# Cryopreservation of Testicular Stem Cells

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# Fundamental Aspects

In this presentation:

Explain the fundamental aspects of (cryo)preservation methods

Review methods for freezing somatic cells, and (spermatogenic) stem cells

Show how the fundamental knowledge can help us to understand the methods and to improve them

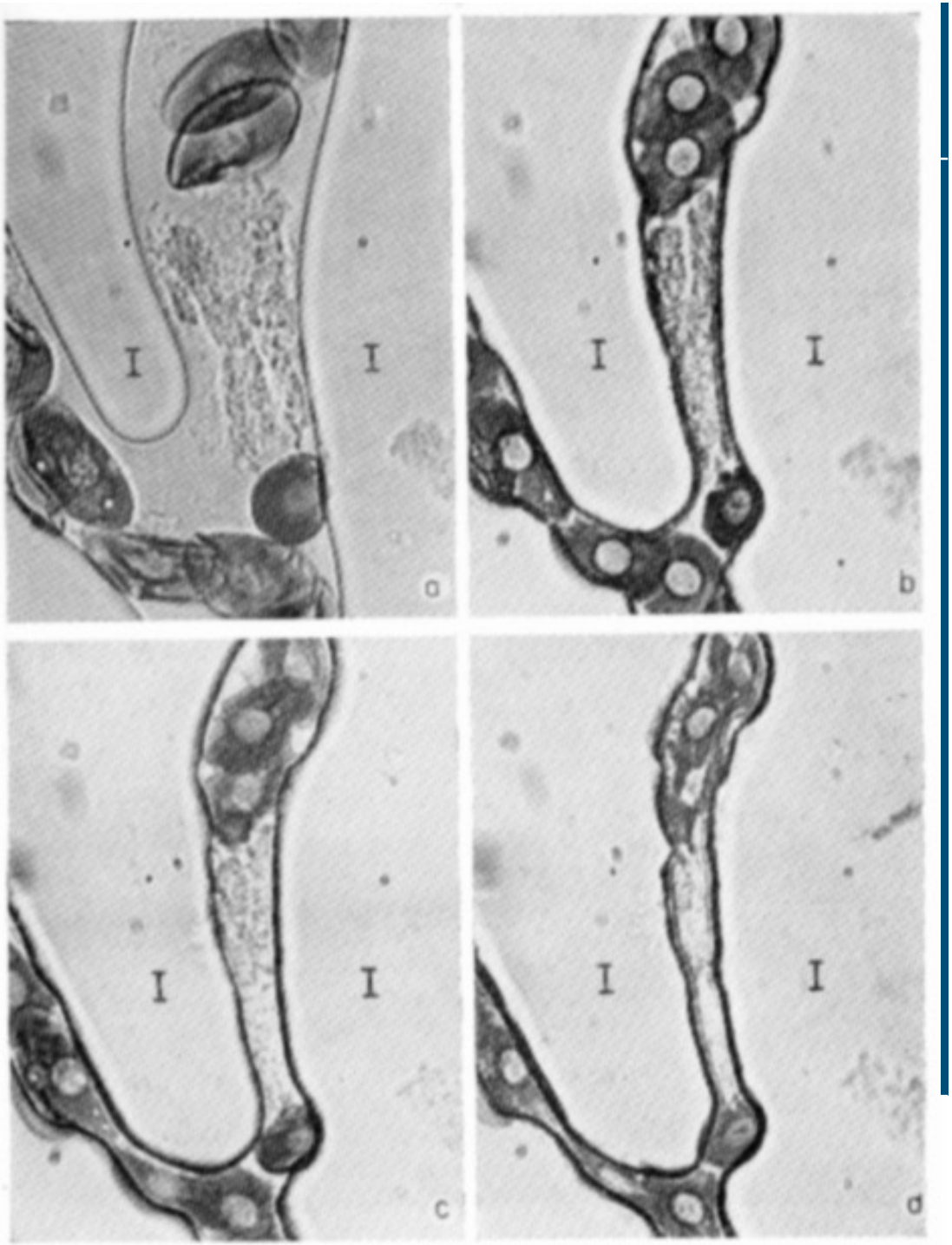
# Slow Freezing

Erythrocytes at

-1,5 -5;

- 7 -10 ºC.

Rapatz & Luyet, 1960

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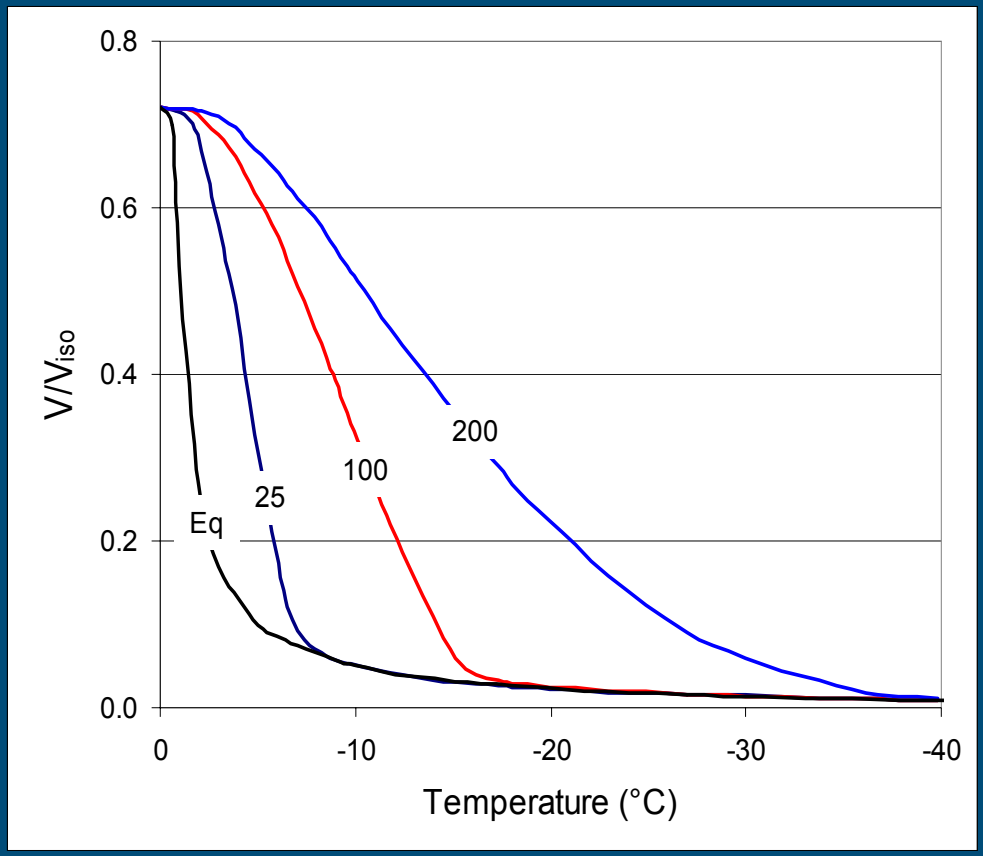
# Slow Freezing




Mazur, 1963; Mazur and Koshimoto, 2002; see also Liu et al., 1997

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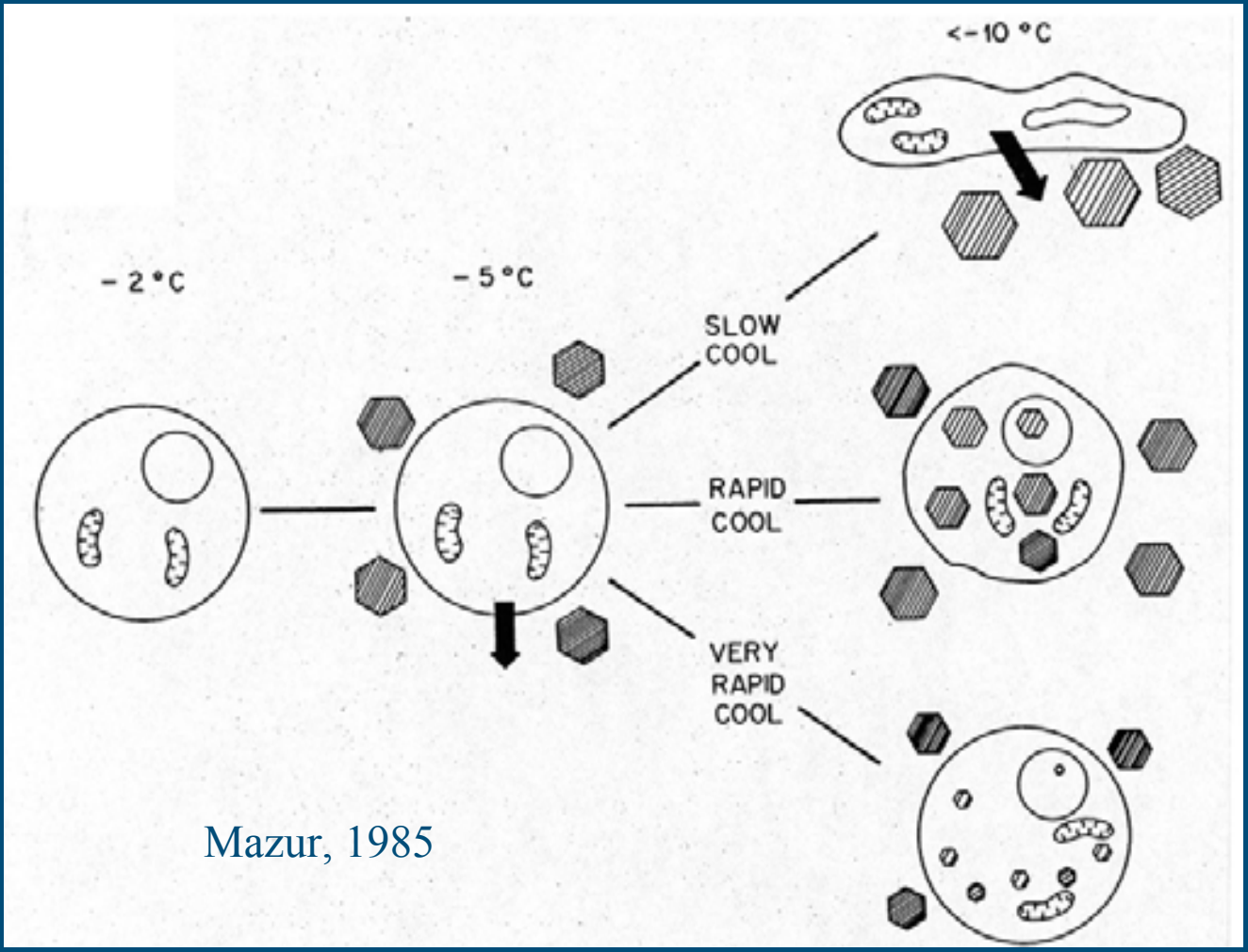
# Slow Freezing




Mazur, 1985

# Slow Freezing

Water freezes (extracellularly) as pure ice

An unfrozen fraction remains that contains all solutes

- The volume of unfrozen fraction ↓
- Water content ↓
- Solute (salt) concentration ↑
- Osmotic pressure ↑
- Viscosity ↑

While IIF is prevented!

At some point of temperature and concentration → Glass transition

# Glass transition

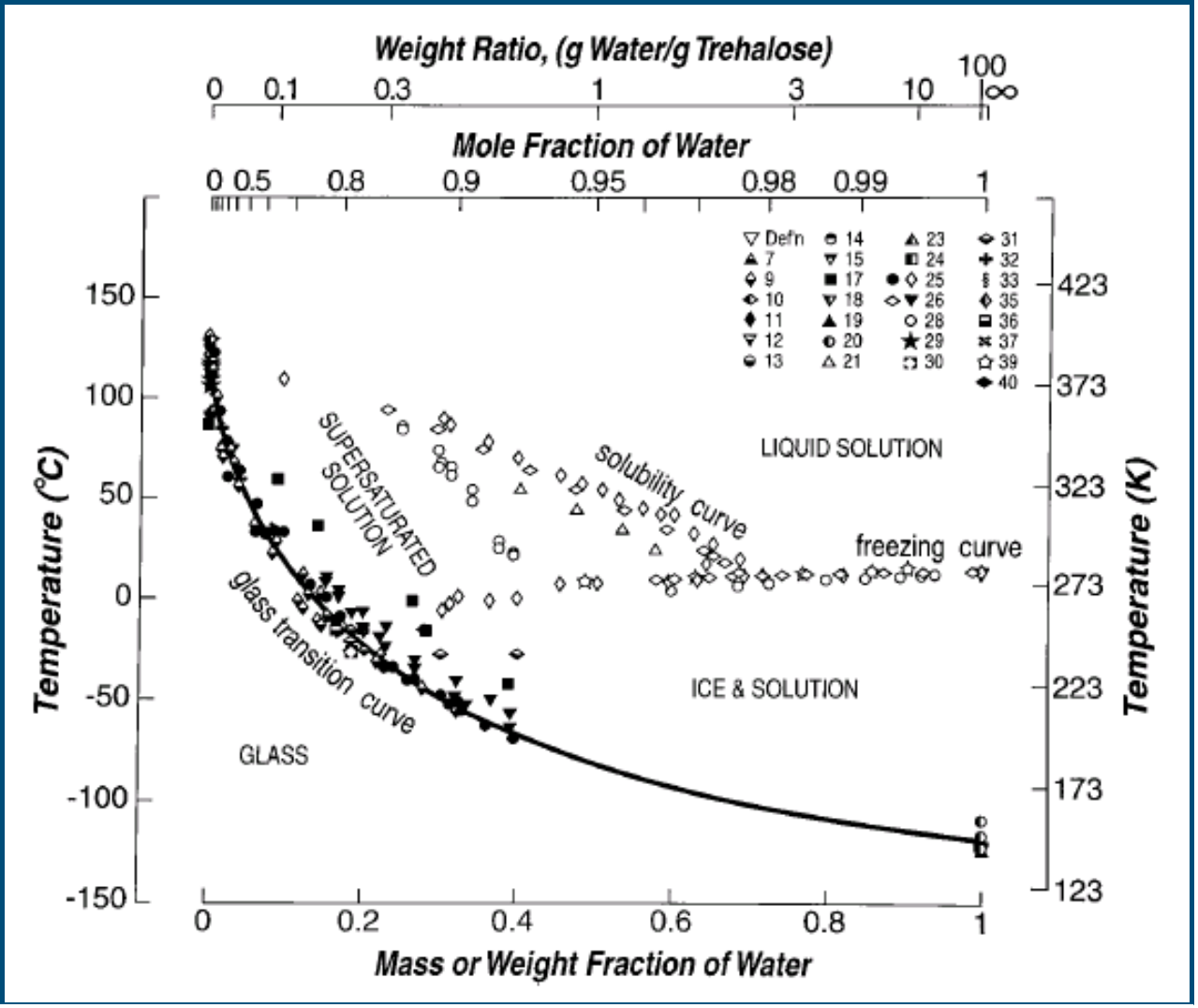
For any type of long-time storage you must go to glass transition!

For cold storage you must go to glass transition while preventing IIF

# Fundamental Aspects

Phase diagram trehalose-water

Chen et al. 2000

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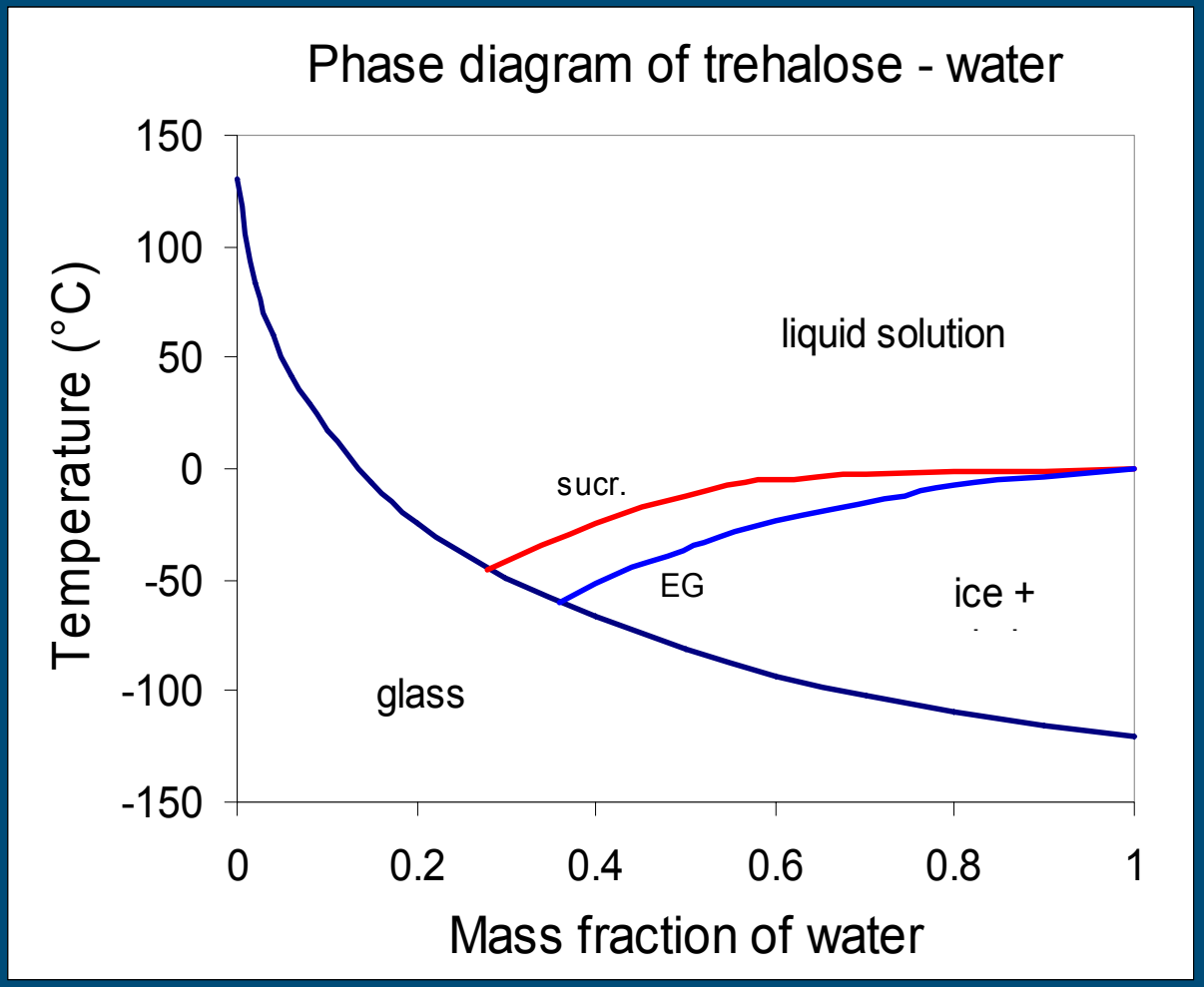
# Fundamental Aspects

Phase diagram

trehalose-water

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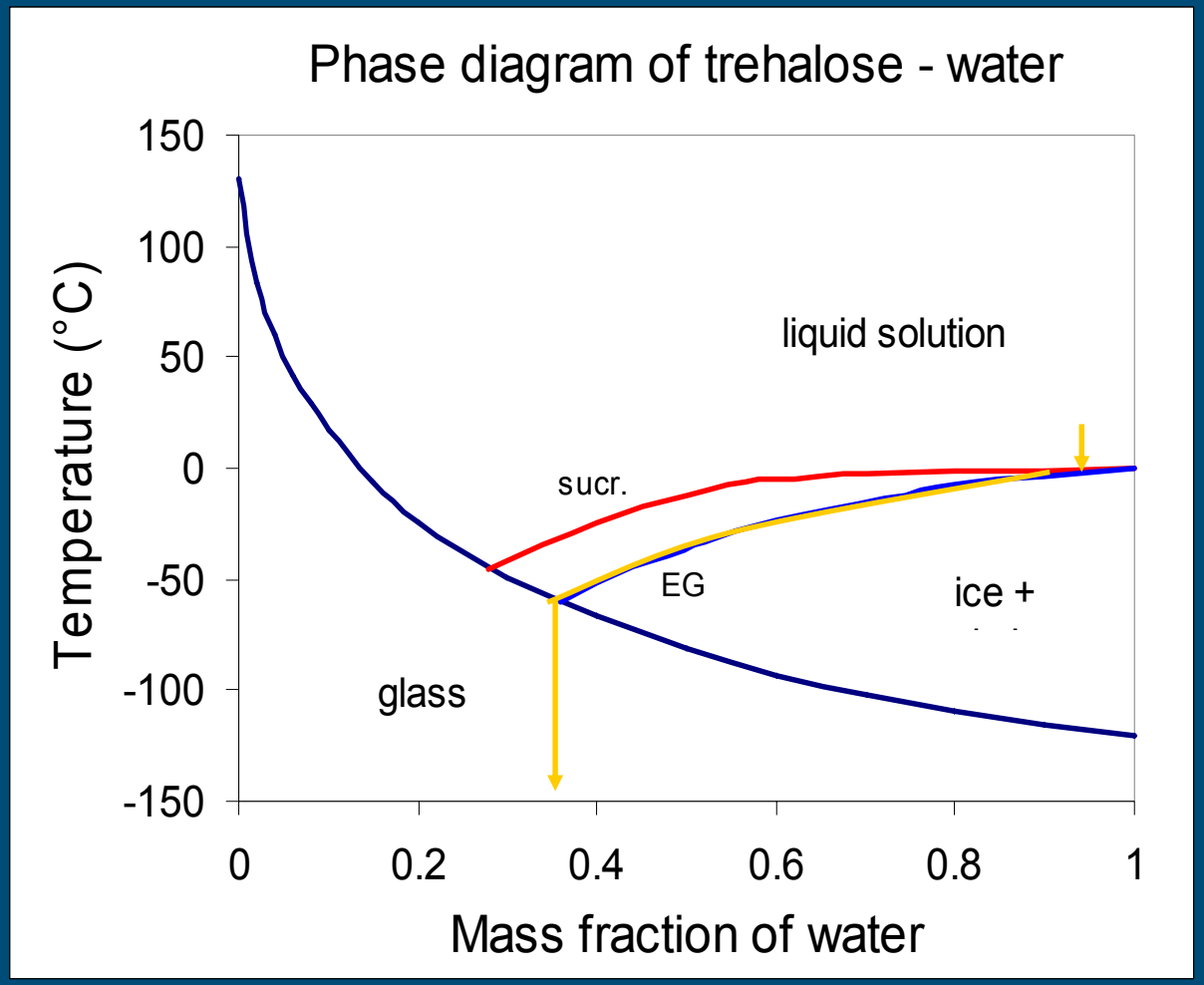
# Slow-freezing

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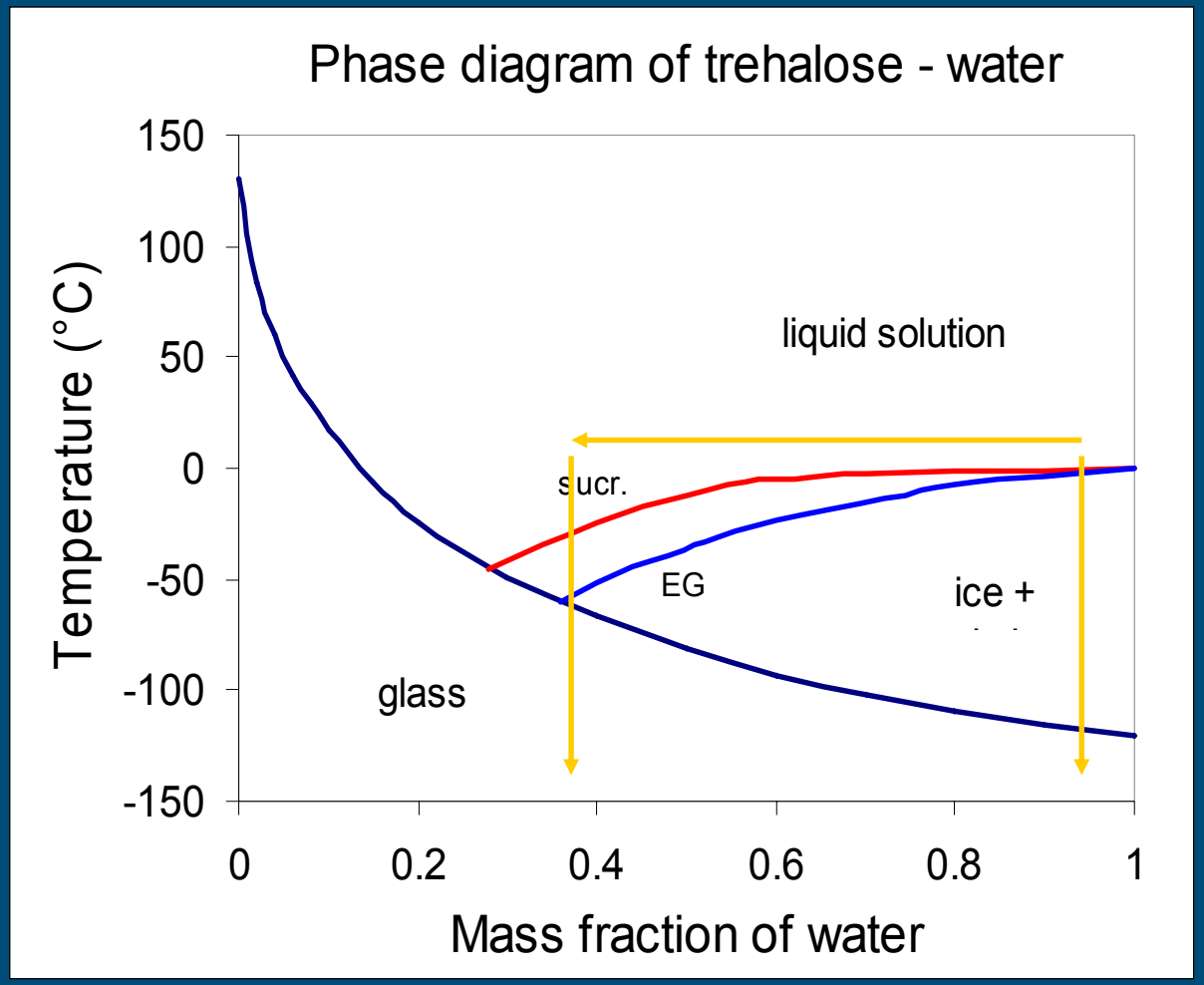
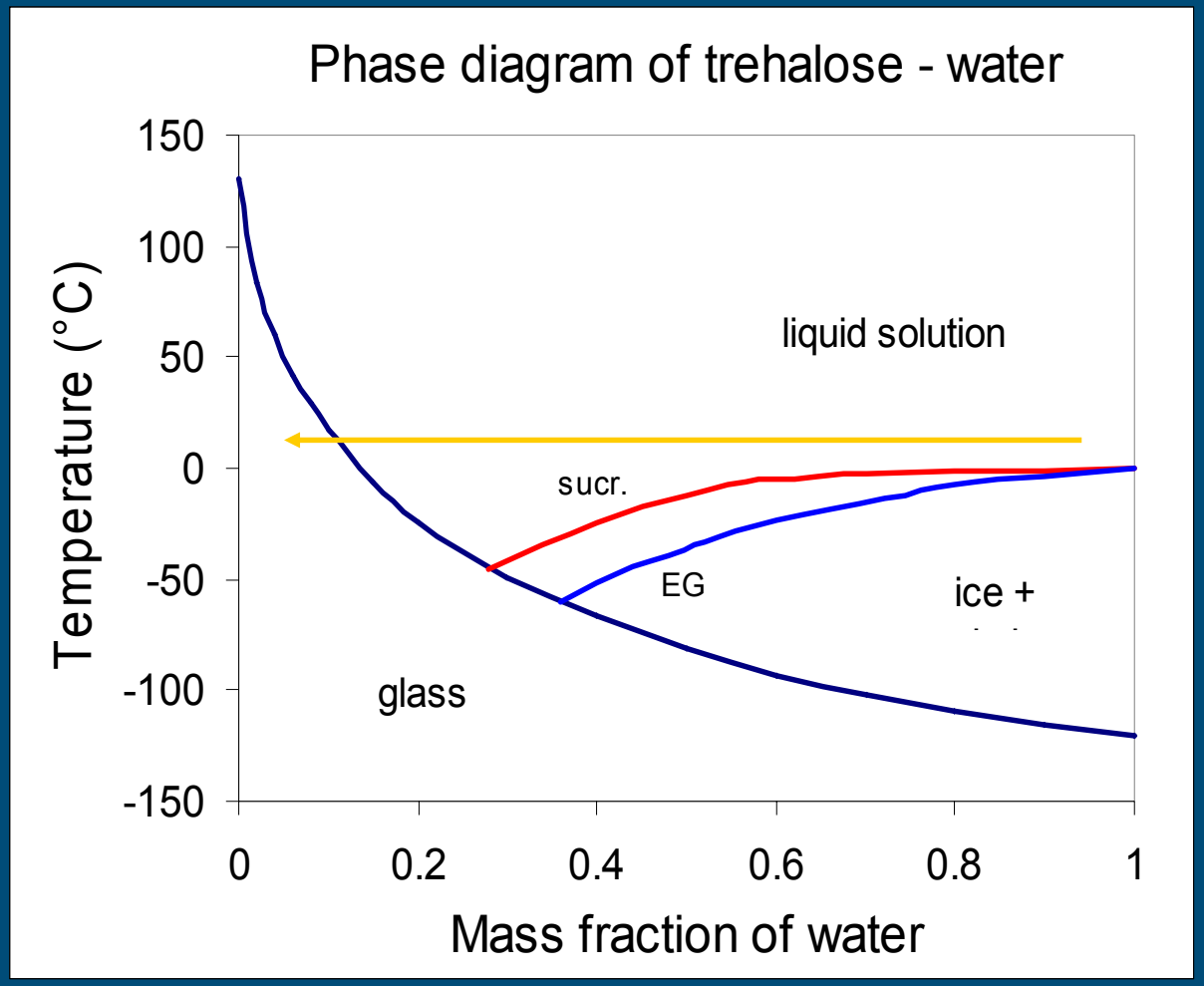
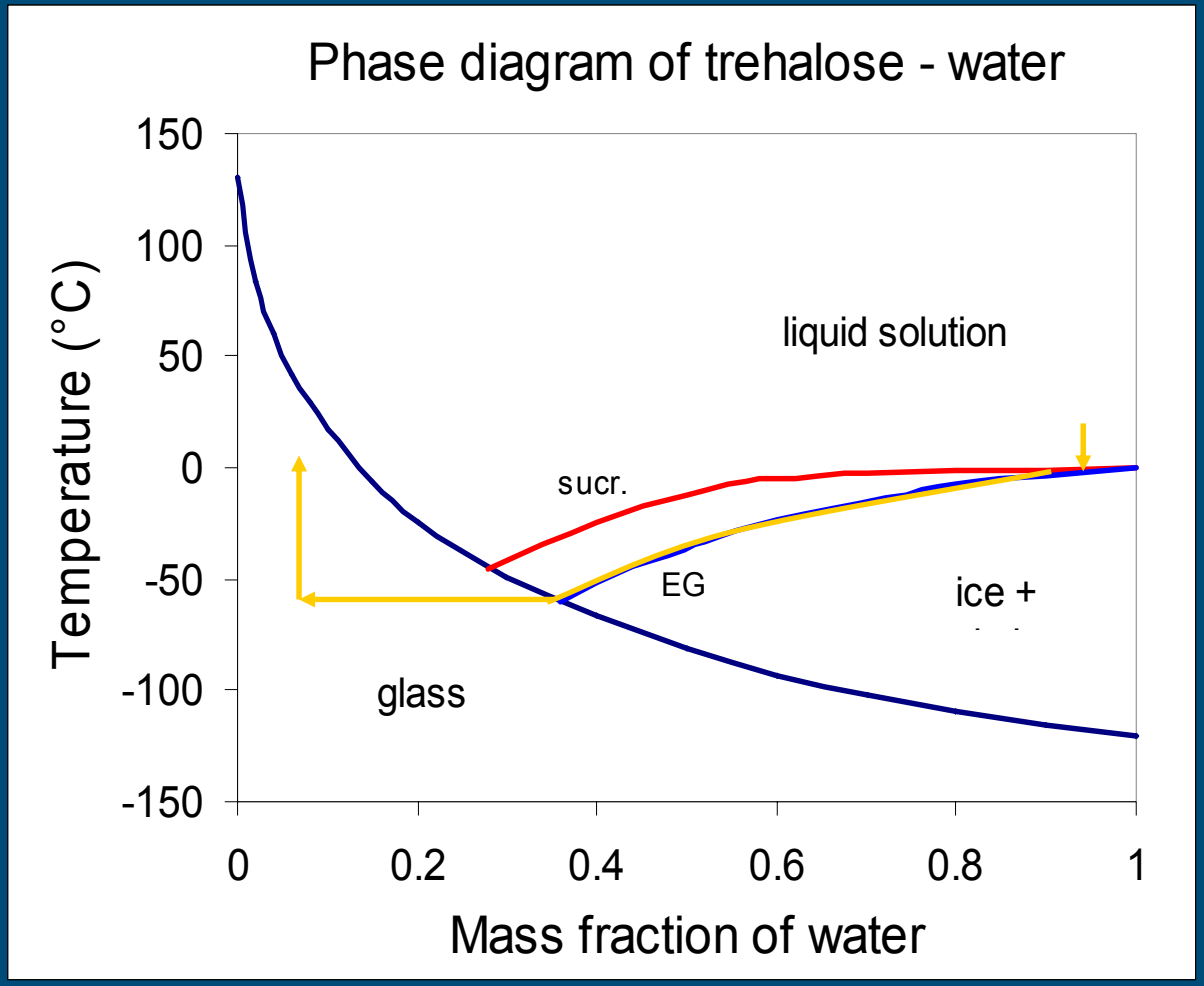
# Vitrification



# Drying (and/or increase solutes)



# Freeze Drying

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# Fundamental Aspects

The common denominator of slow-freezing, vitrification, freeze drying and drying is

the phase diagram shown earlier

and the fact that any storage requires glass transition

A glass is stable because (by definition) molecules have lost the ability of translation movement.

Still, a glass at very low temperatures is much more stable than a glass at ambient temperatures.

# Cryoprotective Agents (CPAs)

To reach glass transition, water content must be low

Or….Solute concentrations must be very high.

Negative effects of very high salt:

- Salt loading
- Destabilisation of proteins
- Cells shrink excessively

By replacing part of the solutes by **permeant** solutes this can be alleviated

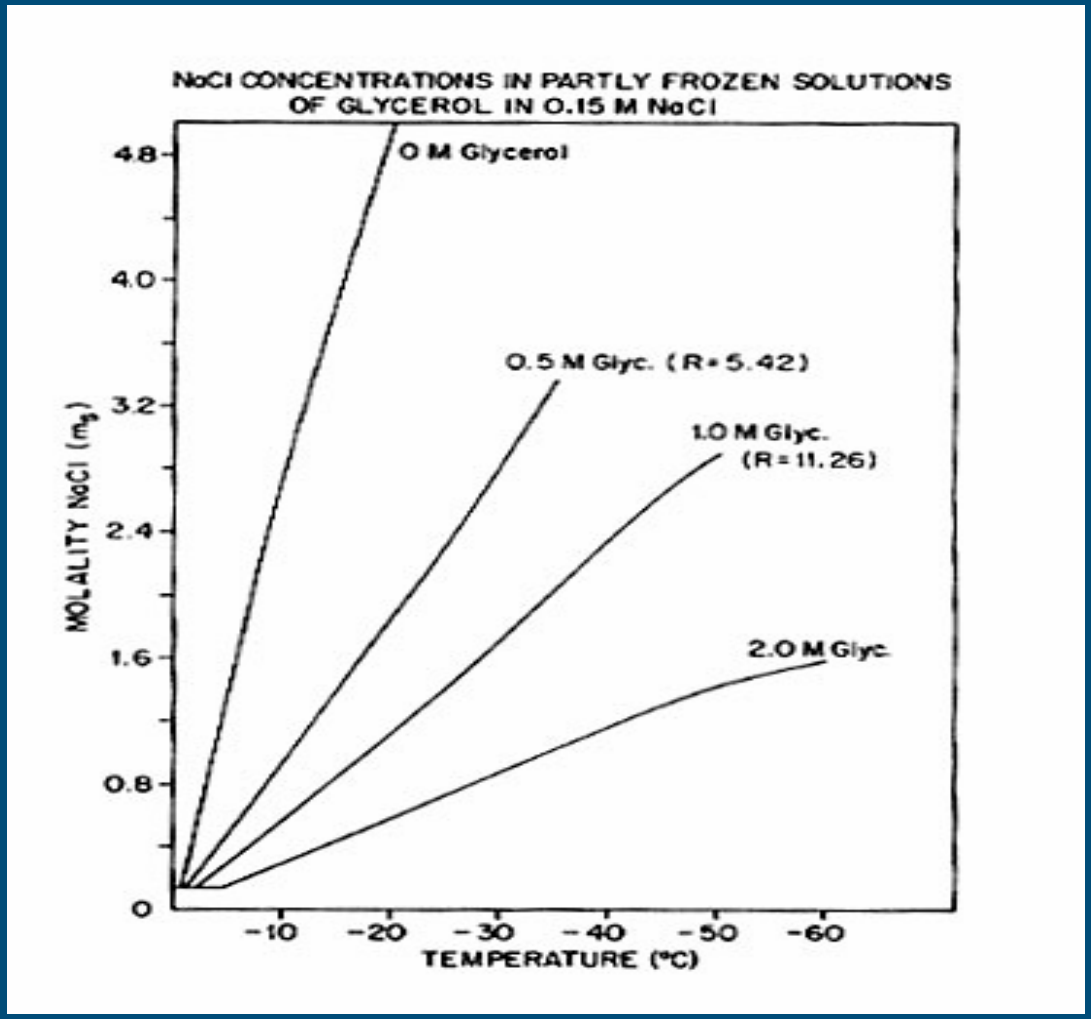
# CPAs

Examples of such compounds:

| | |
|---|---|
| propane triol | glycerol |
| propane diol | propylene glycol (PG) |
| ethane diol | ethylene glycol |
| butane diol | |
| ethanol | |
| methanol | |
| dimethyl sulfoxide | DMSO |

# CPAs

Mazur &
Rigopoulos, 1983



# CPAs

Impermeant CPAs can be used in addition to permeant CPAs

For instance macromolecules that increase the viscosity

- PVP
- PVA
- Ficoll
- Hexa-ethyl Starch (HES)

# Slow Freezing: The Cooling Rate

## Why “Slow” freezing?  and  How slow?

The purpose of slow freezing is:

Increase solute concentration to glass transition while preventing IIF

IIF is prevented by cooling slow enough to allow the cells to dehydrate enough to prevent intracellular supercooling.

The question arises: How slow is “slow-freezing”

# Cooling Rate

Two-Factor Hypothesis (Mazur et al, 1972)

<u>At too high rates</u>

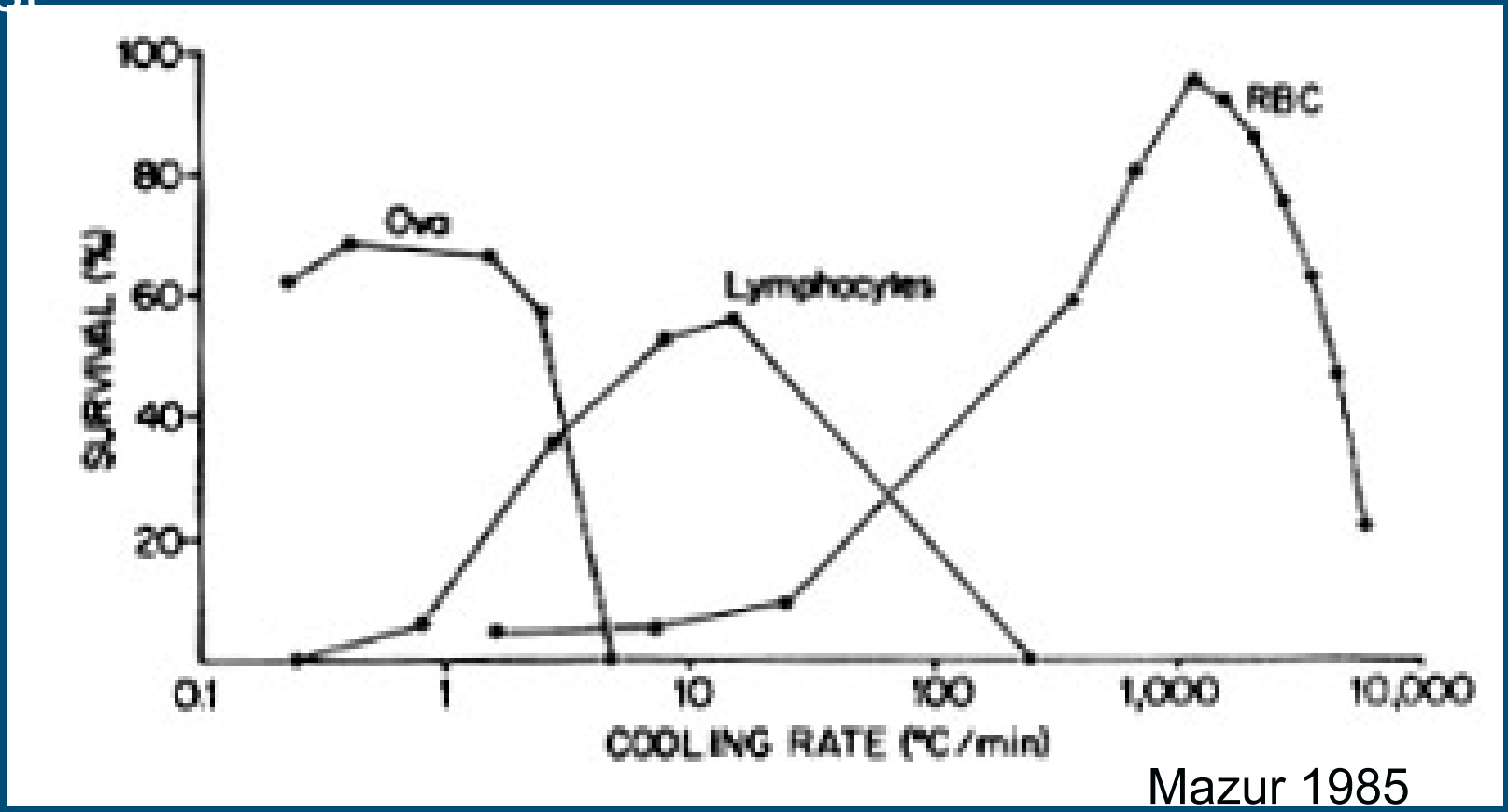
- IIF
- (or pore erosion at high water efflux)
- (or too rapid (ultra)structural changes)

<u>At too low rates</u>

- Cells shrink too much
- Cytoplasm too high salt
- Unfavourable conditions last longer

# Cooling Rate

Optimal rate somewhere between ‘too slow’ and ‘too fast’




Mazur 1985

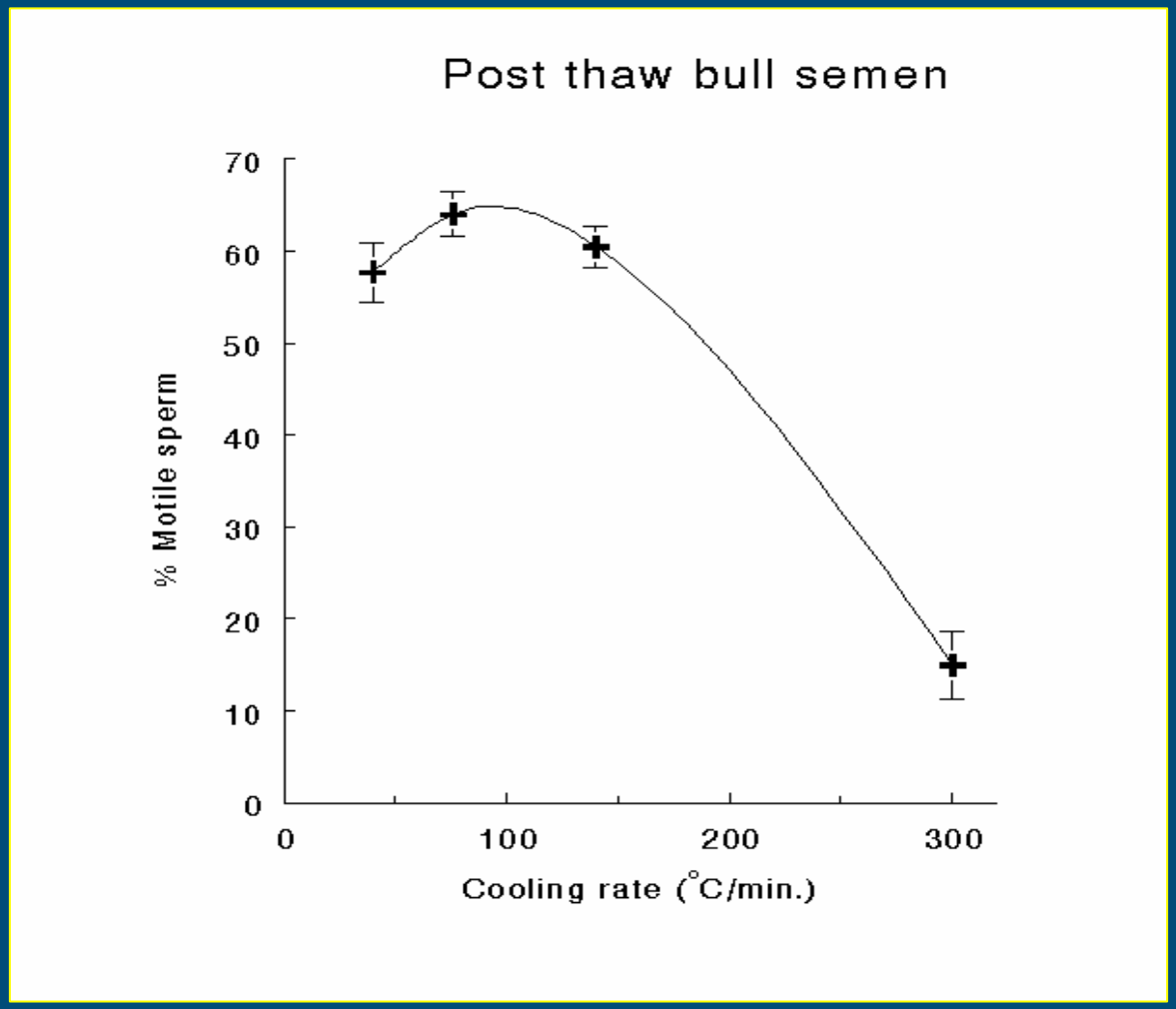
# Cooling Rate: Theoretical Approach

At optimal cooling rate, the rate and level of dehydration are optimal.

The rate of dehydration of the cells depends on the following cell (water) characteristics:

| | |
|---|---|
| Cell water volume | $V_w$ |
| Membrane surface Area | $A$ |
| Membrane hydraulic conductivity | $L_p$ |
| Membrane permeability for cryoprotectant | $P_s$ |

# Cooling Rate: empirical approach




Woelders et al. 1997

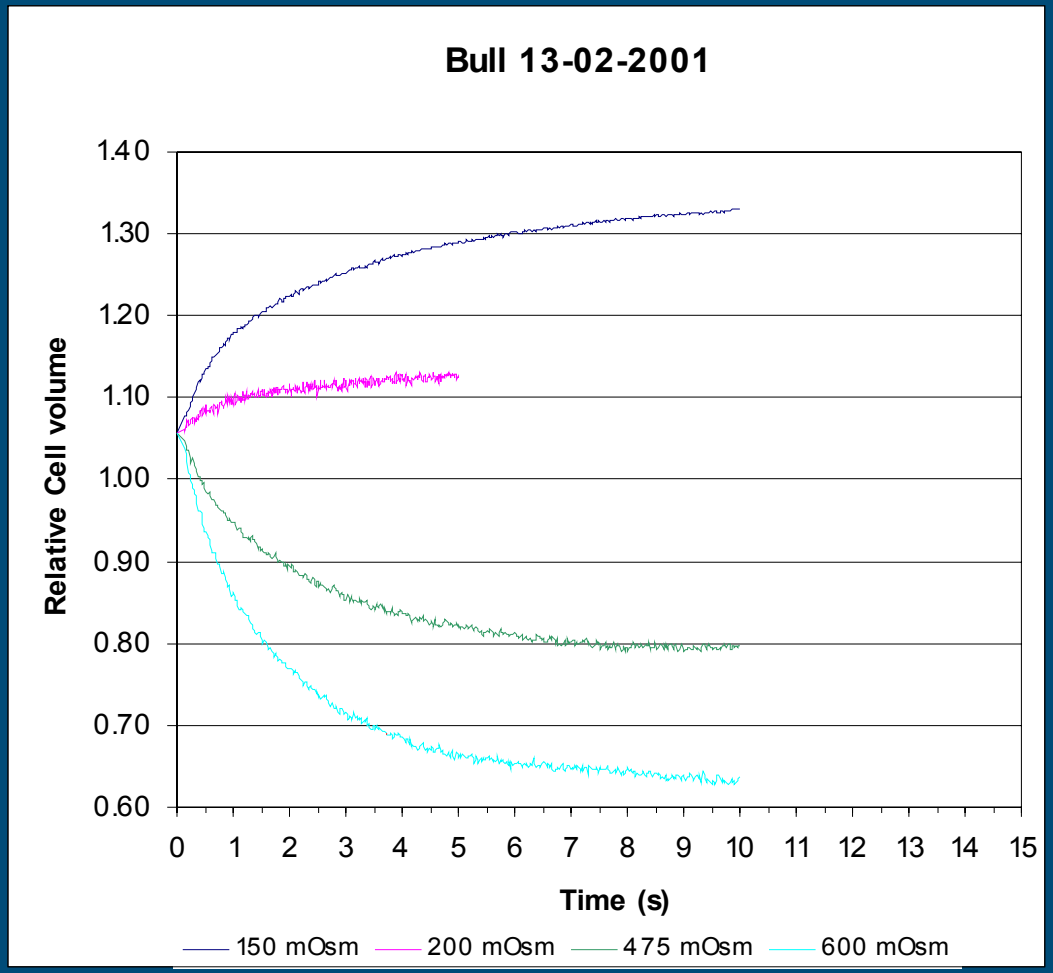
# Cooling rate; theoretical approach

However, the rate of dehydration can also be modelled mathematically

One must know $V_w$, A, $L_p$ and $P_s$ (and $E_a$ for $L_p$ and $P_s$)

# Membrane Permeability Parameters

$$dV/dT = L_p.A.R.T.\Delta M$$

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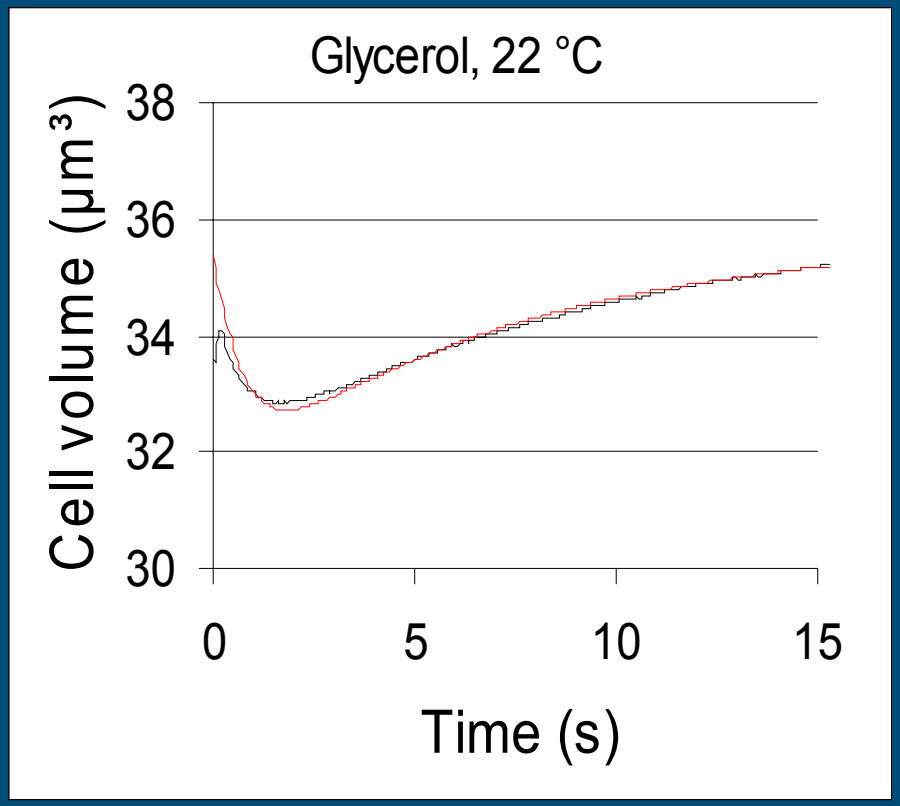
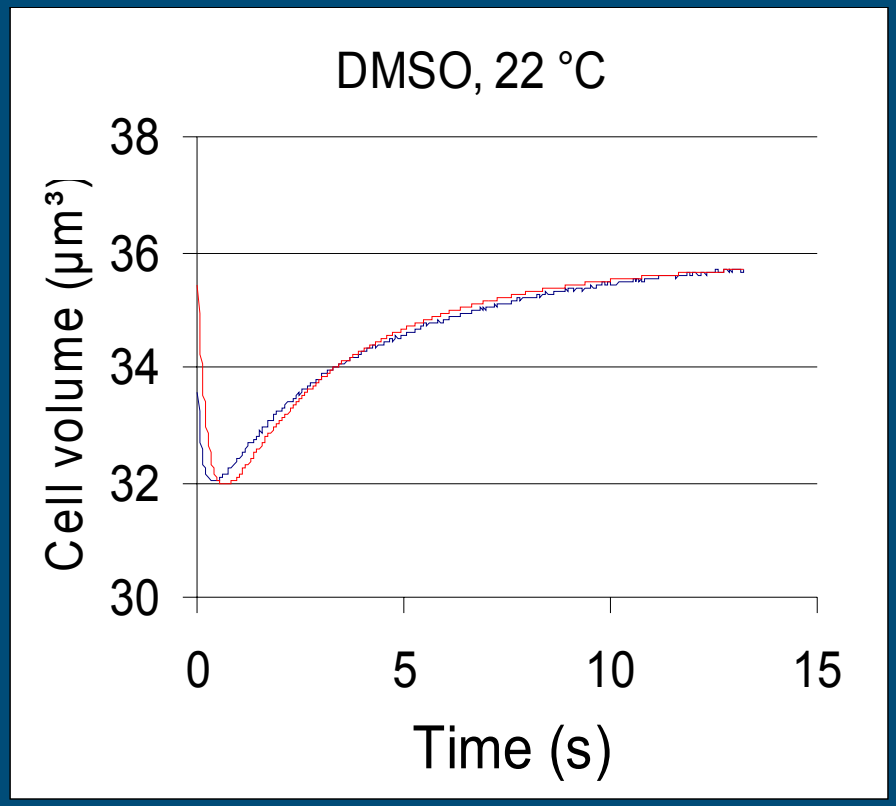
# Membrane Permeability Parameters

Measured volume changes of bull spermatozoa after adding cryoprotectant

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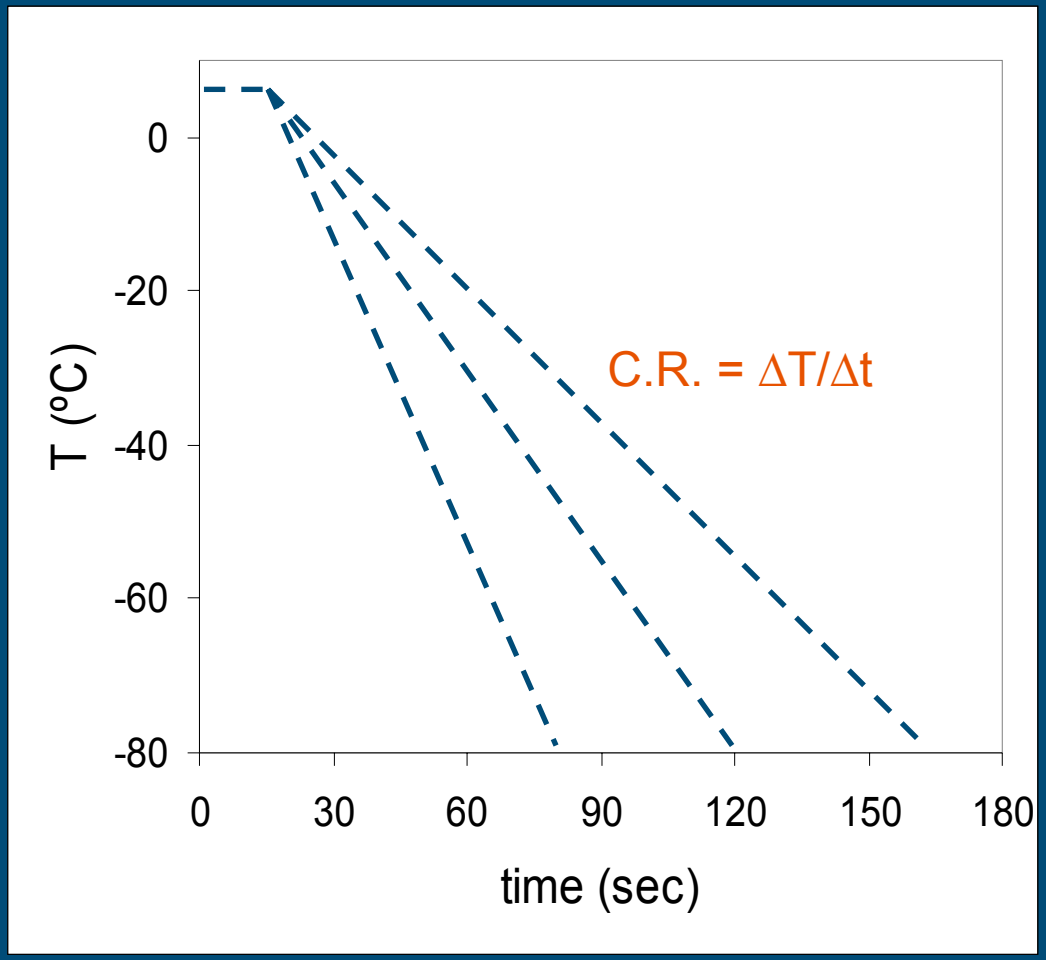
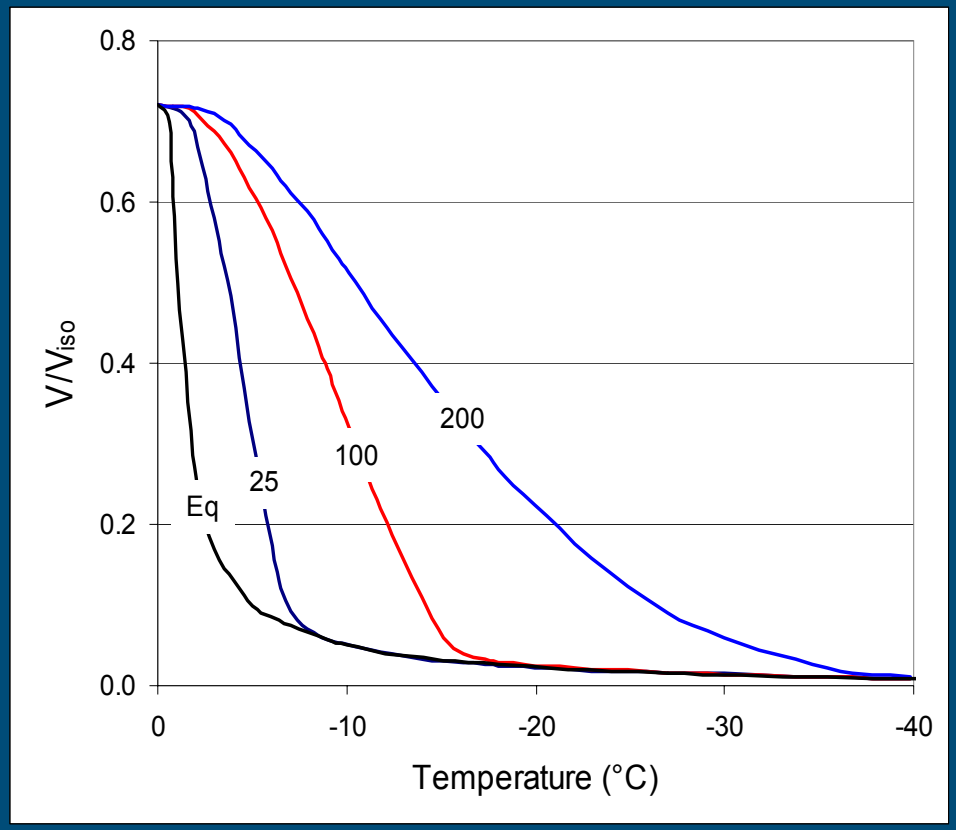
# Theoretical Model

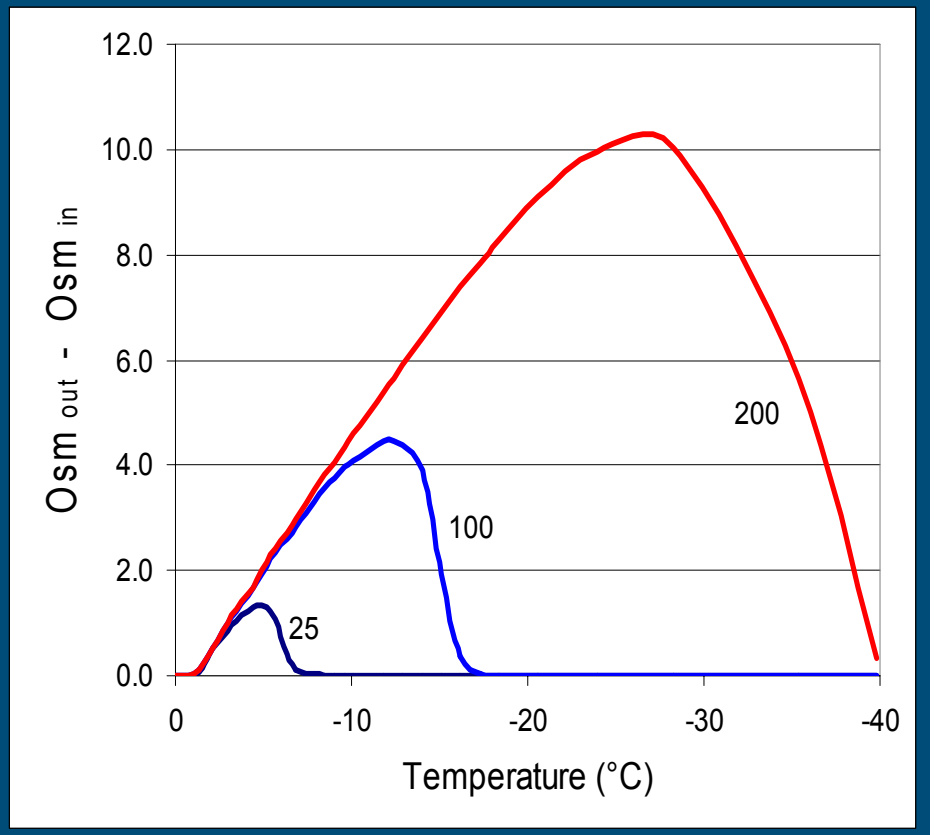
| | |
|---|---|
| Mazur 1963 | Assumed linear cooling (constant cooling rate) |
| Liu et al. 2000 | Also assumed linear cooling, but included movement of CPA |
| Woelders 2003 | Model includes movement of CPA and is not confined to linear cooling |

# Model assuming linear cooling



# Model assuming linear cooling




Mazur, 1963; Mazur and Koshimoto, 2002; see also Liu et al., 1997

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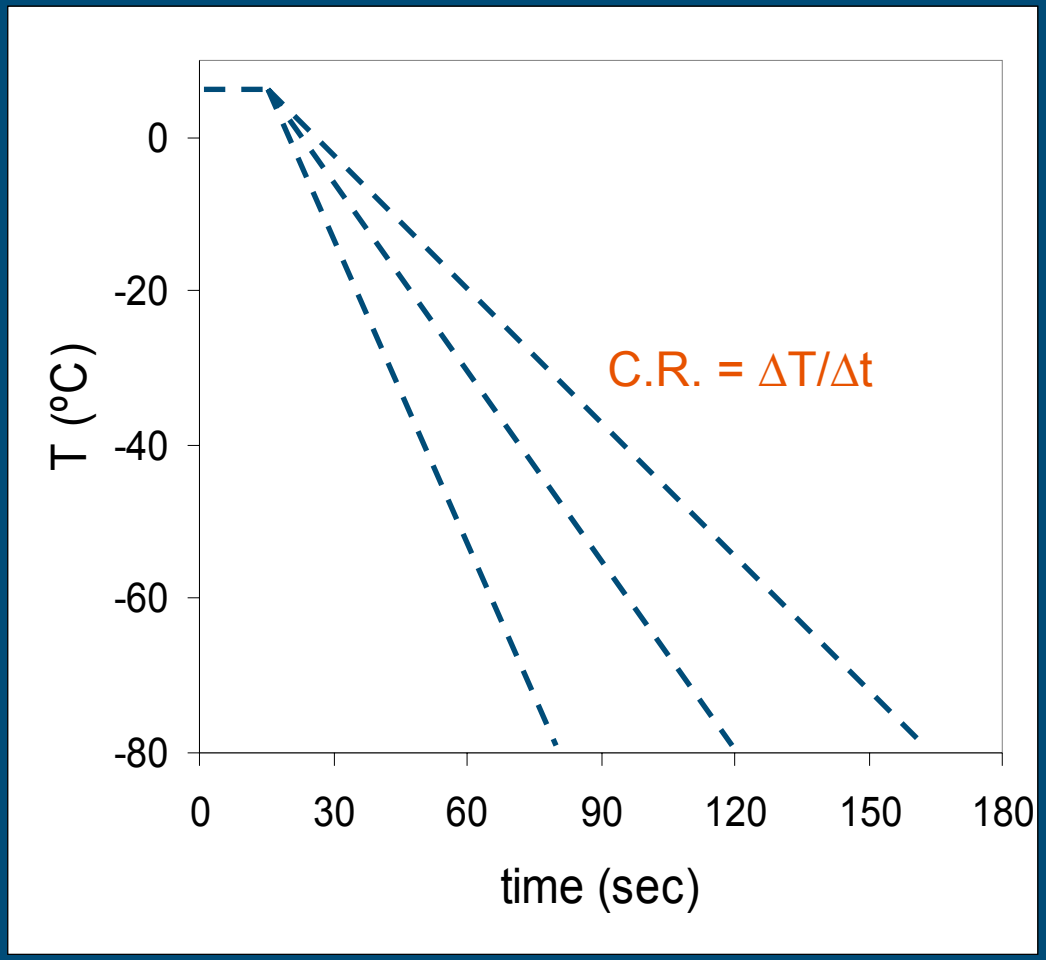
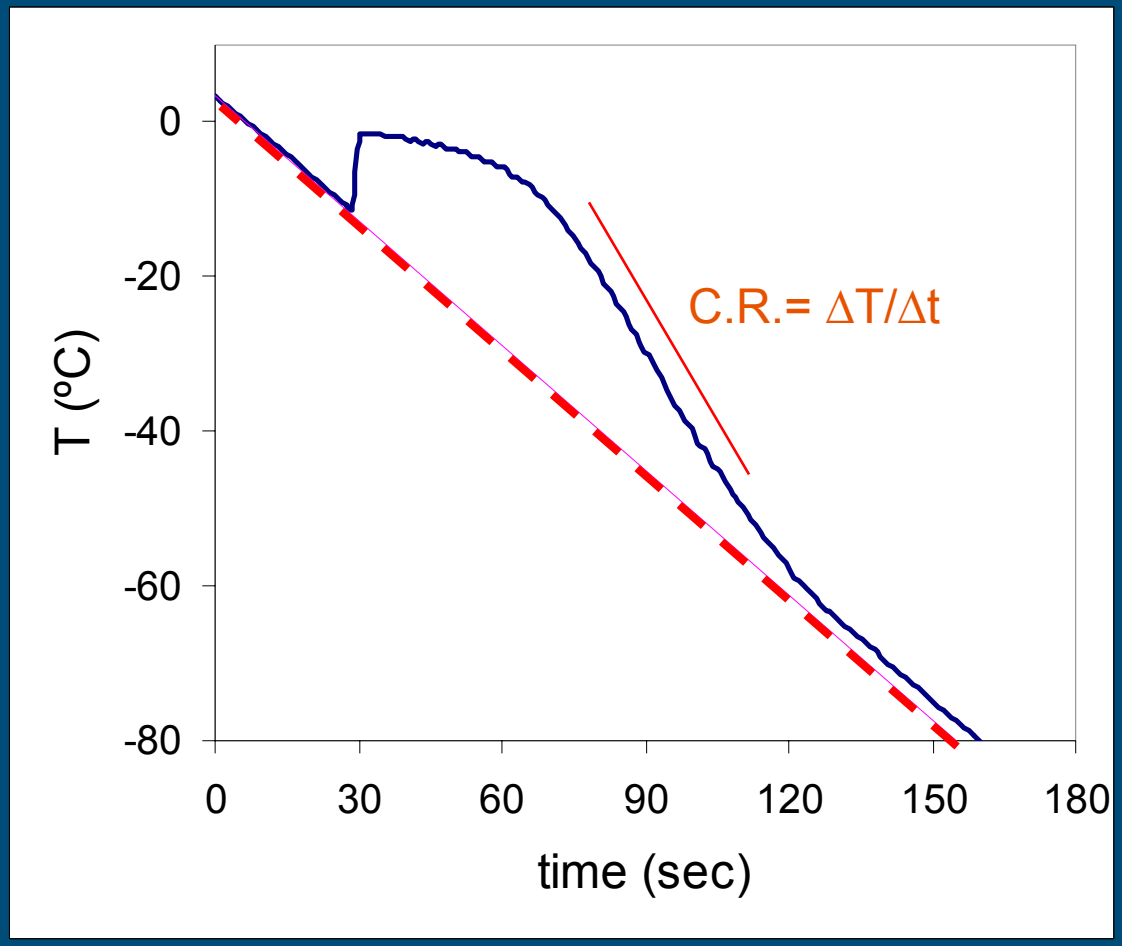
# Model assuming linear cooling



# But what is cooling rate?



# New Theoretical Model

Instead of starting with a constant rate, we have started with the following premisses :

- Cooling rate must be as high as possible
- But, IIF must not occur
- Membrane flux of water and CPA should be not too strong
- Transmembrane osmotic pressure difference should be limited

These boundary conditions can be expressed mathematically

# New Theoretical Model

$$\frac{dV_w}{dt} = L_{p(T)} ART \frac{p}{1.86}$$

$$\frac{dV_{CPA}}{dt} = P_{s(T)} A \left( \frac{\bar{V}_{CPA}\, c_{CPA(ini)}}{c_{s(ini)} + c_{CPA(ini)}} \times \frac{-\theta}{1.86} - \frac{V_{CPA(T)}}{V_{W(T)}} \right)$$

$$\mathrm{CR}_{(T)} = L_p \cdot ART \cdot \mathrm{p} \cdot (\theta + \mathrm{p})^2 / (N^{\mathrm{i}} \cdot 1.86^2)$$

$$L_{\mathrm{p}(T)} = \mathrm{e}^{\left[ \ln\left(L_{\mathrm{p}(295)}\right) + \frac{E_a}{R \cdot 295} - \frac{E_a}{RT} \right]} \qquad P_{\mathrm{s}(T)} = \mathrm{e}^{\left[ \ln\left(P_{\mathrm{s}(295)}\right) + \frac{E_a}{R \cdot 295} - \frac{E_a}{RT} \right]}$$

$c$ = molar conc.

$m$ = molal conc.

p = permissible supercooling

$E_a$ = activation energy

$L_p$ = hydraulic conductivity

$P_s$ = permeability CPA

$\theta$ = Celsius temperature

Woelders et al, 2004

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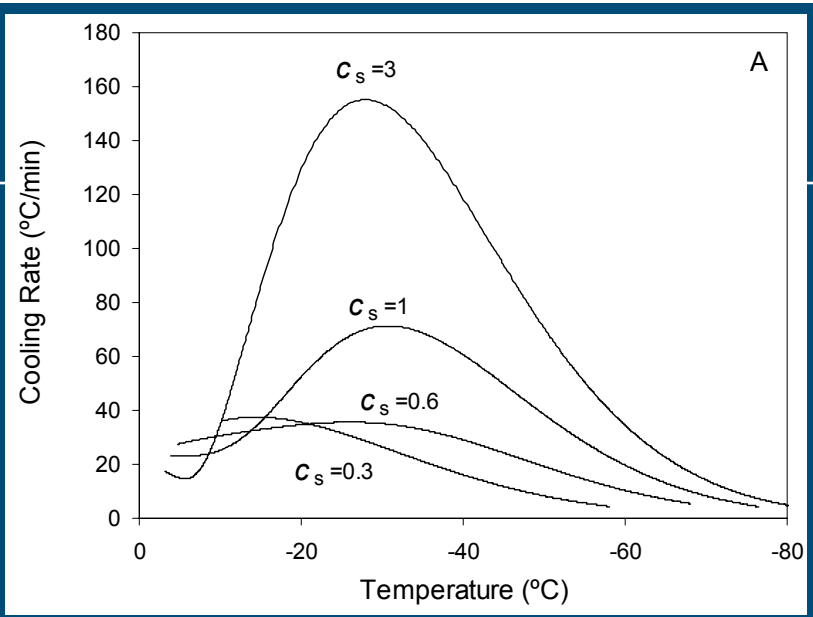


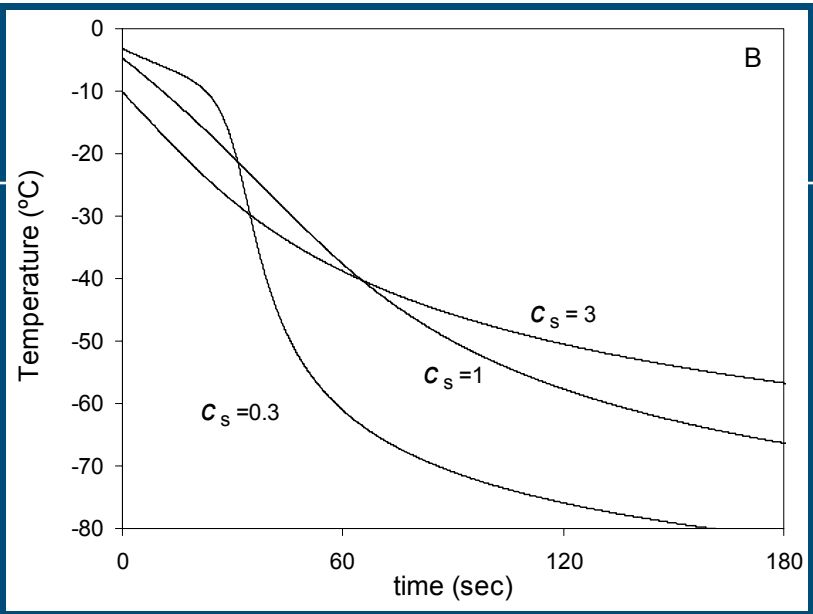


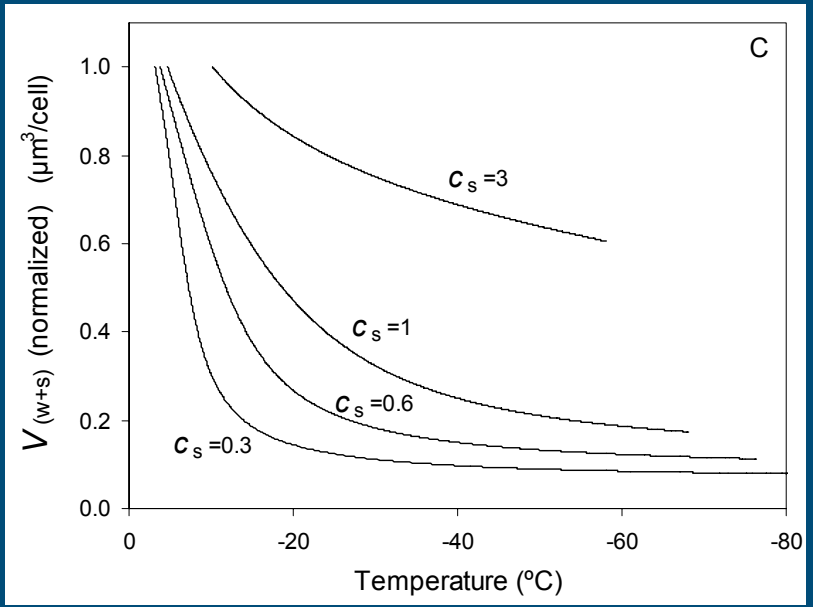


Woelders and Chaveiro unpublished

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# Somatic cells and stem cells

Method for freezing somatic cells:

- Cells in culture
- Add 10% DMSO or other CPA
- Put sample in cryovial in styrofoam box
- Place in –80 °C refrigerator

There are only a few studies in which controlled rate freezers were used.

# Stem cells

Some publications report the use of controlled rate freezers.

These studies point to optimal cooling rates varying from 0.5 to 5 °C/min.

For hematopoietic precursor cells, theoretical modelling of the freezing process resulted in significantly increased grafting, plus a reduction of the DMSO load for the patient (Woods, et al., 2000).

As shown in earlier slide, theoretical modelling predicts an interaction between CPA concentration and cooling rate.

# Spermatogenic stem cells

As early as 1954 there have been reports of successful cryopreservation of testicular tissue.

Recently (Res et al. 2000) such frozen-thawed tissue was used to obtain spermatozoa for ICSI in humans.

Cryopreservation of non-purified spermatogonial cell populations was demonstrated in 1996 by Avarbock *et al.*, using cells from mouse testes.

The same was done in a number of large domestic animals (boars, bulls, and stallions) (Dobrinski *et al.* 2000), and human (Nagano et al., 2002).

Spermatogenesis recovered after transplantation of frozen thawed testis cells in recipient mice

# Spermatogenic stem cells

In the above mentioned studies, non-controlled rate freezing was used

Little effort seemed to have been done to optimise the procedures as to freezing medium composition, cryoprotectant concentration, and cooling rate.

Using a controlled rate freezer, Izadyar *et al.* [2002] investigated the role of cooling rate and medium composition in cryopreservation of purified bovine type A spermatogonia.

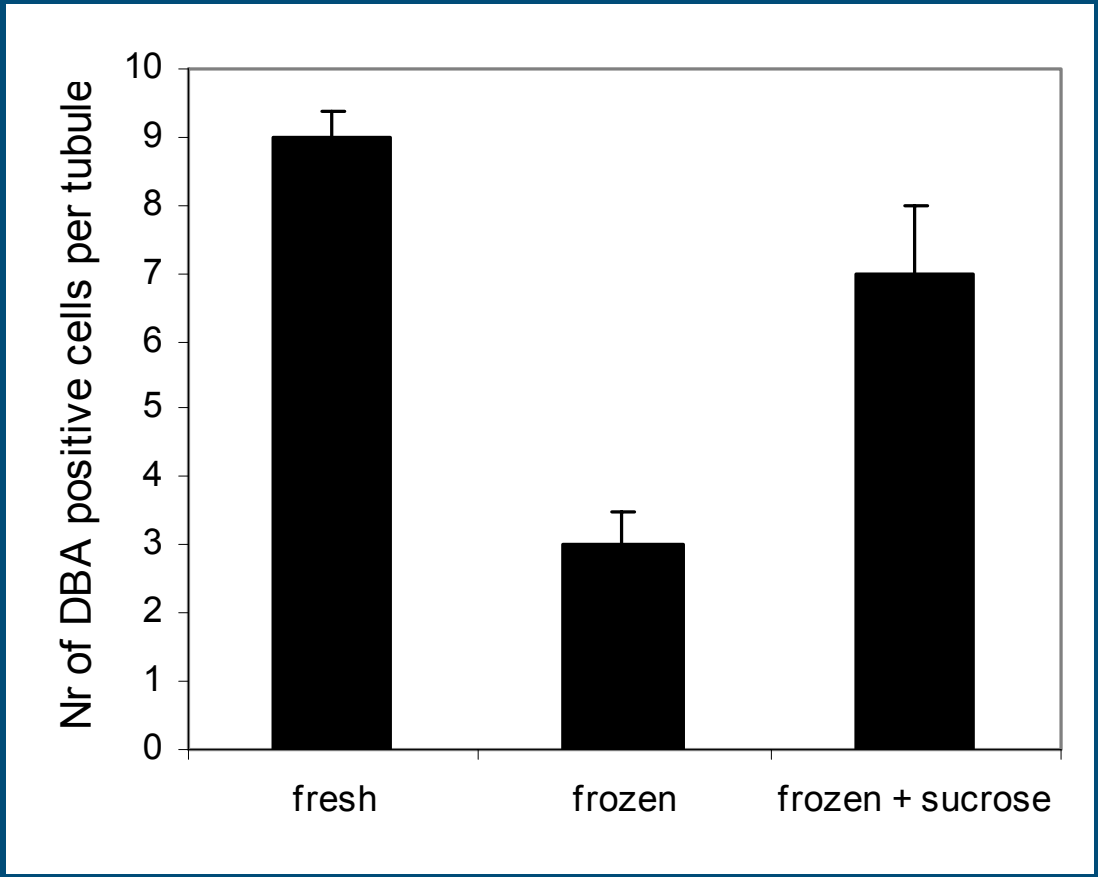
# Spermatogenic stem cells

Purified bovine type A spermatogonia (Izadyar et al. 2002)

| Additions to MEM-BSA | % live pre-freeze | % live post-thaw |
|---|---|---|
| 10% FCS | 81.0 ± 3.6 | 35.5 ± 3.3 |
| DMSO + 10% FCS | 84.0 ± 2.8 | 49.3 ± 4.7[a] |
| DMSO + 20% FCS | 82.0 ± 4.1 | 48.5 ± 5.2[a] |
| DMSO + 10% FCS + 0.07 M sucrose | 83.0 ± 3.5 | 68.3 ± 2.8[b] |
| DMSO + 10% FCS + 0.14 M sucrose | 84.0 ± 2.4 | 66.5 ± 3.2[b] |
| DMSO + 10% FCS + 0.21 M sucrose | 85.5 ± 3.7 | 65.8 ± 4.2[b] |

# Spermatogenic stem cells

Purified bovine type A spermatogonia (Izadyar et al. 2002)



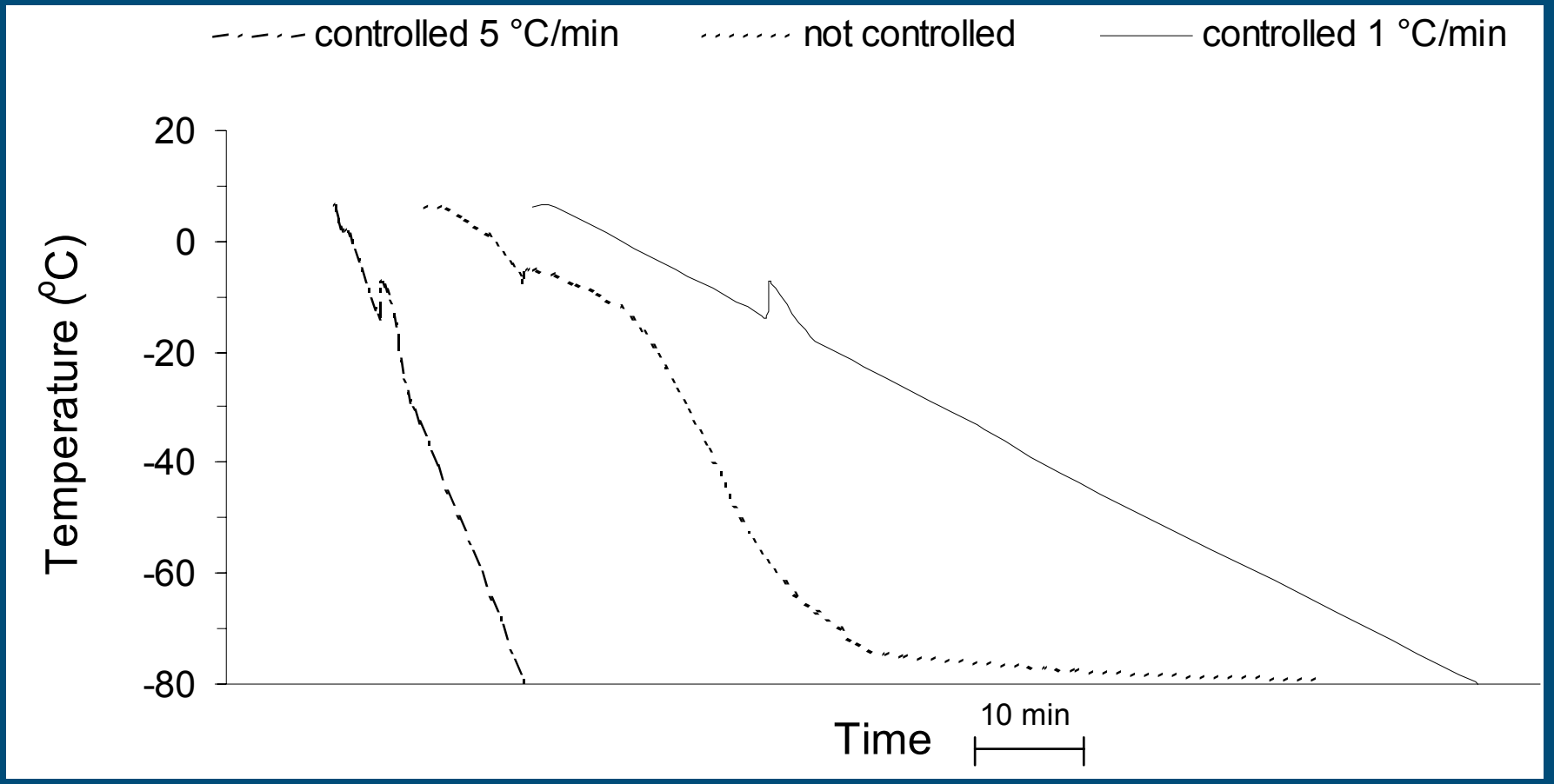
# Spermatogenic stem cells

Purified bovine type A spermatogonia (Izadyar et al. 2002)

| CPA | % live pre-freeze | % live post-thaw | | |
|---|---|---|---|---|
| | | Controlled 1 °C/min | Controlled 5 °C/min | Non-controlled |
| DMSO | 77.3 ± 6.2 | 54.9 ± 3.9[a] | 38.9 ± 4.3[b] | 59.7 ± 4.8[a] |
| glycerol | 77.5 ± 6.6 | 34.3 ± 4.6 | 28.6 ± 3.9[b] | n.d. |

# Spermatogenic stem cells

Purified bovine type A spermatogonia (Izadyar et al. 2002)



# Spermatogenic stem cells

Spermatogonial stem cells of mice, boars, bulls, stallions and humans have been frozen successfully.

The cells can be frozen using the slow freezing procedure in culture medium with 10% (v/v) DMSO + 10% FCS.

Post-thaw ability of purified type A spermatogonia (from bulls) to colonize recipient testes was strongly improved by using 0.07 sucrose in the freezing medium.

A non-controlled rate freezing method is convenient and can give good results. However, packaging and other conditions must be carefully selected to obtain the ‘just-right’ cooling curve.

# Spermatogenic stem cells

For optimal recovery of fertility of patients by transplantation of frozen-thawed spermatogonia, the best possible freezing protocol should be selected and should be standardized.

A theoretical approach as proposed by Woelders and Chaveiro [2003;2004] is needed to predict the optimal shape and steepness of the non-linear freezing curve

This then can be validated and implemented as a non-linear freezing programme in a controlled rate freezer.