VITRIFICATION

PRESENTATION NO. 99

Search for a Safe Least Toxic Vitrification Solution: VS14?

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Vitrified object

PROLOGUE

- **Declaration:**
  - I have no financial interest in the VS14 method of vitrification

  Appreciation:
  - College of Reproductive Biology
  - Dr Bill Boone
  - The Scientific Committee
  - Travel grant from LabIVF
  - My employers for granting conference leave
- **Pardon:**
  - For the insults I am about to hurl against the English Language

Work on Vitrification Late 1980' to early 1990's

Begining early 1990's vitrification began to show promise as an efficient method of cryopreservation and as a potential replacement for controlled rate freezing

Vitrification

'Parvus error in principio magnus est in fine'

Aristotle's reiteration of Plato's warning

"A small mistake in the beginning is large in the end".

- 1980-early 1990's Vitrification research appeared **headed in right direction**
- Then all of sudden vitrification research **reached a crossroad,** then headed down a path that was un- thinkable
- Resulting in the application of non-aseptic cryo techniques in healthcare.
- Some jumped on to the bandwagon-applied non-aseptic technique in healthcare
- For more than 10yrs transplantable human tissues exposed to the elements (embryos, oocytes, etc)
- **Not GLP/GCP - Matter of grave concern**
- **Now many retracting**

Vitrification in mid 1990's

Go right for closed

Go left for open

See

1.Riha et al. Zivoc Vir 1991;36:113–120,
Cited In: Vajta G, Kuwayama M. Theriogenology 65:236-244 (2006).
2.Martino et al. Biol Reprod 54:1059–1069 (1996)
3. Vajta et al. Cryo-Lett 18:191–195 (1997)
4. Lane et al., Fertil Steril 72:1073–1078 (1999)
5. Lane et al., Nature Biotechnol 17(12): 1234-1236 (1999)
6. Vajta and Kuwayama. Theriogenology 65:236-244 (2006)

It is possible to get excellent results with simple, safe, closed (aseptic) methods of vitrification using the 0.25ml straw

No reason why we have to
go down the path of non-asepsis

See :

Kasai, M., Komi, J.H., Takakamo, A. *et al.* (1990). *J Reprod. Fertil*., **89**, 91–97.
Ali J. PhD Thesis 1992 Australian National University, Canberra.
Ali J, Shelton JN. J Reprod Fertil 1993c; 99:65 –70.
Ali J, Shelton JN. J Repro. Fertil 1993b; 98:459–465.

Vitrification Hazardous

- World Congress on Human Reproduction, **Tokyo, Japan**, Oct 1987
- Vitrification attempted – DMSO - Trounson
- Observation: Teratogenic in mouse
- Photographic evidence proved the point
- Inference: Vitrification hazardous
- Challenge to identify/develop a safe VS

Pioneered human embryo cryopreservation
Trounson A, Mohr L.. *Nature.* 1983;305(5936):707–709

Work on Vitrification Late 1980' to early 1990's

- Progress in vitrification hampered although first described in 1937 (Luyet)
- TOXICITY main concern
- TO VITRIFY A SOLN - HIGH SOLUTE CONC. ~40V/V
- High solute concentration of 40% in VS is usually TOXIC
- Rall and Fahy, 1986 – used Acetamide (toxic)forced to use low temp (cold cryoprotectant solutions) to avoid toxicity

Selection of vitrification solution

- It must vitrify when cooled by plunging into liquid nitrogen
- It must not devitrify during warming
- or fracture on cooling or warming
- It must be non-toxic to embryos.

[Toxicity is temperature dependent; cryoprotectant must not be toxic at the temperature at which embryos and media are held during the stages of cryopreservation].

Factors affecting vitrification

Solute concentration
Cell dehydration, degree of
Volume of vehicle
Cooling rate & warming rate
Others

1. Solute concentration

for vitrification to occur in a 0.25ml straw

Cryoprotect	M Conc .	v/v
Sucrose	2.0M	(68.4%)
B. Glycol	3.0M	(27%)
Methanol	No vit	N.A.
P. Glycol	4.0M	(30.4%)
DMSO	5.0M	(39.1%)
Glycerol	5.0M	(46.1%)
E. Glycol	6.5M	7.0M (37.5%)

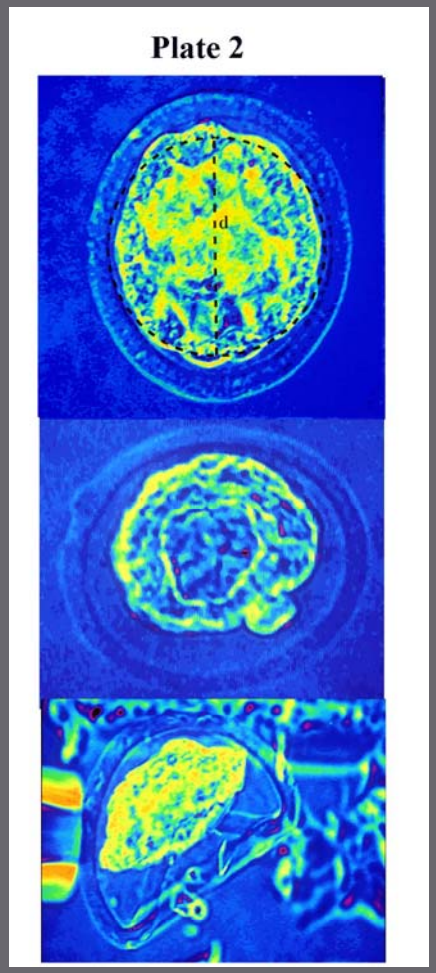
- (Ali, 1992; Ali and Shelton, 1993a)

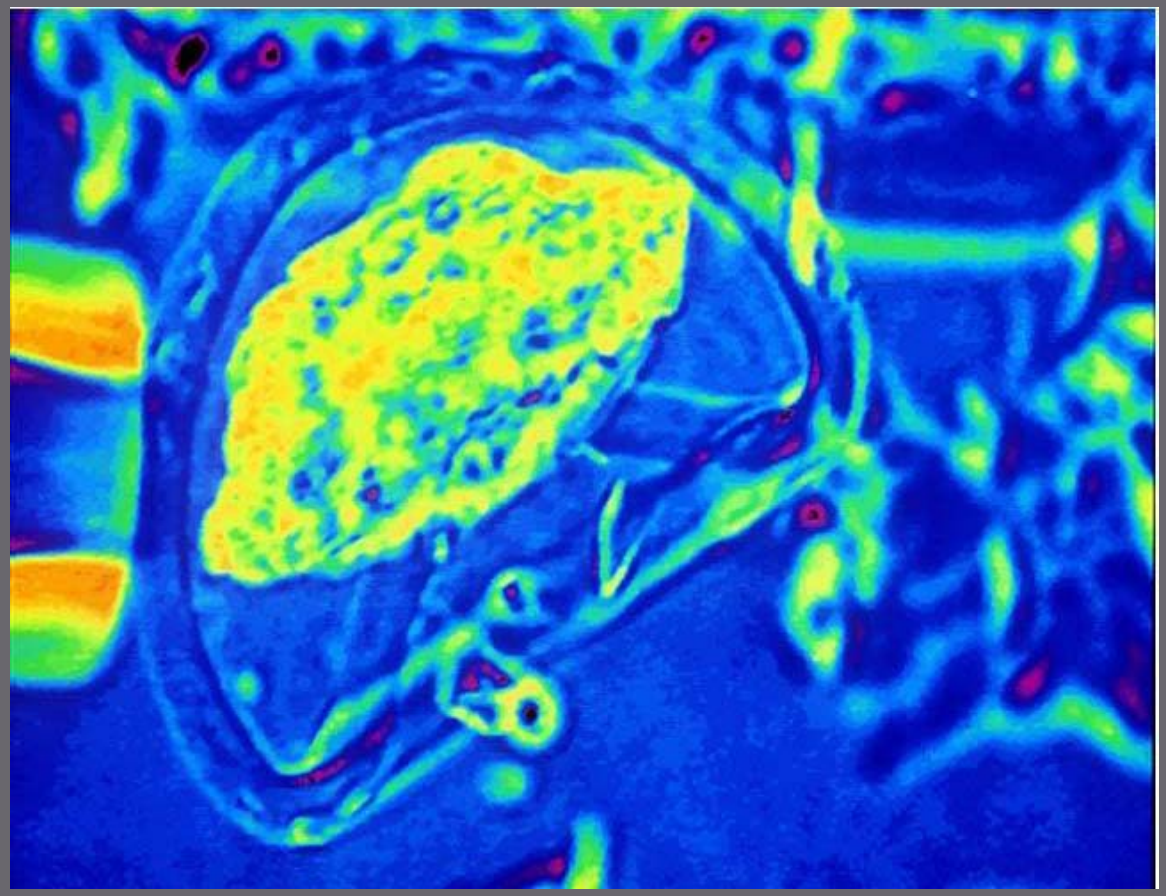
2. Dehydration

and its impact on intracellular solute concentration

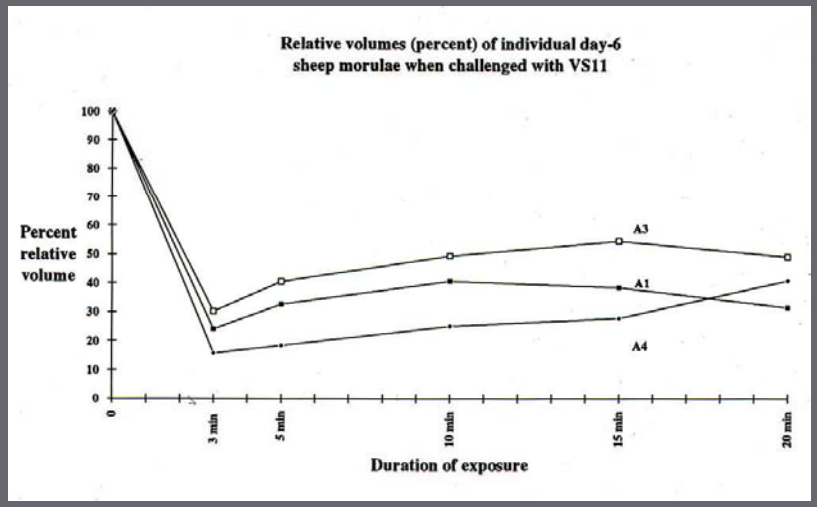
- Vitrification solutions - highly concentrated
- Causes rapid dehydration of cells
- Permeability of water 5000x faster than most solutes
  - Jackowski S, Leibo SP, Mazur P., 1980
- Rapid dehydration increase intracellular solute concentration
- Increase in intracellular solute concentration for intracellular vitrification
- High intracellular solutes promotes & contributes to intracellular glass formation

Severe dehydration after exposure to VS
Ali and Shelton, 1993



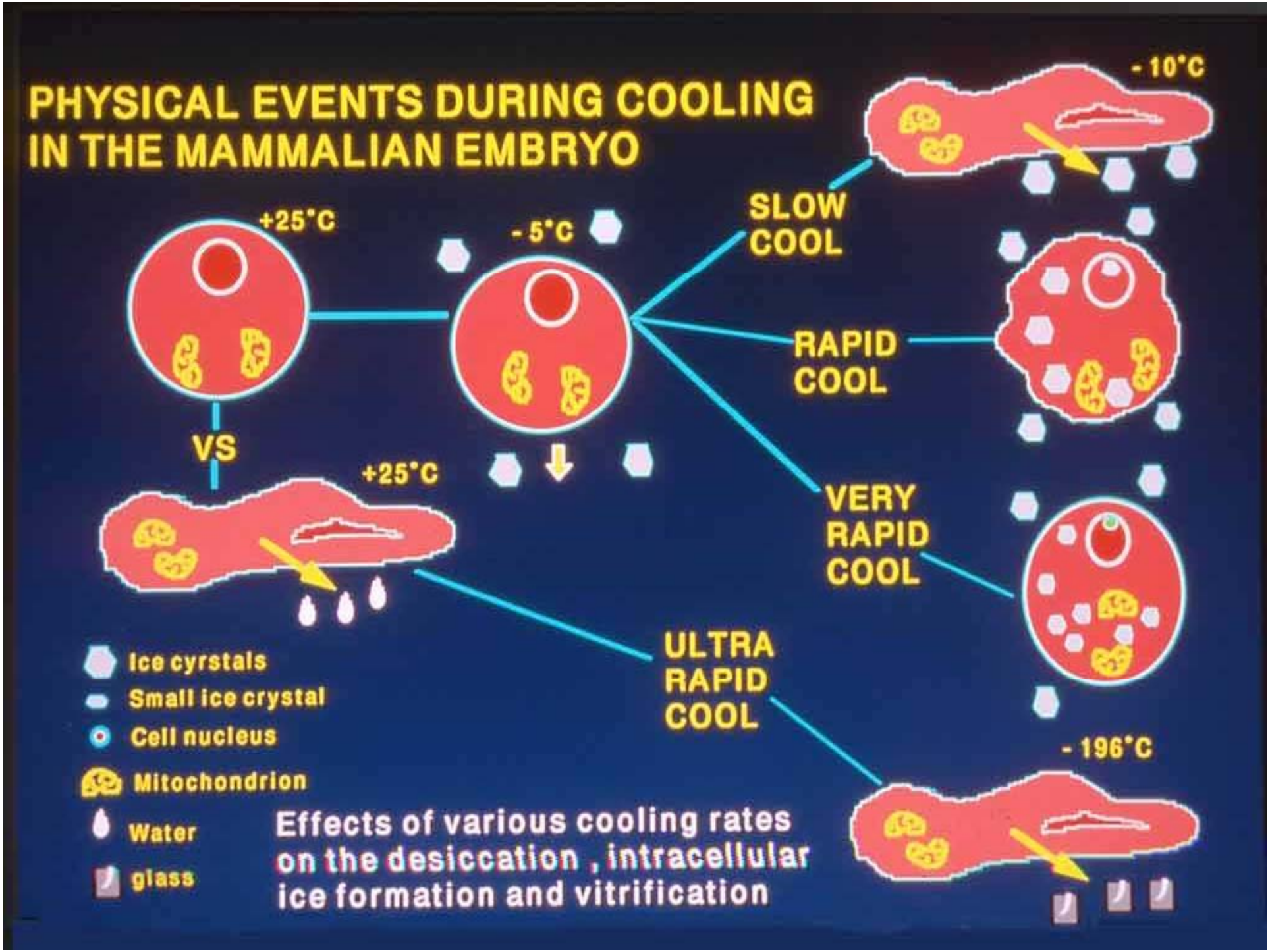


Volume changes after exposure to VS



- **Volume decreases instantaneously due to very high solute concentration**
- **Embryo usually attains a volume 15% of original**
- **Severe dehydration essential for ice-free cryopreservation**

Ali and Shelton, 1993


PHYSICAL EVENTS DURING COOLING
IN THE MAMMALIAN EMBRYO
+25°C
-5°C
-10°C
SLOW
COOL
RAPID
COOL
VS
+25°C
VERY
RAPID
COOL
ULTRA
RAPID
COOL
-196°C
Ice cyrstals
Small ice crystal
Cell nucleus
Mitochondrion
Water
glass
Effects of various cooling rates
on the desiccation , intracellular
ice formation and vitrification

3. Volume of vehicle

- Use of tiny / smaller vehicles a strategy for using cryoprotectant solution that lacks sufficient solute concentration to vitrify in straws during cooling and remain vitrified during warming
  - Promotes faster cooling but disadvantageous - difficult to maintain asepsis
- Larger vehicles need cryoprotectants of higher solute concentration to vitrify and remain vitrified during warming
  - Cooling takes longer -disadvantage - require high solute conc that is often toxic to cells/embryos

Cooling and warming rates

[must be ultra rapid to prevent ice nuceli formation and growth]

- Cooling rate for a 0.25 mL straw when plunged directly into liquid nitrogen from room temperature is about 2500oC/min.

  (Rall, 1987)

- The warming rate for a 0.25mL straw from − 196oC to 25oC is about 1000oC/min.

  (Rall et al., 1986)

- If the volume is reduced to about 1 µL it will be possible to increase the cooling rate to about 15 000–30 000oC/min.

  (Martino et al., 1996; Vajta et al., 1997; Arav & Zeron, 1997)

- Same rules apply for warming rates. Higher warming rates will prevent the formation and growth of ice nuclei during warming. A warming rate in the order of 4460oC/min has been used to successfully warm vitrified embryos

VITRIFICATION

Chemo- and Osmotoxicity

- the main problems because

have to use high concentrations of chemicals to achieve vitrification

Vitrification Vs Toxicity

Cryoprotect	Conc Vitrific	Conc. Toxic
Sucrose	2.0M	2.0M (68.4%)
B. Glycol	3.0M	2.0M (27%)
Methanol	No vit	N.A.
P. Glycol	4.0M	4.0M (30.4%)
DMSO	5.0M	5.0M (39.1%)
Glycerol	5.0M	5.0M (46.1%)
E. Glycol	6.5M	7.0M (40.4%)

- (Ali, 1992; Ali and Shelton, 1993a)

Toxicity Studies - Ethylene glycol

Table 5.1:	Toxicity of molar concentrations of ethylene glycol on day-4 mouse morulae at 25 degreesC				
Molarity of ethylene glycol	Percentage morulae that survived				
		Duration of exposure (mins)			
	control	5 mins	10 mins	20 mins	
5.0 x/n	96.0 (71/74)	98.6 (72/75)	89.7 (70/78)	97.4 (74/76)	6
6.0 x/n	72.1 (62/86)	71.4 (40/56)	63.2 (36/57)	73.1 (57/78)	5
7.0 x/n	92.8 (77/83)	61.8 (42/68)	41.1 (30/73)	2.7 (2/74)	4
8.0 x/n	97.9 (46/47)	41.7 (20/48)	0 (0/49)	0 (0/49)	3

- EG least toxic cryoprotectant
- Toxic only at 7M Conc

Toxicity of cryoprotectants

Table 3.2 The durations of exposure (5, 10, or 20 min) at which molar concentrations of cryoprotectant are toxic to day-4 mouse embryos

Molar concentration	*Cryoprotectant*					
	EG	*Gly*	*DMSO*	*PG*	*Meth*	*BG*
2.0		20	>20	>20		5
3.0		20	>20	10	>20	5
4.0			20	5	>20	
5.0	>20	10	5	5	>20	
6.0	>20	5	5		10	
7.0	5					
8.0	5					

EG, ethylene glycol; Gly, glycerol; DMSO, dimethyl sulfoxide; PG, propylene glycol; Meth, methanol; BG, butylene glycol.

Ali and Shelton, 1993

Designing vitrification solutions

Many types, eg:

Binary: (Water + 1 cryoprotectant)
Ternary: (water + 2 cryoprotectants) – most common
Quatenary: (Water + 3 cryoprotectants) – areas of res
Pentanary: (Water+ 4 cryoprotectants) – areas of res

and so forth

More cryoprotectants in VS : A strategy for reducing toxicity of the VS

eg: 1.0M DMSO +1.5M PROH + 1.5M Gly + 2.0MEG + 0.5 SUC

3,044 Ternary cryo solutions were investigated

- Alanine
- Butylene glycol
- Dextran
- Dimethyl Sulphoxide
- Ethylene glycol
- Ficoll
- Glycerol
- Methanol
- Propylene glycol
- PVP
- Polyethylene glycol
- Sucrose

Designing ternary vitrification solution

Table 4.1: Appearance of straws containing different molarities of ethylene glycol and glycerol after direct plunging into liquid nitrogen or cooling on a styrofoam boat followed by plunging into liquid nitrogen, and warming at +25C.

ETHYLENE GLYCOL (in M Concentrations)

GLYCEROL (in M Concentrations)

		0.0		0.5		1.0		1.5		2.0		2.5		3.0		3.5		4.0		4.5		5.0		5.5		6.0	
	Mode	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
0.0	C	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	I	M	I	M	I	M
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
0.5	C	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	MI	M	MI	M	I	M	T	M	T	I
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
1.0	C	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	I	M	TI	M	T	M	T	TI	T	TI
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
1.5	C	M	M	M	M	M	M	M	M	M	M	M	M	I	M	I	M	TI	M	T	M	T	I	T	T	T	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	MI	M
2.0	C	M	M	M	M	M	M	M	M	MI	M	I	M	TI	M	TI	M	T	M	T	I	TK	T	T	T	TK	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	TI	I	TI	T
2.5	C	M	M	M	M	M	M	MI	M	I	M	I	M	I	M	T	M	T	I	T	T	T	T	T	T	T	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	I	M	T	T	TK	TK
3.0	C	M	M	MI	M	MI	M	MI	M	I	M	T	M	T	I	T	T	T	T	T	T	T	T	T	T	T	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	TI	TI	T	T	T	TK	TK	TK
3.5	C	M	M	MI	M	MI	M	MI	M	T	M	T	M	T	MI	T	T	T	T	T	T	T	T	T	T	T	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	MI	M	TI	TI	T	TK	TK	TK	TK	TK	T	TK
4.0	C	MI	M	MI	M	I	M	T	M	T	M	T	I	T	T	TK	T	T	TK	T	TK	T	T	TK	T	TK	TK
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	TI	TI	TK	T	TK	T	TK	TK	TK	T	TK	T
4.5	C	I	M	T	M	T	M	T	MI	T	MT	T	T	T	T	T	T	T	T	T	T	TK	T	T	T	T	T
	W	M	M	M	M	M	M	M	M	M	M	MI	M	T	T	T	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK
5.0	C	T	M	T	M	T	I	T	TI	T	T	T	T	T	T	T	T	TK	T	TK	T	T	T	TK	T	TK	T
	W	M	M	M	M	M	M	M	M	I	TI	TI	TI	TI	T	TI	TI	TI	TI	TI	TI	TI	TI	TI	TI	TI	T
5.5	C	T	M	T	MI	TK	TI	T	T	T	T	T	T	T	T	T	T	TK	T	T	T	TK	T	TK	T	TK	T
	W	M	M	M	M	M	M	TI	TI	TI	TI	T	T	TI	TI	TI	TI	T	TI	TI	T	TI	TI	TI	TI	TI	TI
6.0	C	T	TI	TK	T	TK	T	T	T	TK	T	T	T	T	T	TK	TI	TK	T	T	T	TK	T	T	T	T	T
	W	MI	M	TI	MI	T	T	TI	T	T	TI	TI	TI	TI	T	TI	TI	TI	TI	TI	TI	T	TI	T	TI	TI	TI

KEY

C = Cooling to -196C

W= Warming to +25C

D = Direct plunging into liquid nitrogen

S = Boat cooling followed by plunging into liquid nitrongen

M= Milky /crystallization

I = Intermediate

T = Transparent /vitrification

K = Fractured

Designing ternary vitrification solutions

Table 4.10: Appearance of straws containing different molarities of dimethyl sulphoxide and 1,2-propanediol after direct plunging into liquid nitrogen or cooling on a styrofoam boat followed by plunging into liquid nitrogen, and warming at +25C.

DIMETHYL SULPHOXIDE (in M Concentrations)

1,2-PROPANEDIOL (in M Concentrations)

		0.0		0.5		1.0		1.5		2.0		2.5		3.0		3.5		4.0		4.5		5.0		5.5		6.0	
	Mode	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
0.0	C	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	I	M	T	M	T	M	T	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
0.5	C	M	M	M	M	M	M	M	M	M	M	M	M	M	M	MI	M	MI	M	TK	M	TK	TK	TK	T	TK	TK
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	TI	I	?	TK
1.0	C	M	M	M	M	M	M	M	M	M	M	M	M	MI	M	MI	M	TI	M	T	TI	TK	TK	TK	TK	TK	TK
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	T	TK	T	TK	TK	TK
1.5	C	M	M	M	M	M	M	M	M	MI	M	MI	M	MI	M	T	M	T	TI	T	T	TK	T	T	T	TK	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	TI	TI	T	TI	T	TI	T	T
2.0	C	M	M	M	M	M	M	M	MI	MI	M	I	M	T	TI	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T
	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	TK	TK	?	TK	T	TK	TK	TK	TK	TK
2.5	C	M	M	M	M	MI	M	I	M	T	M	T	MI	TK	TI	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK
	W	M	M	M	M	M	M	M	M	M	M	M	M	TK	M	TK	I	TK	TK	T	T	T	T	T	TK	T	TK
3.0	C	MI	M	I	M	I	M	T	M	T	MI	TK	T	T	T	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	T
	W	M	M	M	M	M	M	M	M	M	M	M	I	I	I	TK	TK	TK	TK	T	?	?	?	T	TK	TK	TK
3.5	C	I	M	I	M	T	M	TK	MI	T	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	TK	TK	T
	W	M	M	M	M	M	M	M	M	MI	MI	I	I	T	TK	T	TK	?	TK	?	TK	?	TK	TK	TK	TK	TK
4.0	C	T	M	T	MI	T	TI	T	T	T	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	TK
	W	M	M	M	M	M	M	M	M	TK	I	T	TK	?	TK	?	TK	?	TK	?	TK	T	TK	?	TK	?	TK
4.5	C	T	MI	T	TI	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T	TK	T
	W	M	M	M	M	I	I	TK	TK	TK	TK	?	TK	?	TK	TK	TK	TK	TK	?	TK	?	TK	?	TK	?	TK
5.0	C	T	T	TK	TK	T	TK	TK	TK	TK	TK	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK
	W	MI	MI	I	I	TK	TK	TK	TK	T	?	T	TK	?	TK	T	?	?	?	?	?	?	?	?	?	?	?
5.5	C	T	T	TK	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK
	W	I	I	T	TK	TK	?	T	?	?	?	?	?	?	TK	?	?	?	TK	?	?	?	?	?	?	?	?
6.0	C	TK	T	TK	TK	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	T	TK	TK	TK	TK	TK	TK	TK	TK	TK	TK
	W	?	TK	?	TK	?	TK	T	?	?	?	?	?	?	?	T	TK	T	?	?	?	?	?	?	?	?	?

KEY

C = Cooling to -196C

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The Ideal Vitrification Solution?

- Out of 3,044 solutions investigated for vitrification & toxicity properties only **one** appeared promising, that is:

  5.5M ethylene glycol + 2.5M glycerol (VS1)

- BUT VS1 toxic to some developmental stages of mouse/sheep embryos
- So VS1 was further modified – another 14 modifications
- Total of 14 modifications made from VS1 i.e: VS2 to VS15
- Of these only VS14 appeared MOST PROMISING
- 5.5 ethylene glycol + 1.0M sucrose (VS14)
  (Ali, 1992; Ali and Shelton, 1993a,b,c)

VS1 and VS11 Toxic to some developmental stages of mouse and sheep embryos

Mouse embryos

VS	Treatment	Stage of embryo development: SO blastocysts, early blastocysts and morulae	SO 8 cell	SO 4 and 2 cell	SO 1 cell	F_1 1 cell
VS1	Control	89.6	87.2	85.3	40.0	87.1
	Exposed	91.9	36.8	8.0	7.3	78.1
	Vitrified	92.6	21.6	0	0	66.7
VS11	Control	100	94.6	89.7	40.0	90.9
	Exposed	98.8	75.4	41.8	37.2	86.4
	Vitrified	94.4	91.2	19.0	16.3	0

Table 1. Percentage survival *in vitro* of day-6 sheep embryos exposed* to or vitrified with VS1 and VS11

Vitrification solution	Treatment	Stage of development: Blastocyst	Early blastocyst	Morula
VS1	Control	100 (6)**	96.4 (28)	59.0 (39)
	Exposed	100 (4)	64.3 (14)[a]	59.5 (42)
	Vitrified	11.1 (9)[b]	24.2 (33)[c]	26.8 (41)[b]
VS11	Control	88.9 (27)	87.5 (24)	71.7 (46)
	Exposed	75.0 (20)	80.0 (5)	68.0 (20)
	Vitrified	11.5 (26)[c]	81.8 (22)	85.7 (42)

*Durations of exposure were 1 min for blastocysts, 2 min for early blastocysts and 3 min for morulae. **Numbers in parentheses are numbers of embryos in the group. [a]$P < 0.05$ compared with controls; [b]$P < 0.005$ compared with controls; [c]$P < 0.0001$ compared with controls.

Further improvement needed to enhance survival post vitrification

Final modifications to VS1

3,044 cryoprotectants solutions

↓

VS1: 5.5M Ethylene Glycol + 2.5M Glycerol

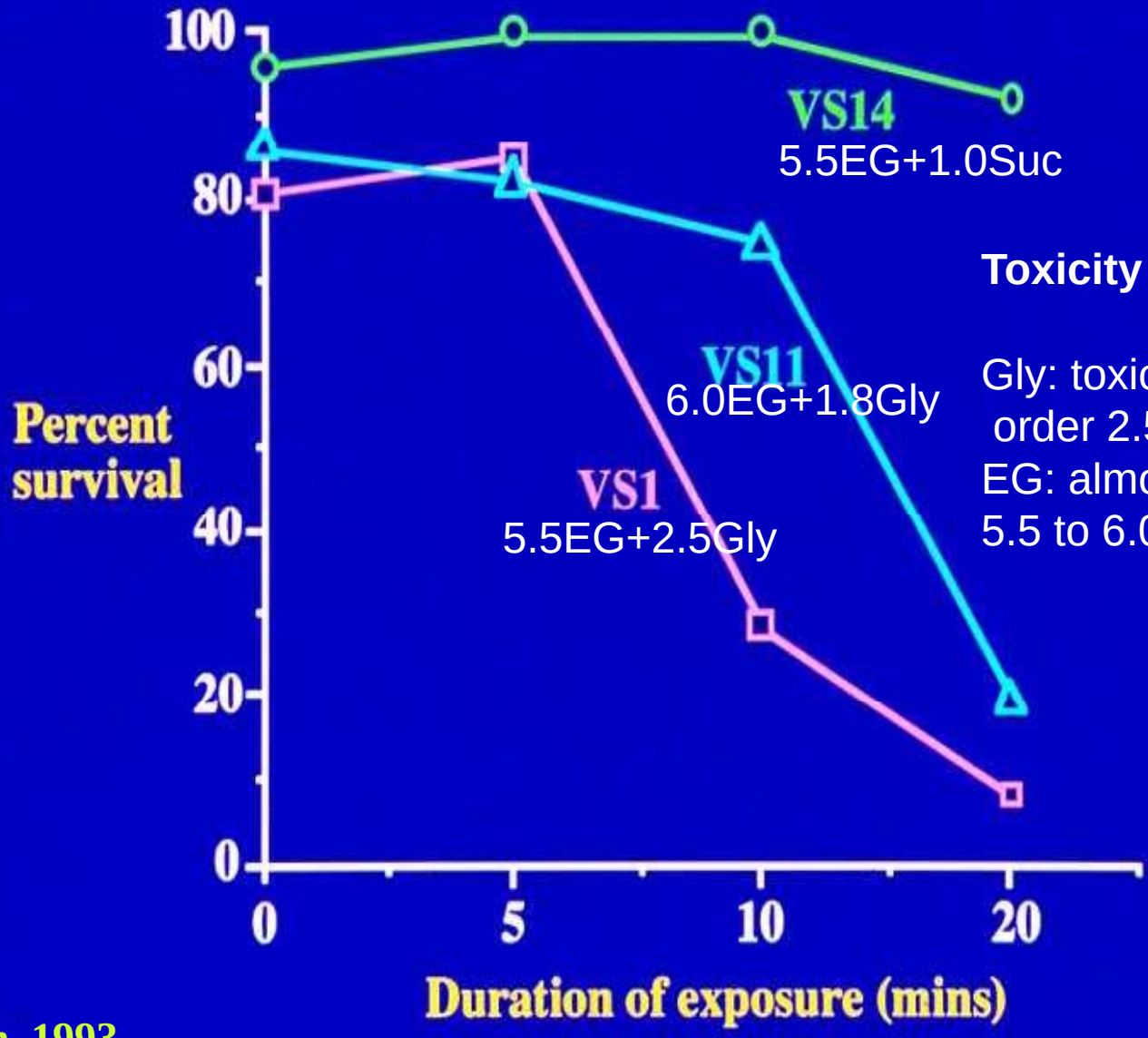
↓

VS11: 6.0M Ethylene Glycol +1.8M Glycerol

↓

VS14: 5.5M Ethylene Glycol + 1M Sucrose

Fig. 1: Survival of day-4 mouse morulae after exposure to vitrification solutions at 25C.



Toxicity related to:

Gly: toxicity in reducing order 2.5:1.8:0

EG: almost constant 5.5 to 6.0M

Ali and Shelton, 1993

Survival of vitrfied mouse embryos.
Ali and Shelton, 1993

In Vitro Survival	VS1	VS11	VS14
Developmental stage	5.5E+2.5G	6E+1.8G	5.5E+1S
SO Mice			
D-4; Blastocyst	86% (NS)	93% (NS)	97% (NS)
D-4; Early Blast	95% (NS)	96% (NS)	97% (NS)
D-4; Comp morulae	88% (NS)	93% (NS)	98% (NS)
D-3; 8-cell	22% (S)	91% (NS)	87% (NS)
D-3; 4-cell	0 (S)	12% (S)	70% (NS)
D-2; 2-cell	0 (S)	23% (S)	71% (NS)
D-1; 1-cell	0 (S)	16% (S)	19% (S)
D-1; 1-cell;F1	67% (NS)	0% (S)	92%(NS)

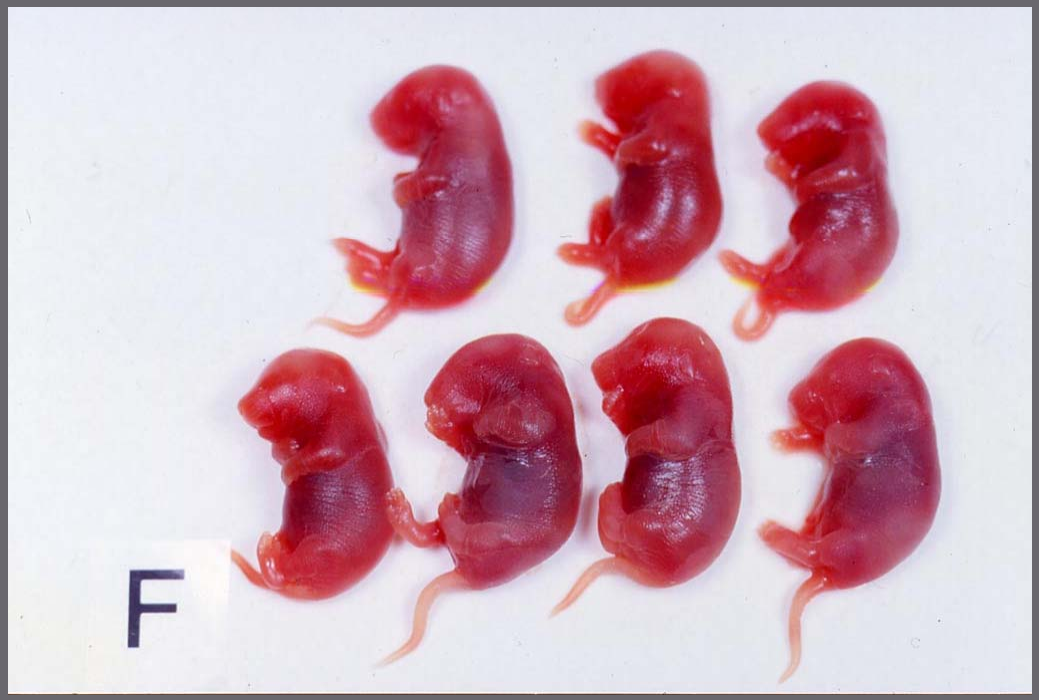
In Vivo Survival (SO)		Control	VS1	VS11	VS14
D-4	Embryos	19.1%	18.4%(NS)	24.3%(NS)	26.1%(NS)
	n=(x)	(125)	(33)	(116)	(31)

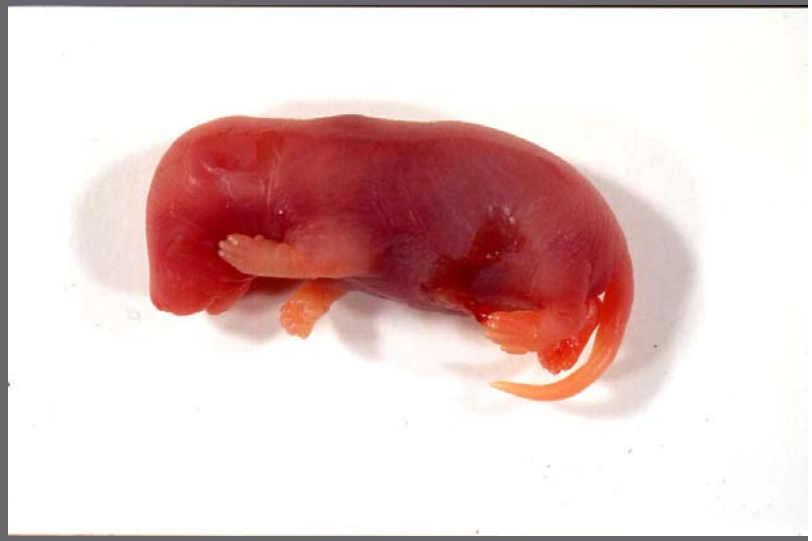
Survival of day 6 sheep embryos in VS11 and VS14

Table 3.8 Viability of day-6 sheep embryos vitrified with VS11 or VS14 and transferred to surrogates

Vitrification procedure	*Dilution procedure*	*Stage of development*	*Percentage developed to live fetuses*	*Percentage of surrogates pregnant*
One-step VS11	With sucrose	Morulae	7.9	15.8
		Early blastocysts	2.4	4.4
		Blastocysts	0	0
	Without sucrose	Morulae	13.3	26.7
		Early blastocysts	0	0
		Blastocysts	0	0
Total one-step VS11			(8/158) 5.1	(8/79) 10.1
Two-step VS11	With sucrose	Morulae	55.2	78.6
		Early blastocysts	10.0	20.0
		Blastocysts	62.1	78.6
		Expanded blastocysts	50.0	50.0
Total two-step VS11			(37/72) 51.4	(25/35) 71.4
One-step VS14	With sucrose	Morulae	50.0	100.0
		Blastocysts	100.0	100.0
		Expanded blastocysts	0	0
Total one-step VS14			(5/10) 50.0	(3/5) 60.0

Teratogenicity not noted in mouse fetuses derived from vitrified embryos





Ali and Shelton, 1993

Vitrified mouse embryos are viable and can result in normal young ; Ali and Shelton , 1993

Ali and Shelton, 1993

Mice derived from vitrified embryos reproduce and give birth to normal young

Ali and Shelton, 1993

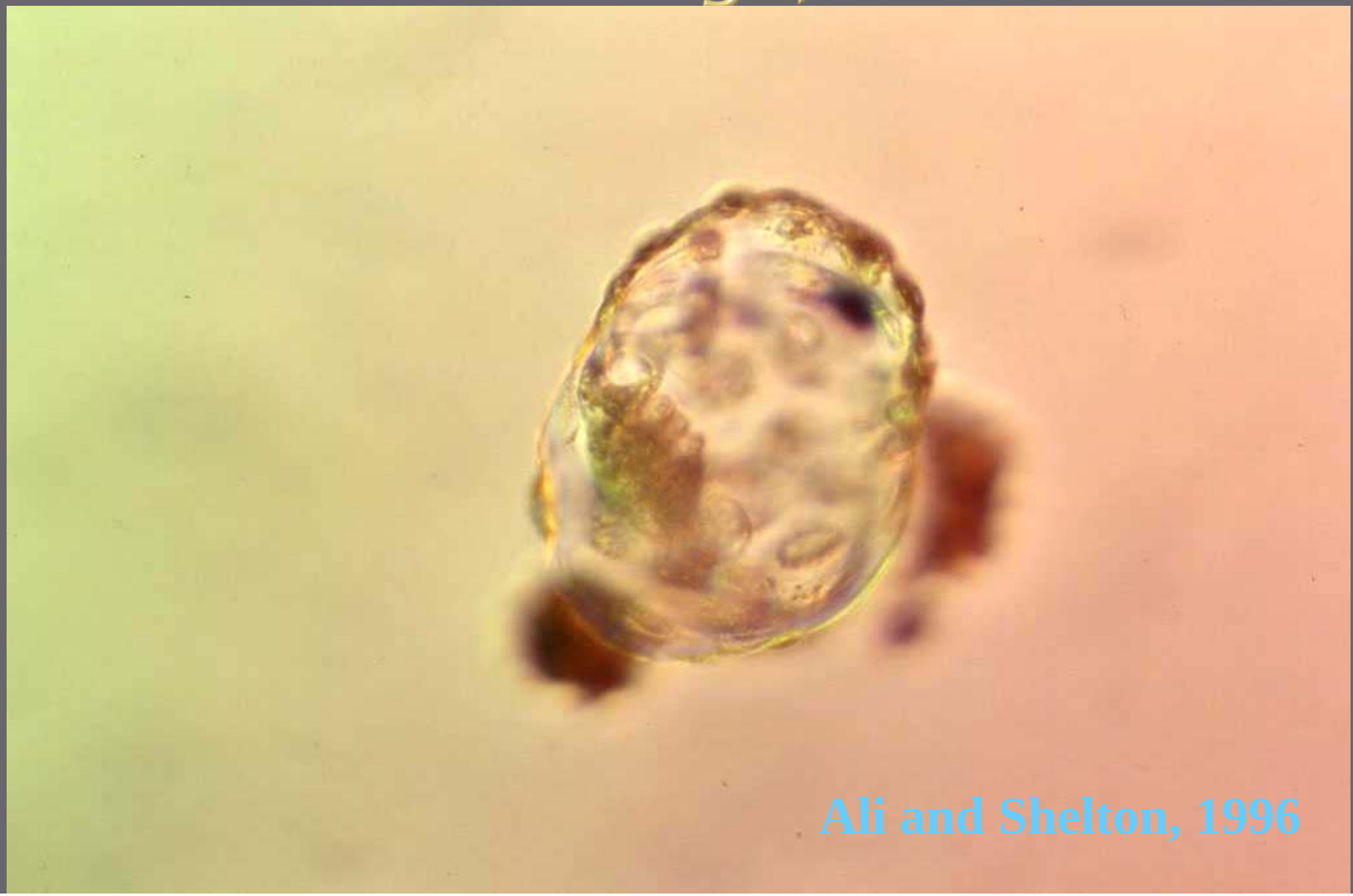
Ali and Shelton, 1993

Developmental competence of VS14 vitrified human day 2 1PN and 3PN embryos

Developmental Stage	Fresh	Vitrified
Survival (Day-2)	-	100 %
	(n=39)	(n=24)
1st cleavage (D-3)	89.7 %	83.3 %
	(n=35)	(n=20)
Morula/Comp Mor (D-4)	60.0 %	75.0 %
	(n=23)	(n=18)
Early cavitation	41.0 %	37.5 %
	(n=16)	(n=9)
Expanded blastocyst	frozen	16.7 %
		(n=4)
Hatched blastocyst		4.2 %
		(n=1)

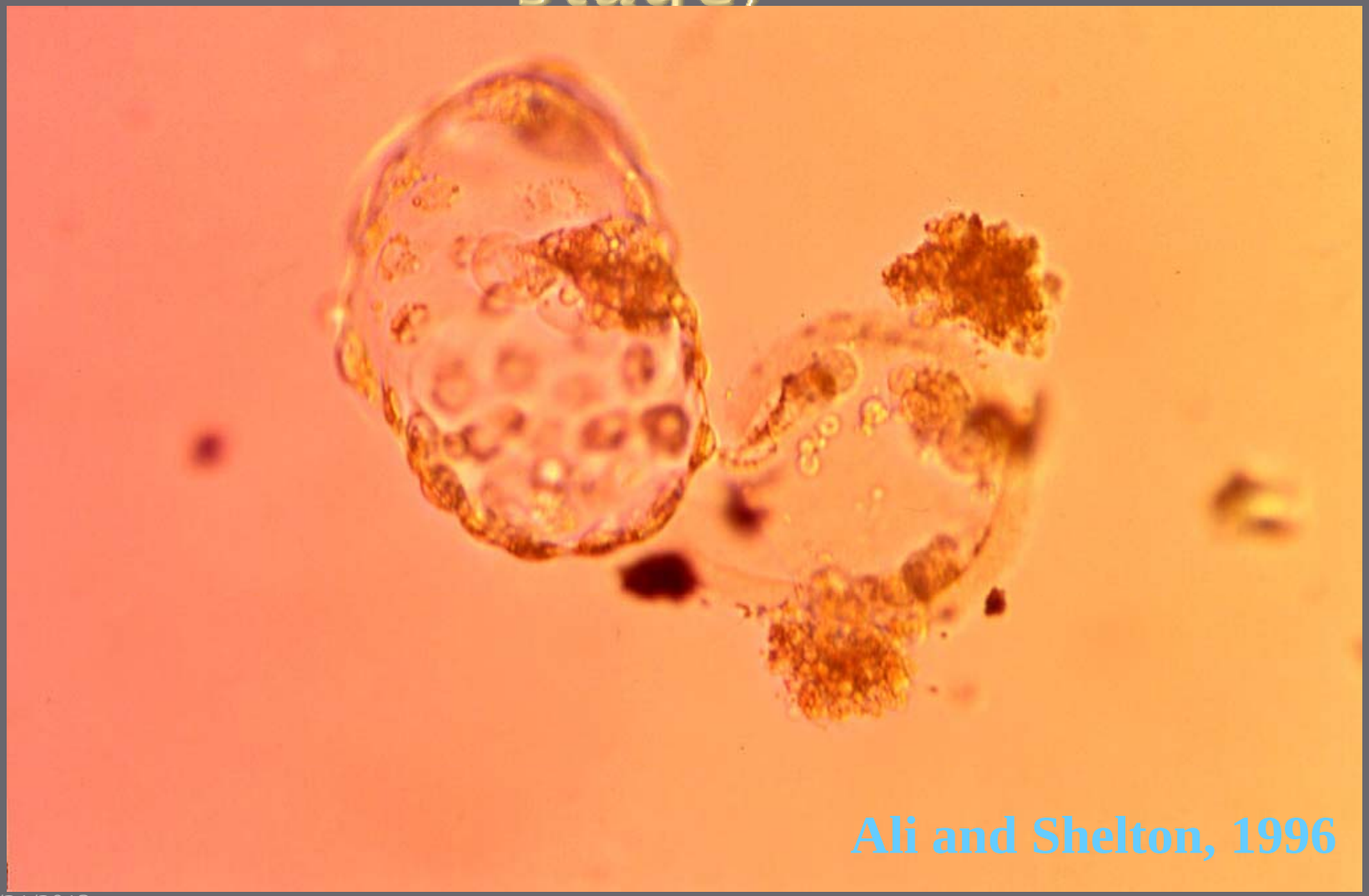
(Ali, 1996)

Hatching human blastocyst (Vitrified at day 2 cleaved stage)



Ali and Shelton, 1996

Hatched human blastocyst (Vitrified at day 2 cleaved stage)



Clinical Trial on VS14

Clinical Trial: VS14 NOT Approved by Ethics Committee – 1996. Unable to do any further work on VS14 since 1996

But numerous workers around the world used VS14 successfully to cryopreserve a wide range of human/animal:

Oocytes (mature/immature) with live births
Embryos (zygote, day2/3 & blastocysts –live births
Ovarian tissue, and
cell lines.
ESC lines have been vitrified successfully with VS14

Refs next slide

VS14 successfully utlized by other workers worldwide

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Making vitrification safer

- Use closed aseptic vitrification – incl. the 0.25ml straw
- Eliminate use of hazardous donor serum proteins in vitrification solutions to prevent transmission of disease

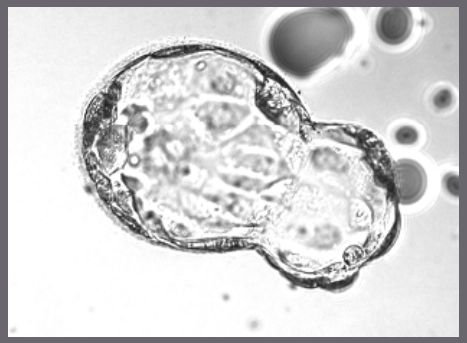

Fig: A hatching blastocyst vitrified using VS14 devoid of added serum proteins – a protein-free VS

- Vitrification methods must comply with GLP/GCP

Vitrification now cryo method of choice in IVF

For good reasons:-

- Rapid
- Economical
- Less demanding on manpower resources
- Above all – could be as efficacious as or better than conventional methods

Comparison between closed and open vitrification

Authors	Entity cryo-ed	Outcome / Technique		Recommendations/ Inference
		Open	Closed	
Panagiotidis et al., 2013 RBMOnline	Blastocyst Human	Vitrisafe Open Comparable	Vitrisafe Closed Comparable	Both technic OK
Hashimoto et al. 2013 Assist Reprod Genet.	Blastocyst from cryopreserved zygotes Human	Open vitrif sys Comparable	Closed Vitrif Sys Comparable	Both technic OK
AbdelHafez et al., 2011 BMC Biotechnol.	Cleavage stage Blastocyst Human	Cryotop Comparable	Cryotip HVS Comparable	Open Crytop comparable to Closed HSV but not Cryotip lower survival with Cryotip
Valbuena et al., 2012 Fertil Steril.	Blastomeres Mouse & Human	Cryotop poor survival	Cryotip Better survival	Closed better than open technic
Paffoni et al., 2011 RBM Online.	Oocytes Human	Better efficiency	less efficient	Comparison were between two separate expts with fresh controls
Bonetti et al.,2011 Fertil Steril	Oocytes MII Human	Cryotop more efficient Ultrastructure Better preserved	Closed less efficient Ultrstructure Less preserved	Open better than closed
Ramezani et al. 2005 Cryo Letters	2-cell embryos Mouse	OPS Less efficient	Closed pulled straw better Closed convent. straw Less efficient	Closed pulled straw better than open pulled straw or closed conventional straw
Chen et al., 2001 Hum Reprod	Oocytes Mouse	OPS Less efficient	Closed pulled straw better Closed convent. straw Less efficient Girds Less efficient	

Overall

- In general closed system appears more efficient than open system for embryos and blastomeres
- For human eggs the open system appears more efficient
- For human eggs more work is needed to developed efficient closed system of vitrification
- It may be possible to use conventional straws if an efficacious vitrification solution can be designed for human oocytes
- After more than a decade of exposure of transplantable tissues to infectious agents and hazardous chemicals it is heartening to see people re-considering aseptic techniques!!

CONCLUSION

- **Excellent outcome with vitrification; Vitrification poised to replace conventional freezing techniques within this decade**

- **Closed conventional straw VS14 method works; no need to subject tranplantable embryos and oocytes to potentially hazardous agents in LN2**

- **Need more research to vitrify oocytes in closed systems**

- **Need for more research on quartenary & pentanary VS**
- **Need for research to eliminate use of hazardous donor proteins in VS**

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