitrification

Outcomes Following Cryopreserveration at the Zygote Stage

- Implantation rates are similar to those reported for transfer of fresh sibling zygotes
- Implantation rates are generally much lower than those reported for transfer of fresh/cryopreserved cleavage stage or blastocyst stage embryos

Summary

- Select only normally fertilized oocytes for cryopreservation at the zygote stage
  - May limit numbers preserved according to existing viability scoring criteria
- Cryopreserve 16-20h post-insemination during G2 Phase of cell cycle
  - Do not cryopreserve at syngamy
- Cryopreservation does not result in a significant reduction in viability but not all zygotes will survive the procedure
- Vitrification and slow cooling protocols can be used to cryopreserve zygotes
  - Recent results indicate that vitrification may be the superior procedure for cryopreservation at the zygote stage

Conclusions

Although the zygote stage of development can be successfully cryopreserved with high post-thaw survival, there is little ability to select the one or two most viable zygotes in the cohort for transfer resulting in:

- Significant workload for the laboratory
- Potential delays to achieving pregnancy

Recommended only in countries that prohibit cryopreservation at later stages of development when there is further opportunities to select those embryos likely to survive cryopreservation with a high probability of viability