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Drying Biological Material for use in Assisted Reproductive Technology

This thesis is submitted
for
the degree of Doctor of Philosophy

by

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Errata

Throughout the text "de Castro" → "**Garcia** de Castro"

p. xvii second line: change "Assisted" → "assisted"

p. xvii line 11: delete extra full stop after "bags."

p. xvii para. 4: "Guo *et al* (1998), Puvlev *et al.*, (2001), Wolkers *et al.* (2001)" → (Guo *et al.*, 1998; Puvlev *et al.*, 2001; Wolkers *et al.* 2001)"

p. xvii para. 4: "Previous work has utilised complex protocols **or drying protocols** to introduce trehalose into the cells." → ""Previous work has utilised complex protocols to introduce trehalose into the cells."

p.1 "Freeze-drying has been used to preserve delicate biological and biochemical materials which would otherwise be highly unstable at room temperature including, microorganisms (Conrad *et al.*, 2000; Miyamoto-Shinohara *et al.*, 2000; Perry, 1995; Popescu *et al.*, 1997) bio-molecules (Anchordoguy *et al.*, 2001; Bridges and Taylor, 2001; Cortesi *et al.*, 2000; Franks, 1998; Hinrichs *et al.*, 2001), foodstuffs (Kouassi and Roos, 2000; Lavelli *et al.*, 2001; Restani *et al.*, 1997) pharmaceuticals (Castellanos-Serra *et al.*, 1999; Goudey-Perriere and Perriere, 1998; Haese *et al.*, 1999; McEvoy *et al.*, 1998; Metcalf and Codd, 2000; Schwarz and Mehnert, 1997; Ward *et al.*, 1999; Worrall *et al.*, 2000), biological products (Colaco *et al.*, 1995; Ford and Dawson, 1993; Hancock and Dalton, 1999; Hopkinson *et al.*, 1996; Kreilgaard *et al.*, 1998; Mouradian *et al.*, 1985; Page *et al.*, 2000; Surrey and Wharton,.." → "Freeze-drying has been used to preserve delicate biological and biochemical materials which would otherwise be highly unstable at room temperature including, microorganisms (Conrad *et al.*, 2000; Miyamoto-Shinohara *et al.*, 2000; Perry, 1995; Popescu *et al.*, 1997), bio-molecules (Anchordoguy *et al.*, 2001; Bridges and Taylor, 2001; Cortesi *et al.*, 2000; Franks, 1998; Hinrichs *et al.*, 2001), foodstuffs (Kouassi and Roos, 2000; Lavelli *et al.*, 2001; Restani *et al.*, 1997), pharmaceuticals (Castellanos-Serra *et al.*, 1999; Goudey-Perriere and Perriere, 1998; Haese *et al.*, 1999; McEvoy *et al.*, 1998; Metcalf and Codd, 2000; Schwarz and Mehnert, 1997; Ward *et al.*, 1999; Worrall *et al.*, 2000), biological products (Colaco *et al.*, 1995; Ford and Dawson, 1993; Hancock and Dalton, 1999; Hopkinson *et al.*, 1996; Kreilgaard *et al.*, 1998; Mouradian *et al.*, 1985; Page *et al.*, 2000; Surrey and Wharton, .."

p. 2 para. 3: "a range microorganism" → "a range **of** microorganisms"

p. 2 para. 3: not done, the definition of cryptobiosis is explained in the text.

p. 5 para. 3: (Crowe and Madin, 1975; Womersley, 1989) → (Crowe and Madin, 1975).

p. 5 para. 3: "water content **felt**" → "water content **fell**"

p. 6 para. 2: "sequence of genes encoding for"→ "sequence of genes **code for**"

p. 6 para. 2: ""Browne et al. (2002) showed that a sequence of genes encoding for a strongly hydrophilic protein (late embryonic abundant LEA) **rather than a sugar** was upregulated in response to desiccation stress (they were deprived of water) in the nematode (*Aphelenchus avenae*). They speculated that this protein could act as a hydration buffer, molecular chaperone, or membrane stabiliser." → "Browne et al. (2002) showed that a sequence of genes encoding for a strongly hydrophilic protein (late embryonic abundant LEA) was upregulated in response to desiccation stress (they were deprived of water) in the nematode (*Aphelenchus avenae*). They speculated that this protein could act as a hydration buffer, molecular chaperone, or membrane stabiliser.

p. 6 para. 3 and throughout the thesis: "de Castro" → "**Garcia** de Castro"

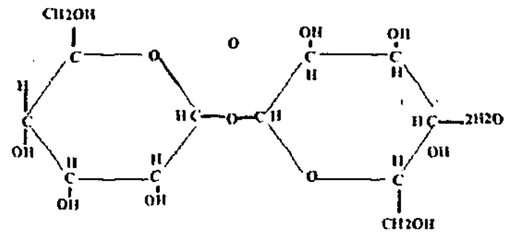
p. 6 last sentence: *"ie."* → " ***e.g.***"

p. 8 sub-section 1.5.2, para. 1: "(De Haas *et al.*, 1990)" → "(**Hottiger, *et al.*, 1987**).

p. 8 Sub section 1.5.2. para. 1: added these sentences at the end of the paragraph. "Panek (1985) interpreted these findings to mean that the trehalose must be inside and outside the cell to stabilise them. However, Coutinho *et al.*, (1988) showed that intracellular level of trehalose is not important, because yeast strain with low level intracellular trehalose apparently exhibit similar desiccation tolerance to the wild type. Further work by Eleutherio *et al.* (1993) showed that a specific trehalose carrier seems to enable the sugar to protect the yeast cell membrane by translocating trehalose from the cytosol to the extracellular environment. *Saccharomyces cerevisiae* mutant strains which lack the trehalose carrier did not survive after dehydration they accumulated endogenous trehalose Furthermore, when carrier mutants were dehydrated in the presence exogenous trehalose the cells became more resistant showing increased survival."

p. 9. Last para.: "may protect **a** plant" → "may protect plant".

p. 10 Revised Trehalose picture



p. 11 para. 1: "Concentrations of trehalose in desiccation tolerant plants can reach 35% of the dry weight of the cells (Crowe *et al.*, 1984 and Wiemken, 1990)" → has been changed to "Concentrations of trehalose in desiccation tolerant **yeasts** can reach 35% of the dry weight of the cells (Crowe *et al.*, 1984 and Wiemken, 1990)"

p. 11. Last para.: "however at **a** high" → "however at high"

p. 13 Section 1.8. last para: Add sentence: ""Although there were some *in vitro* evidence suggesting hydrogen bonding of trehalose with protein, the *in vivo* case is less convincing."

p. 13 para. 5: "(de Castro *e: al*, 2000a)" → "(**Garcia** de Castro, *et al.*, 2000b)"

p. 14 section 1.10.1, last para.: "These results have been subjected to some debate (Guo, 2000 and de Castro *et al.*, 2000a)" → "These results have been subjected to some debate (**Levine**, 2000 and de Castro *et al.*, 2000a)"

p. 15 para. 1.: "The introduction of a low concentration (0.2 M) of trehalose permitted long-term post-thaw ..." → "The introduction of **0.2 M** of trehalose permitted long-term post-thaw ..."

p. 15 para. 3: Delete "Guo *et al.* (2000)"

p. 17 last para.: "attempts sperm" → "attempts **at** sperm"

p. 20 para. 3: "such as -phenylalanine" → "such as phenylalanine".

p. 25 para. 3: "that a freezing" → "that freezing"

p. 26 line 1: "single spermatozoon" → **(not changed)**

p. 31 para. 1: "amphiphatic" → "amphipathic".

p. 32 para. 3: "The packaging method for liquid semen in New Zealand is the Minitub straw sealed at both ends with glass beads Minitub, Germany (Vishwanath and Shannon, 2000)" → "The packaging method for liquid semen in New Zealand is the Minitub straw sealed at both ends with glass beads **(Minitub, Germany)** (Vishwanath and Shannon, 2000)."

p. 34 Heading section 1.18: "Fibroblast" → "Fibroblast **preservation**"

p. 35 para. 1: "for loss cells" → "for **lost** cells"

p. 35 para. 4: "to protect cell" → "to protect **cells**"

p. 36 Table 1.2. col. 3 row 5: "Trehalose" → "**Intracellularly produced** trehalose"

p. 36 Table 1.2. col. 4 row 5: "Viable **and proliferate** cells" → Viable cells"

p. 36 Table 1.2. col 4 row 6: Viable cells" → "Viable **and proliferate** cells"

p. 36 Table 1.2. added row; of Bloom *et al.* (2001), Garcia de Castro, A. and Tunnacliffe, A. (2000) and Chen, *et al.*, (2001) results. See below

Cell type	Drying method	Excipient	Results	References
LMTK cells	Air dry	Trehalose	Cells not survive desiccation	Garcia de Castro and Tunnacliffe (2000)
Human 293H cells	Air dry	Glycan	Recover viable and dividing cells	Bloom *et al* (2001)
NIH 3T3, murine fibroblast	Nitrogen drying	Trehalose	Recover cells with intact membranes	Chen *et al.* (2001)

p. 36 last para.: "*otS-A* and *otS-B*" → "***ots-A* and *ots-B***"

p. 37 last line: "Wokers" → "**Wolkers**"

p. 51 last para.: "medium the absence" → "medium **in** the absence"

p. 53 para. 1: "chromatin decondesation" →"chromatin decondensation"

p. 65 para. 1: "Single spermatozoon" → "A single spermatozoon"

p. 72 para. 3: "those group A" → those **in** group A"

p. 72 para. 4: "Following after rehydration of spermatozoa from group A, we found high the proportion spermatozoa with physical damage such as bent and broken tail." → "Following rehydration**, a high proportion of** spermatozoa from group A, **had** physical damage such as bent and broken tail.

p. 72 last para. : "ICSId" → "**injected**"

p. 76 para. 1: "none those" → "none **of these**".

p. 83 section 4.2.: "The vials were then placed in a speedy-vac (Dynavac) at 1 mTorr pressure"→ "The vials were then placed in a speedy-vac (Dynavac) at 1 **Torr** pressure"

p. 83 para. 2: "Pictures were **manipulated** using Confocal Assistant ver 4.02." → "Pictures were **analysed as TIFF or JPEG images as this enhanced the image quality** using Confocal Assistant ver 4.02. **(Todd Clark Brelje, University of Minnesota)**."

p. 84 last para.: "was attributable" → "was attributable **to**"

p. 93 para. 3: the second"-15°C" I different line → read as "-15°C"

p. 94 para. 3: "-non permeabilised" → "non-permeabilised"

p. 102 para. 2: " Preliminary observation compared the efficacy of sugars, capacitance and media on cell viability after electroporation." → "Preliminary observation compared the efficacy of sugars, capacitance and media on cell viability after electroporation."

p. 105 Fig. 5.2.: Label for x-axis "**voltage**"

p. 113 Fig. 5.6. legend to Fig. 5.6.

A. Light microscopy of viable plated cell and non-viable cell (spherical);
B. Epifluorescent microscopy of viable-plate cell (not bright) and non-viable cell (bright),

C. Layered picture with viable-permeable and non viable cell and viable-non-permeable.
Fig 5.6. PI retention by viable plated cell after 24 h in culture.
changed →
Fig 5.6. PI retention by viable plated cells after 24 h in culture.
A. Light microscopy of viable plated cell and non-viable cell (spherical);
B. Epifluorescent microscopy of viable-plate cell (not bright) and non-viable cell (bright),
C. Layered picture with viable-permeable and non viable cell and viable-non-permeable.

p. 124 para. 3: "to3" → "**to 3**"
p. 125 para. 3: "However cells obtained frozen residues" → "However cells obtained **from** frozen residues"
p. 126 Table 5.4. notes: "1 mTorr" → "1 **Torr**"
p. 126 para. 1: "as a multisteps process" → "as a **multistep** process"
p. 129 para. 2: Add this sentence at the end of paragraph. "Chen *et al.*(2001) found that intra and extra cellular trehalose gave a beneficial effect on membrane integrity of dried mammalian cells."
p. 129 para. 2: "and dry the cells using air *drying*" → "and **dried** the cells using air "
p. 129 para. 4, last line: "Woelker" → "Wolkers".
p. 139 sub-section 6.3.2. para. 2: "super cooling" → "**supercooling**"
p. 176 last para.: "alonethere" → "**alone there**"
p. 178 para. 1: "might due" → "might **be** due"
p. 179 para. 3: "Wolker" → "Wolkers"
p. 184 para. 2: "Removal of water using vacuum pressure at 1 mTorr for 8 h.." → " Removal of water using vacuum pressure at 1 **Torr** for 8 h..
p. 187 last para.: "(Puhlev, 2001)" → "(Puhlev ***et al.***, 2001)
p. 244 insert ref. Bloom, F.R., Price, P., Lao, G.F., Xia, J.L., Crowe, J.H., Battista, J.R., Helm, R.F., Slaughter, S., and Potts, M. (2001). Engineering mammalian cells for solid-state sensor applications. Biosensor Bioelectron., 16, 603-608.
p. 247 "de Castro, A.G., and Tunacliffe, A. (2000)" → "**Garcia** de Castro, A. and Tunnacliffe, A. (2000)"
p. 247 delete ref. "De Haas, G.H., Dijkman, R., Van Oort, M.G., and Verger, R., (1990). Competitive inhibition of lipolytic enzymes. Biochim. Biophys Acta 1043, 249-257."
p. 251 insert ref.: "Hottiger, T., Boller, T and Wiemken, A., (1987). Rapid changes of heat and desiccation tolerance correlated with changes in trehalose content in *Saccharomyces cerevisiae cells* subjected to temperature shifts. FEBS lett. 220, 113-5115.
p. 254 insert ref.: Levine, F., (2000). Nat. Biotech. 18, 473.
p. 256 "Merryman (1966)" → "Meryman (1966)"

I recommend the PhD be accepted after attention to the following points:

1. There are many typographical errors, some of which are noted on the attached sheet, which should be corrected.

Introduction (Chapter 1)

2. On page 4, last para (and on the following page), the candidate introduces the water replacement hypothesis, but should make clear that it is only a hypothesis. There is very little *in vivo* evidence to support it (eg. Clegg et al. 1982 may be the only evidence, and this could be open to other interpretations). It also seems odd to introduce the WRH without discussing other hypotheses, chiefly the vitrification hypothesis (although this is mentioned elsewhere), and the various solution properties of disaccharides.
3. Page 5: Womersley (1989) refers to nematodes, not tardigrades.
4. Page 6, Browne et al. (2002): This paper does not suggest that an LEA protein is produced in place of disaccharides, but together with them. The concept of a "bioglass" is discussed.
5. On page 8, para 2, the correct ref is: Hottiger, T., Boller, T. & Wiemken, A. 1987 Rapid changes of heat and desiccation tolerance correlated with changes in trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. *FEBS Lett.* **220,** 113-115.
6. Panek's work is arguably not conclusive, particularly with respect to intracellular trehalose. The candidate should consult Coutinho, C., Bernades, E., Felix, D. & Panek, A. D. 1988 Trehalose as cryoprotectant for preservation of yeast strains. *J. Biotechnol.* **7,** 23-32. Here strains with low levels of intracellular trehalose apparently exhibit similar desiccation tolerance to wild-type (see for example, Figure 1), which argues that intracellular trehalose levels are not important. Some of these same "low level trehalose producers" were used in Eleutherio et al. 1993 (Eleutherio, E. C. A., De Araujo, P. S. & Panek, A. D. 1993 Role of the trehalose carrier in dehydration resistance of *Saccharomyces cerevisiae*. *Biochem. Biophys. Acta* **1156,** 263-266) and do not lack the ability to synthesis trehalose, as stated in para 2, p. 8. The candidate should be more circumspect in interpreting Panek's work, after closer examination of the primary literature.
7. The disaccharide structures on p. 10 leave the position of the ring oxygens in some doubt. As drawn, the ring is composed of six carbons instead of five carbons and an oxygen.
8. On page 11, para 1, it is stated (at the end) that "concentrations of desiccation tolerant plants can reach 35% of the dry weight of the cells." This can't be correct, since most plants use sucrose. Does the candidate mean yeast?
9. On page 12, section 1.8, we return to the WRH and the candidate rightly refers to Webb's pioneering work, but also to "evidence based on stereomodels". This was first done by Warner (1962) and should be reference appropriately (Warner, D. T. 1962 Some possible relations of carbohydrates and other biological components with the water structure at 37°. *Nature* **196,** 1055-1058).
10. Again in section 1.8, consideration of point 2 above should be made: although there is some *in vitro* evidence suggesting hydrogen bonding of trehalose with proteins, the *in vivo* case is less convincing.
11. Section 1.10 introduction: the reference to Guo et al. (2000) implies that mammalian cells were genetically engineered, when they were not – only infected with a genetically engineered viral vector. Garcia de Castro and Tunnacliffe (2000) do genetically engineer the cells, as mentioned. Why is Eroglu et al. (2000) and their inducible poration technique not mentioned here?
12. Section 1.10.1: the (second) reference to "Guo 2000" should be Levine 2000 *Nat. Biotech.* **18,** 473.
13. Section 1.10.2 and elsewhere throughout the thesis (eg. p. 35, last para; p. 93; p. 128, para 3). There is reference to Eroglu et al. (2000) introducing "low" concentrations of trehalose into porated mammalian cells. Actually, these are "high" concentrations. The candidate should consider the range of trehalose concentrations found in various anhydrobiotes: 20 mM in tardigrades, 100 - 150 mM in nematodes, up to 400 mM in yeast (but typically lower). So the 0.2 M (ie. 200 mM) of Eroglu is on the high side.
14. Section 1.10.4, p. 15: I don't think Guo et al. (2000) used extracellular trehalose, as implied by the first sentences in this section. Puhlev et al. (2001) did do this, though.
15. There is a degree of repetition throughout the thesis, with a particular example being mention of the papers by Wakayama and Yanagimachi (1998) and Kusakabe et al. (2001) multiple times: on pp. 2, 17 (twice), 18, 33 (twice), 51/52, 53/54, 79 (twice), 80, etc., as well as in the Abstract. If a point is made too often, it loses its force!
16. The heading to section 1.18 needs to be more informative.
17. Table 1.2 (p. 36) is a little misleading:
    - Guo et al. (2000) do not demonstrate or claim proliferation of dried and rehydrated cells (although Puhlev et al. do);

- the air drying methods used by different authors are lumped together as equivalent, when they are quite different eg. Matsuo (2001) talks about partial dehydration, while Guo et al. (2000) claim complete dryness;
- Puhlev et al. and Gordon et al. claim (among other things) dehydration without any biochemical modifications to the cell – it is not clear, under the "Excipient" column, whether intracellular or extracellular trehalose is meant (although strictly an excipient is something added to the drying cocktail and can therefore only ever be extracellular – so Guo et al. do not use any drying excipient);
- The candidate may not be aware of Bloom et al. who report admittedly preliminary results in semi-review format (Bloom, F. R., Price, P., Lao, G. F., Xia, J. L., Crowe, J. H., Battista, J. R., Helm, R. F., Slaughter, S. & Potts, M. 2001 Engineering mammalian cells for solid-state sensor applications. *Biosensors Bioelectron.* **16**, 603-608), but where some success is claimed after overlaying mammalian cells with a glycan derived from cyanobacteria;
- if the Table aims to list "attempts" at drying mammalian cells, it should include Garcia de Castro and Tunnacliffe (2000) and Chen et al. (2001)

Chapter 3

18. The introduction to Chapter 3 should include something about the physiological events following ICSI, given that normal fertilisation is reviewed on p. 53. I may have missed it, but I also couldn't find a review of the ICSI process and its development anywhere. Surely this is of central importance to the thesis?
19. The candidate is rightly concerned about gas permeability of foil bags on p. 66. "Oxygen molecules are smaller than CO_2." Water molecules are smaller than either of these, or nitrogen. Was consideration of the water permeability of the various containers of dried material.
20. I couldn't find mention of Tables 3.5, 3.6 and 3.7 in the text anywhere. Is there a section of text missing from page 67?
21. It is hard to assess this Chapter as a whole, since the initial positive results are not reproducible. It's not entirely clear how many attempts were made to repeat this result, but the repeated failure to do so must cast doubt on the first experiments. Perhaps the sperm were not dry? Nowhere in the thesis is this question addressed: how dry is dry? This is a common problem, I think, with any cell drying approach and is probably (in my opinion) responsible for the results from the Levine and Potts labs. Normally, of course, an unrepeatable result would not be reported, even in a thesis, and I recommend that it should be omitted, when point 22 (below) is taken into account.

Chapter 4

22. It is not clear to me why Chapter 3 and Chapter 4 are presented separately. That they cover very similar ground is clear from the fact that all sections in Chapter 4 are very short. In addition, the results of Chapter 4 seem to be more reproducible than those of Chapter 3, and therefore the (negative) Chapter 3 material could be accommodated in a combined chapter without giving an overall negative impression. I strongly recommend combining Chapters 3 and 4, with omission of the unreproducible experiments from Chapter 3.
23. Table 4.2 suggests that dried sperm chromatin decondenses at 3 hr after injection, but Figure 4.2 at the 3 hr time point shows that sperm chromatin looking exactly as it does at 1 hr (cf. fresh sperm experiment, where the 1 hr picture shows very clear decondensation).
24. Again, it wasn't entirely clear how many times the experiments were repeated. Are these "one-offs"? How reproducible is this procedure? This question is, of course, central if a new technique is being claimed.
25. The Methods section (4.2; p. 83) suggest that a "speedy-vac" can attain a 1 mTorr vacuum. Can this be correct? Our high specification vacuum/freeze dryer rarely gets below 30 mTorr pressure.

Chapter 5

26. Section 5.1.2: again, Guo et al. (2000) and Puhlev et al. (2001) are credited with genetic engineering of human fibroblasts. This is not correct: see point 11 above.
27. What level of vacuum is achieved in the freeze dryer (section 5.2.6.1.3; p. 99)? This is an important point since it is suggested (p. 125) that the vacuum is not strong enough to perform proper freeze drying.
28. It is rather puzzling how electroporation can be carried out in media/salt solutions such as DMEM. In our experience, we have needed to use media with very low conductivity to prevent arcing in the cuvette.
29. The x-axes in Fig. 5.2 are not labelled.
30. The legend to Fig. 5.6 requires the last line to come first.
31. Page 129: Chen et al. (2001) make a very particular claim: intracellular trehalose, above a certain threshold (100 mM), can preserve the integrity of cell membranes if stored at low temperature. They do not see viability maintained. Levine's group is alone in claiming this (if we discount the rather strange report from Bloom et al. 2001, which has not been substantiated elsewhere). The candidate should not be reticent about his results, since he is, after all, in the consensus group!

Chapter 6

32. It is a pity that intracellular trehalose was not measured at any point in this Chapter or elsewhere. Relatively straightforward techniques are available to do this without recourse to HPLC or GC. It is possible that, in a number of experiments where no significant effect of trehalose was observed, this was due to low intracellular concentrations being achieved.
33. There should be discussion, somewhere in the thesis, of what cryoprotectants are thought to do.
34. Page 179: DMSO might be used in freeze-drying experiments. In our experience, freeze-drying can remove it, although it needs to be cleaned out of the condenser afterwards!

Chapter 7

35. On page 183, the experiments of Chapter 3 are discussed. Perhaps, given that the air drying protocol does not seem to give rise to fully functional sperm, the candidate should question the results of Wakayama and Yanagimachi (1998) and Kusakabe et al. (2001)? Has any other group been able to repeat their work? Why didn't the candidate try this method, given that he had access to a freeze-dryer (and that there is nothing sophisticated about the approach used by Wakayama and Yanagimachi (1998) and Kusakabe et al. (2001))? It seems to me more likely that spermatozoa cannot be dried (or freeze-dried) than that they can.
36. Table 7.1 again implies that Guo et al. (2000) genetically engineered human cells. See points 11 and 26.
37. On page 187, last para and following, and possibly throughout the thesis, the candidate seems to misunderstand the measurement of pressure, as it relates to a vacuum. It is not the vacuum which is being measured, but the pressure. So it is stated that the "equipment used in this study could only draw a vacuum of 1 mTorr, while others (Wolkers et al. 2001) used 30 mTorr." A vacuum of 1 mTorr is *higher* than that of 30 mTorr (although Wolkers et al. actually state 20 mTorr!), although the pressure is lower: ie. a higher vacuum means lower pressure. So I am curious to learn how 1 mTorr was achieved (as mentioned in point 25). If it was achieved, that's a very fine vacuum indeed, and would be sufficient for all the experiments intended! This needs some clarification throughout the text.
38. The controversy surrounding mammalian cell drying is raised again on p. 188, and I reiterate point 31 above. In fact, this is a bit of a damp squib: the consensus seems to be that mammalian cells cannot be dried in a viable state simply by engineering high levels of intracellular trehalose into them (by whatever method). The candidates results are in line with this consensus. In our experience, replicating mammalian cells can be dehydrated to some degree and survive, and some trehalose might improve this survival – this is essentially what Matsuo (2001) is doing – but real desiccation is lethal. Wolkers' platelets might be an oddity, since they are non-replicating cell fragments and therefore arguably similar to liposomes, which are stabilisable by trehalose. [Although it's perhaps worth mentioning that Quadrant Healthcare scientists spent several years trying to dry electroporated platelets containing plenty of trehalose without the success claimed by Wolkers et al. The best that was achieved was some partially functional platelet fragments.]

Summary

Overall, Chapters 3 and 4 are the least convincing of the thesis, while Chapters 5 and 6 are much more thorough and represent publishable work of a higher standard. The thesis would be greatly improved by moving Chapters 5 and 6 to the front, and combining and editing Chapters 3 and 4 into a final experimental chapter. A generally more sceptical approach thoughout might be beneficial.

It might be of interest that Bronshtein's group at Universal Preservation Technologies have informally reported similar findings on electroporated mammalian cells, ie. they do not survive drying. I am not sure whether they intend to publish this work (although I have urged Bronshtein to do so previously), but it would be sensible for the candidate to proceed rapidly with preparation of manuscripts for publication, from the work of Chapters 5 and 6, if this has not already been done.

Addendum: Responses to examiner's question/comments

Introduction (Chapter 1).

1. I agree this is only a hypothesis. I have inserted the word "may". Accumulating certain sugars **may** help anhydrobiotic organisms to replace the water around polar residues in membrane phospholipids and proteins, thereby maintaining their integrity in the absence of water (Crowe *et al.*, 1996). .

2. I agree that the sentence is ambiguous, "*Browne et al. (2002) showed that a sequence of genes encoding for a strongly hydrophilic protein (late embryonic abundant LEA)* ***rather than a sugar*** *was upregulated in response to desiccation stress (they were deprived of water) in the nematode (Aphelenchus avenae). They speculated that this protein could act as a hydration buffer, molecular chaperone, or membrane stabiliser.*"
   I replaced it with the following sentences.
   "*Browne et al. (2002) showed that genes encoding for a strongly hydrophilic protein (late embryonic abundant LEA) was upregulated in response to desiccation stress (they were deprived of water) in the nematode (Aphelenchus avenae). They speculated that this protein could act as a hydration buffer, molecular chaperone, or membrane stabiliser.*"
3. I agree that more information from other workers, Coutinho *et al.* (1988) and Euletherio *et al.* (1993) should be included at the end of the paragraph.
   "However, Coutinho *et al.*, (1988) showed that intracellular level of trehalose is not important because one yeast strain with low levels of intracellular trehalose apparently exhibited similar desiccation tolerance to the wild type. Further work by Eleutherio *et al.* (1993) showed that a specific trehalose carrier seems to enable the sugar to protect the yeast cell membrane by translocating trehalose from the cytosol to the extracellular environment. *Saccharomyces cerevisiae* mutant strains which lack the trehalose carrier did not survive dehydration even though they accumulated endogenous trehalose. Furthermore, when carrier mutants were dehydrated in the presence exogenous trehalose the cells became more resistant."
   See errata list for p. 8.

4. Yes I agree, yeast is the most correct word. However I also refer to the resurrection plants, *Craterostigma platagineum* and *Selaginella lepidophylla* as "plants".
   "Concentrations of trehalose in desiccation tolerant plants can reach 35% of the dry weight of the cells (Crowe *et al.*, 1984 and Wiemken, 1990)", → has been changed to
   "Concentrations of trehalose in desiccation tolerant **yeasts** can reach 35% of the dry weight of the cells (Crowe *et al.*, 1984 and Wiemken, 1990),"
   See errata list for p. 11.

5. Warner, D.T., 1962. Some possible relationships of carbohydrates and other biological components with the water structure at 37°. Nature, **196**: 1055-1058.
   In his paper, D.T. Warner used *Scyllo*-inositol as the model and Dreiding stereo models to speculate about possible relationships between carbohydrates and other molecules such as water and peptides. Hydroxyl groups may contribute an important role bonding the hydrophilic and hydrophobic groups from peptides to stabilise peptide structures. This model may extend to water replacement hypothesis as described by Webb and Crowe. Even though Warner reviews stabilising biological system and viable tissue during freezing and thawing, it does not specifically address preservation of dry biological materials.
   The following sentence has been added at the end of the last paragraph in section 1.8:
   "Although there is some *in vitro* evidence suggesting hydrogen bonding of trehalose with protein, the *in vivo* case is less convincing."
   See errata list for p. 13.

6. Guo et al. (2000) were "inserting" *ots*A and *ots*B genes into cells (that do not produce trehalose) using viral vector as the vehicle and allowing those cells to produce their own trehalose. This can be classified as transgenic (see, "1.10.1. Genetic engineering/transgenic approaches"). I assumed that was not a mistake if I classified Guo *et al's.* (2000) work as genetic engineering.
7. I did not include Eroglu et al. (2000) in this paragraph, because they studied snap freezing not drying. However, Eroglu et al. (2000) was mentioned in sub section 1.10.2.
8. Corrected, only F. Levine wrote the reply in Nat Biotechnol. 2000 May;18(5):473. This mistake had been corrected, see errata list for p. 14 section 1.101
9. I cited the word ("*low*") from their abstract (Eroglu, *et al.*, 2000). In view of the examiner's comments it is appropriate to delete this word. See errata list for p. 15.
10. Delete "Guo et al. (2000) see Errata list for p 15.
11. Repetition of Wakayama and Kusakabe's papers. This thesis was initiated by Wakayama and Yanagimachi's (1998) work on sperm drying. And only their group (Yamagimachi) has repeated and obtained consistent results. The approach taken in my experiment was heavily influenced by their studies. The repetition resulted from a desire to acknowledge their background work appropriately.
12. Heading cha. :d to " Fibroblast preservation" see errata list for p. 34.
13. In this table I try to summarise the general methods which have similarity, with details in paragraph pp. 36-38.
14. See errata list for p. 36.
15. Yes, thank you, I did not see that paper (Bloom *et al.*, 2001) when I wrote my thesis and I like to add their finding into table 1.2.
    See errata list for p. 36.
16. Garcia de Castro, A. and Tunnacliffe, A. (2000) and Chen, *et al.*, (2001) were added.
    See errata list for p. 36.

Chapter 3

7. Basically ICSI and normal fertilisation initiate similar physiological events after the spermatozoon penetrates/placed inside the egg cytoplasm. Hewitson *et al.* (2000) showed similarities between IVF and ICSI in terms of the cytoskeletal dynamics of oocytes after the spermatozoa underwent oolemma binding and fusion. Currently ICSI has become a universal technique and is widely used to overcome problems in male infertility (Ramalho-Santos, *et al.*, 2000). Page 53, para. 1 explains normal fertilisation which correlates with the physiological events after ICSI
   Hewitson, L., Simerly, C., Dominko, T., and Schatten, G. (2000). Cellular and molecular events after in vitro fertilization and intracytoplasmic sperm injection. Theriogenology 53, 95-104.
   Ramalho-Santos, J, Sutovsky, P, Simerly, C, Oko, R, Wessel, G.M, Hewitson, L, Schatten, G. (2000). ICSI choreography: fate of sperm structures after monospermic rhesus ICSI and first cell cycle implications. Hum. Reprod. **15**, 2610-20

8. At that time I was not aware of "water molecule penetration", because I concentrated on the oxidation effect. Clearly this aspect needs to be explored further.
9. There are no detailed descriptions of sperm morphology, however details on the classification of spermatozoa morphology can be read in the WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. Cambridge University Press, Cambridge. WHO (1987).
10. Several attempts were made to repeat the results, however none of them gave a positive results. The question about the dryness and the possibility of oxidation damage during drying, storage or rehydration need to be answered. The limited resources and equipment made the purchase of equipment impossible. However we tried to minimise inconsistencies in each replicate. In further work is needed.
    This chapter will not be omitted or merged with Chapter 4 (see point 22).

Chapter 4

11. Chapter 3 and 4 are two independent chapters. Chapter 4 concentrated on the events that happened inside the cytoplasm following ICSI, to explain the fertilisation failures following ICSI using dried spermatozoa.
12. Figure 4.1 shows that at 1 and 3 hr dried spermatozoon retain their shape, while the fresh spermatozoon underwent syngamy. By 3 hr they had formed an "aster" and the second polar body had been extruded.
13. This experiment used eggs that had been injected with a sperm. To assess the rate of sperm head decondensation groups of oocytes were fixed 0, 15 min, 30 min, 1 hr, 2 hr and 3 hr after ICSI. The eggs were fixed using 3% Glutaraldehyde in 1 M Na-Cacodylate buffer, then stained with 100µg/mL propidium iodide (PI) then stored at 4°C in the dark. This procedure prevented any continuous observation on the same eggs/embryos.
14. The display (unit)on the gauge on our FD machine is in "Torr", **not** "mTorr". The text should therefore read Torr. See errata list for p 83, 126 and 184.

Chapter 5.

15. See answer for point 11.
16. Our Dynavac FD machine gives pressures from 20 Torr to 0.1 Torr. In the experiment the pressure was started at 20 Torr, and reached a maximum of 1 Torr. We never got below 1 Torr.
17. There are several types of electroporation medium. In my experiments, I used high conductivity media including
    DMEM,
    Cytomix (120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4, 25mM HEPES, 2mM EGTA and 5mM $MgCl_2$).
    mHBS (120mM KCl, 10mM NaCl, 20mM HEPES, 10mM ATP).
    The reason was those "high conductivity" media (particularly cytomix and mHBS) are similar to the intracellular compartment) while DMEM is similar to extracellular compartment.
    High conductivity media were not detrimental .
18. See errata list for p. 105.
19. See errata list for p. 113.
20. I don't think Chen *et al.* (2001) stored the cells at low temperatures. Chen *et al.* (2001) looked at the membrane integrity of dried cells. The text..."differ to those of Guo *et al* (2000), Puhlev *et al* (2001) and Chen *et al* (2001) who showed that the presence of trehalose in both intra and extra-cellular protected cells against drying." Has been changed to " differ to those of Puhlev *et al* (2001) and Chen *et al* (2001) who showed that the presence of trehalose in both intra and extra-cellulariy protected cells, or cell membranes, against drying."
    See errata list for p. 129.

Chapter 6

21. Measuring the intracellular trehalose content was a goal, however we were advised by the Department of Chemistry that we could not afford to get equipment. We therefore used propidium iodide as the indicator of membrane permeability as a guide that the membrane were leaky and that trehalose may be present intracellularly.
    The use of **anthrone reaction** may provide an alternative method to measure the intracellular trehalose content.
22. There is a discussion on cryoprotectants in the history of sperm preservation (section 1.15.3, page 28).
23. See paper Greiff, D., Doumas, B.T., Malinin, T.L., and Perry, B.W. (1976). Freeze-drying of solutions of serum albumin containing dimethylsulfoxide. Cryobiology, **13**, 201-205.
    Even though that paper shows both successful and unsuccessful results, when DMSO is not totall, removed it may cause detrimental effects during storage.

Chapter 7.

24. There are several papers published on the use of freeze dried spermatozoa after Wakayama and Yanagimachi (1998) and Kusakabe *et al.* (2000). But most of these papers have been published by Yanagimachi's group. Anther group that has published a similar paper on freeze dried spermatozoa is:
    Keskintepe, *et al.* (2002). Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa. Biol Reprod 2002 Aug;67(2):409-15.
    See response for point 11.
25. The gauge is in "Torr" not "mTorr". See answer for no. 25 and 27 and errata list for p. 83, 126, 184.
26. I agree with the consensus that trehalose alone may not be sufficient, however I am trying to modify the methods/protocol to allow cells to be stored at room temperature or in a dry state.

Finally, thank you and we appreciate for your positive responses and criticisms on my thesis. The experiments to preserve biological materials using fibroblasts as a model are ongoing in our lab.

List of abbreviation and measurement unit.

Abbreviation:

ART: Assisted Reproductive Technology
BSA: bovine serum albumin
CP: cryoprotectant
DMEM: Dullbecco's Modified Eagle Medium
DMSO: di-methyl sulphoxide
EG: ethylene glycol
EP: electroporation
ET: embryo transfer
FBS: fetal bovine serum
HCG or hCG: human chorionic gonadotrophin
ICSI: Intracytoplasmic sperm injection
mHBS: modified Hepes basal solution
PI: propidium iodide
PMSG: pregnant mare's serum gonadotrophin
PN: pronuclear
RT: room temperature
TB: trypan blue
Tg: Glass transition temperature

List of measurement

°C: degree Celcius
μF: micro Farad
μL: micro liter
μM: micrometer
hr: hour
IU: international unit
M: molar
MBar: milli Bar
min: minute
mTorr: milli Torr
N: number
pg.: pico gram
psi: pound per square inch
rpm: rotation per minute
SEM: Standard error of the mean
V: volt

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Chapter 5

List of Publications

Pangestu, M., Shaw, J., Lacham-Kaplan, O., and Trounson, A., 2000. Evaluation of Vacuum Drying as an Alternative to Cryopreservation for Mouse Sperm Storage. Cryobiology 41: 329 (Abstract).

Pangestu, M., Shaw, J., Lacham-Kaplan, O., and Trounson, A., 2000. Evaluation of Evaporative Drying as an Alternative to Cryopreservation for Mouse Sperm Storage. Cryobiology 41: 329 (Abstract).

Pangestu, M., L. Lewin, Shaw, J., Lacham-Kaplan, O., and Trounson, A. 2001. Evaluation of embryo development after ICSI with dried mouse spermatozoa. Theriogenology 55(1): 508 (Abstract).

Abstract

Replacing frozen with dry storage would reduce the cost of storing materials for Assisted reproductive technologies (ART). Significant progress in the last 3 years has made it possible to reliably store functional spermatozoa and viable fibroblasts in a dry state (Wakayama and Yanagimachi 1998; Kusakabe *et al.* 2001). However their dried sperm were not motile and their freeze drying machine was expensive. The first part of this thesis therefore investigated whether sperm could be dried by alternative, less expensive, methods.

Mouse spermatozoa were dried by flushing gas (N_2, 5% O_2 in air or air) over a small drop of sperm (<10µl) within a 0.25 ml straw. No motile spermatozoa were present after rehydration but those dried with a continuous stream of dry nitrogen gas and stored under nitrogen within aluminium foil bags,. did form embryos and could produce live normal offspring following ICSI into mouse oocytes. This result was however difficult to replicate and was therefore not a useful alternative to the established freeze drying protocols. Spermatozoa dried in the presence of oxygen did not generate any cleavage stage embryos after ICSI.

Mouse spermatozoa which were vacuum dried formed embryos after ICSI into mouse oocytes. A small number of embryo transfers were performed but did not give rise to live offspring. These results may be inferior to those obtained by freeze drying because oxygen was present during both drying and/or storage.

The second part of this thesis investigated drying protocols for cells. Mammalian cells and platelets are normally killed by dehydration or by freezing in the absence of a cryoprotectant. However some treatments which introduce the sugar trehalose into the cytoplasm could provide protection from freezing Eroglu *et al.*, (1998), and dehydration Guo *et al* (1998), Puvlev *et al.*, (2001), Wolkers *et al.* (2001). Previous work has utilised complex protocols introduce trehalose into the cells and/or complex drying protocols. This study therefore investigated whether simple procedures for introducing trehalose into cells such as electroporation, or cooling, would allow mouse STO fibroblasts to be frozen or dried. Permeabilization was confirmed by adding propidium iodide and trypan blue, which are normally non permeable dyes, at different times after electroporation.

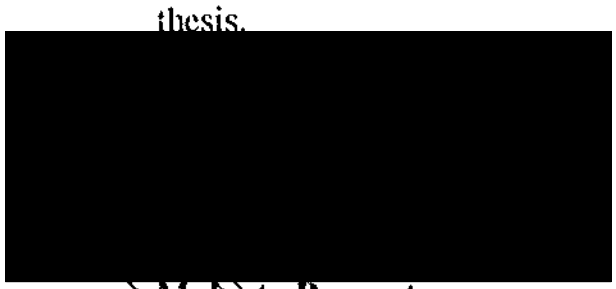
Electroporation settings that allowed PI to cross the membrane, in trehalose containing media allowed cells to be cryopreserved by slow cooling or snap freezing

even in the absence of penetrating cryoprotectants. While viable cells were obtained after partial drying, no viable cells remained in completely dried samples. Cooling in the presence of trehalose or FBS could also provide some protection against snap freezing.

More investigations are required to improve our understanding of how to induce tolerance to anhydrobiotic conditions in non-anhydrobiotic organisms such as mammalian cells. Further experiments may allow simple, inexpensive and effective drying protocols for spermatozoa and somatic cells to be developed.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and affirms that to the best of the candidate's knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.



Mulyoto Pangestu

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Chapter 1. General introduction

1.1. Dry preservation

Preservation by drying is of enormous practical importance and widely used for biological products in clinical medicine, for food stuffs, pharmaceuticals and in agriculture (Crowe and Crowe, 2000). Drying is a particularly valuable method of storage as the storage costs are much lower than frozen storage. Methods are just starting to be developed which allow mammalian cells to maintain their viability following drying storage. Optimisation of these strategies would have implications in many areas of biological sciences and be of considerable interest for assisted reproductive technologies (ART).

Currently cryopreservation and storage in liquid nitrogen provide the only reliable strategy for storage of biological material. The disadvantage of frozen storage is that it requires access to liquid nitrogen and appropriate storage tanks. This can be a problem when work has to be conducted in locations, such as developing countries, where access to liquid nitrogen and appropriate equipment may be limited and/or prohibitively expensive. Dehydration offers an obvious alternative to frozen storage.

Drying strategies, such as freeze-drying that comprises several steps have been used to preserve biological materials. During freeze-drying, first all the water is frozen and then removed by placing the samples in a vacuum as the temperature is gradually raised. Ice crystals that formed during freezing are converted directly to vapor without passing through a liquid phase (sublimates). Freeze-drying has been used to preserve delicate biological and biochemical materials which would otherwise be highly unstable at room temperature including, microorganisms (Conrad *et al.*, 2000; Miyamoto-Shinohara *et al.*, 2000; Perry, 1995; Popescu *et al.*, 1997) bio-molecules (Anchordoguy *et al.*, 2001; Bridges and Taylor, 2001; Cortesi *et al.*, 2000; Franks, 1998; Hinrichs *et al.*, 2001), foodstuffs (Kouassi and Roos, 2000; Laveili *et al.*, 2001; Restani *et al.*, 1997) pharmaceuticals (Castellanos-Serra *et al.*, 1999; Goudey-Perriere and Perriere, 1998; Haese *et al.*, 1999; McEvoy *et al.*, 1998; Metcalf and Codd, 2000; Schwarz and Mehnert, 1997; Ward *et al.*, 1999; Worrall *et al.*, 2000), biological products (Colaco *et al.*, 1995; Ford and Dawson, 1993; Hancock and Dalton, 1999; Hopkinson *et al.*, 1996; Kreilgaard *et al.*, 1998; Mouradian *et al.*, 1985; Page *et al.*, 2000; Surrey and Wharton,

1995) and desiccation tolerant organisms (Anthony *et al.*, 1996; Attfield, 1987; Beattie *et al.*, 1997, Gu *et al.*, 2001, Oetjen, 1999, Pikal, 1999; Precausta, *et al.*,1999, Souzu, 1999). Wakayama and Yanagimachi (1998) and Kusakabe *et al* (2001) showed that freeze-dried spermatozoa stored and/or transported at room temperature could produce viable offspring after being injected into mouse oocytes.

It is only very recently that cells which are not naturally desiccation resistant have been shown to survive drying. Those protocols have been developed to allow cells to be stored in a dry state at room temperature.

1.2. Natural anhydrobiosis

It is well established that a range microorganism, plants and invertebrates can, under certain conditions, be without any visible signs of life and yet not be dead, for when conditions permit, they return to normal active life (Keilin, 1959). This peculiar state of an organism is referred to in the literature as viable lifelessness, suspended animation, latent life or anabiosis.

The strategies that allow metabolic activities to come temporarily to a halt in these organisms are not understood, though many papers deal with the metabolic problems of anhydrobiotic organisms. Cryptobiosis, or the reversible interruption of the life process has immediate practical importance as it explains the tolerance of certain microorganisms, spores, and cysts to desiccation. This tolerance leads to great longevity and an enhanced potential for dissemination. Dry storage would be of considerable medical and agricultural interest (Fry, 1966), if it could be applied to other desiccation sensitive living systems including mammalian cells.

Organisms are called hypobiotic if they naturally or under experimental conditions exhibit a complete gradation between the states of high biological and metabolic activity through more or less deep dormancy, or torpor due to hibernation, aestivation, diapause, or quiescence associated with a lower but still measurable metabolism (hypometabolism), to the ametabolic latent life or cryptobiotic state (summarised in Figure 1, Monterosso, 1934 in Keilin 1959).

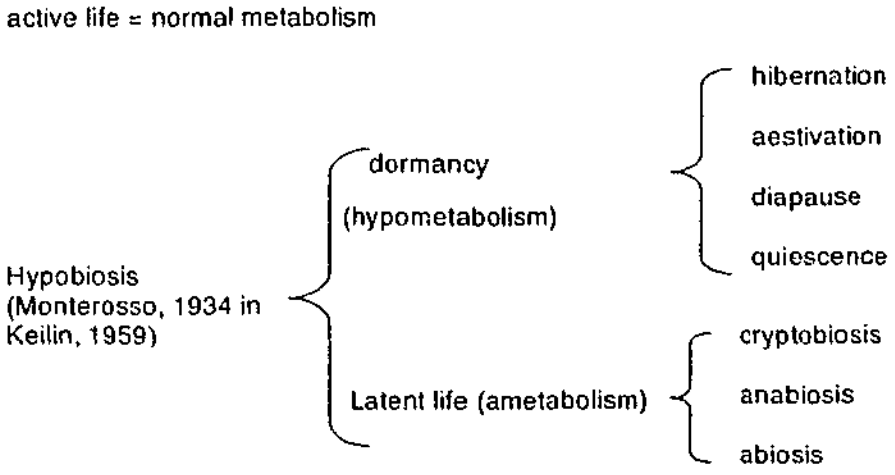


Fig. 1.1. Relationship between different states of organisms

Table 1.1. Summary of the relationship between several hypobiotic states.

	States	Conditions
Hypobiosis	anhydrobiosis	dehydration
	cryobiosis	cooling
	anoxybiosis	lack of oxygen
	osmobiosis	high salt concentration/osmolarity
	combinations all of these	

Source: Keilin (1959).

1.3. Intracellular water in hydrated cells

Water is the most abundant component in living systems. It confers a structural order on membranes and proteins in cells and is involved in every life process. If water is removed from the cells, a series of changes occur: an increase in solute concentration, changes in intracellular pH and ionic strength, the acceleration of destructive reactions, denaturation of proteins and disruption of membranes. These events have the potential to disrupt all synthetic and metabolic pathways and destroy the structural organisation of cells and macromolecules (Bryant and Wolfe, 1992; Roser and Colaco, 1993, Sun and Leopold, 1997). However, numerous organisms are capable of surviving near complete or complete dehydration.

Intracellular water differs from the typical bulk aqueous solution ("bulk water") in that it is filled with macromolecules and membranes. Current controversies on the

physicochemical state of water and solutes in cells revolve around the extent to which macromolecular and membranous surfaces influence the structure of the surrounding water. This effect on water structure, in turn, will determine the solvent properties of intracellular water. Three models have been proposed to describe these properties.

(I) Intracellular water does not differ greatly from bulk water in its solvent properties. This model is the accepted by the majority view (Gary-Bobo and Solomon 1968). It does not deny that the presence of macromolecules perturbs the solution properties of water, but considers these perturbations to be minor.

(II) Intracellular water greatly differs from bulk water including its solvent properties. This model emphasises the similarity of the cytoplasm to hydrated gels, which may selectively extract solutes from a bulk aqueous phase. Ling (1970 and 1973) considered intracellular water to consist of polarised multilayers, a structure imposed by an underlying protein lattice. In this model cell water is abnormal in its solvent properties compared to bulk water.

(III) Intracellular water has heterogeneous solvent properties. Solutes partition between different aqueous solution within the cell, depending on the solvent properties of these various phase and is perturbed by the extensive surface present in cells (Garlid, 1978). Garlid (1978) considered water within membrane in mammalian cells to be limited in extent (30- to 50 Å) representing only a limited fraction (25-30%) of the total intracellular water but it is able to admit solutes. These molecular arrangements, which are thought to be entropically driven, are lost when water is removed.

1.4. Anhydrobiosis or dry biological systems.

Anhydrobiotic organisms can tolerate up to 99.9% of their body water being removed leaving as little as 0.1% water (Keilin, 1959). In mammalian cells this would not be consistent with life as they contain many labile proteins which lose their functional integrity as water is withdrawn (Carpenter, 1994). Accumulating certain sugars help anhydrobiotic organisms to replace the water around polar residues in membrane phospholipids and proteins, thereby maintaining their integrity in the absence of water (Crowe *et al.*, 1996)

Lethal damage to bacteria during drying arises from damage to their cell membranes and denaturation of their proteins. The damage to the cell membranes is believed to arise from phase changes in the membrane phospholipids (Crowe and Crowe, 1988; Leslie, *et al.*, 1995). Such phase changes are especially common in membranes that are rich in phosphatidylethanolamine, such as membranes of *S. typhimurium* (Leslie, *et al.*, 1995). The sugar trehalose is able to prevent such damaging phase changes by becoming associated with phospholipid head groups in place of water molecules (Leslie *et al.*, 1995). Trehalose is also able to stabilise the structure of proteins by replacing the water molecules of the shell of hydration (Crowe and Crowe, 1988; 1989; Leslie *et al.*, 1995)

Anhydrobiotic organisms, include plant seeds, yeast cells, fungal spores, and certain nematodes, rotifers, tardigrades and cysts of some crustacean embryos (for example the brine shrimp, *Artemia*). Under favourable conditions, anhydrobiotic organisms may remain in dry state for decades or even centuries. When water becomes available again, they rapidly swell and resume active life (Keilin, 1959; Leopold, 1986).

Some animals that exhibit anhydrobiosis either require an induction phase, during which important changes occur that appear to be adaptive, or their ability to survive drying is restricted to one phase of the life cycle. Studies on the effects of desiccation (Crowe and Madin, 1975; Womersley, 1989) showed that when tardigrades (*Macrobiotus areolatus*) were dried at 70-97% relative humidity, their water content fell slowly over a period of many hours, after which they could be transferred to dry air. When the animals were then rehydrated, at least 90% recovered. In contrast, when they were initially air-dried at lower relative humidity, they lost water very rapidly and did not survive. During the slow dehydration the animals prepare for more extensive dehydration.

Tardigrades produce a barrel-shaped structure known as a "tun" by contracting on their long axis. Tun formation apparently reduces the subsequent rate of evaporative water loss by folding in a way that protects the intersegmental areas of the cuticle that have a higher permeability to water (Crowe, 1972). Changes in the phase state of cuticular lipids may further decrease the rate of evaporative water loss. Using cryo-scanning microscopy Wright (1989) found that the tun possess extensive deposits of surface lipids that are absent in rapidly dried animals. Further, he found that when these lipids were extracted, the rate of evaporative water loss increased greatly. Womersley

(1989) has however, found a number species of nematodes that do not appear to require slow desiccation and proposed that such nematodes may posses an adaptation to survive drying at all times.

Browne et al. (2002) showed that a sequence of genes encoding for a strongly hydrophilic protein (late embryonic abundant LEA) rather than a sugar was upregulated in response to desiccation stress (they were deprived of water) in the nematode (*Aphelenchus avenae*). They speculated that this protein could act as a hydration buffer, molecular chaperone, or membrane stabiliser.

Yeasts are well known for their ability to survive dehydration, as are many other species of fungus, but they cannot simply be dried out. For instance, bakers' yeast (*Saccharomyces cerevisiae*) must be dried at high relative humidities initially, after which they can be transferred to dry air. Furthermore these yeasts can normally survive drying only in the stationary (non-growth) phase in culture (Panek, 1985).

High intracellular trehalose concentrations improved desiccation tolerance of Gram-Negative bacteria (de Castro, *et al.* 2000b) and in transgenic plants expressing bacterial trehalose synthesis genes (Pilon-Smith, *et al.*, 1998).

Brine shrimp (*Artemia*) produce encysted gastrulae that can be dried immediately after they leave the maternal tissue. The cysts float in salt ponds and can blow up in windrows, where they are harvested commercially. When these dry cysts are placed in water and allowed to develop, they rapidly lose their ability to survive dehydration again (Clegg, 1984; 2001). Eggs that commence development immediately after emergence from the maternal ovisac do not survive dehydration.

Anhydrobiosis is most pronounced in mature seeds and pollen of some but not all plant species. Immature seeds and immature pollen do not survive dehydration (Hoekstra and van Roekel, 1988), nor do seeds survive after they have undergone extensive development following germination (Koster, 1991).

Dehydration tolerance in plants has been investigated using three main approaches: (a) examining tolerant systems, such as seeds and resurrection plants; (b) analysing mutants of model species; and (c) analysing the effects of stress on agriculturally relevant plants. The molecular basis of dehydration tolerance in plants have been reviewed by Ingram and Bartels (1996). "Resurrection plants" ie. *Selaginella lepidophylla*, represent several genera of plant found in xeric (dry) environments

worldwide. The plants can be placed in water, where they rapidly commence photosynthesis, often within minutes, depending on the species. They can be dried and rehydrated repeatedly.

Browne et al. (2002) showed that the same sequence of genes (those encoding for late embryonic abundant LEA protein) were upregulated in response to desiccation stress in plants as in the nematode (*Aphelenchus avenae*). This indicates that these animals and plants use a common protective strategy in response to dehydration, and provides a unifying insight into the mechanism of anhydrobiosis

1.5. Sugars in anhydrobiotes

When drying takes place within a solution the composition of medium in which the cells are dried can influence the outcome (survival). An abundance of colloid will generally help to protect the cells; whereas metabolic end products such as acids and other small organic compounds will have the opposite effect, particularly as the concentration increases with the removal of water. The role of sugars as a stabiliser has been explored. Trehalose is a type of sugar that is naturally produced by most anhydrobiotic organisms (Clegg, 1965; 2001; Clegg and Jackson, 1992; Crowe, *et al.*, 1992; 1997).

Clegg (1965) found that the non-reducing disaccharide, trehalose was the major stored carbohydrate of *Artemia* encysted embryos. However, there was no evidence that trehalose was used for metabolic activity while in that state. Trehalose's ability to limit membrane phase transitions (Wolfe and Bryant, 1999) and to replace water to maintain cellular protein or lipid stability (Crowe *et al.*, 1992, 1993, 1996) was considered very important. The involvement of trehalose in survival after dehydration seems to be found in a large array of phylogenetically dissimilar organisms, ranging from cysts of the brine shrimp *Artemia* to the dessert resurrection plant *Selaginella* and *Archaebacteria* indicating that the ability to accumulate trehalose is an ancient adaptation (Nicolaus, *et al.*, 1988).

1.5.1. Sugars in Animals

The nematode *Aphelenchus avenae* accumulates up to 20% trehalose after slow dehydration (Crowe and Madin, 1975). Crowe *et al.* (1997) found that survival of

animals in the dry state was strictly correlated with their trehalose content, but they also may accumulate glycerol, a potent cryoprotectant, during the induction phase. However, the glycerol was not effective in stabilising biological structures or function during drying (Crowe *et al.*, 1997), while trehalose was remarkably effective (Crowe *et al.*, 1990; 1992). Furthermore Womersley (1989) found that trehalose was nearly always a constituent of anhydrobiotic nematodes, while glycerol may or may not be present, a finding that has been confirmed for tardigrades as well.

1.5.2. Sugars in baker yeast

Bakers' yeast (*S. cerevisiae*) accumulates trehalose in the cytosol, and the presence of this molecule appears to be required for survival of these cells and the absence of water (Beker *et al.*, 1984). In the log growth phase in culture, these cells contain little trehalose and show poor survival after dehydration. In the stationary phase they accumulate trehalose and survival following drying increases. However, cells in the log phase will, if they are subjected to heat shock, rapidly synthesise trehalose and acquire the ability to survive dehydration, again implicating accumulation of trehalose as a key factor in the survival of dry cells (De Hass *et al.*, 1990). Probably the most convincing evidence for a role for trehalose in stabilising dry yeast cells has come from the work of Panek (1985), who isolated mutant strains of yeast that lacked the ability to synthesise trehalose or were able to synthesise trehalose but lacked a membrane transporter for the sugar. Neither strain survives dehydration, but if trehalose is added to the outside of the cells, they survive drying. In the cells that cannot synthesise trehalose, the trehalose transporter must be expressed for the cells to survive. Panek (1985) interpreted these findings to mean that the trehalose must be inside and outside the cell to stabilise them.

It has been proposed that in yeast, trehalose functions primarily as a highly efficient protecting agent to maintain the structural integrity of the cytoplasm under environmental stress conditions (De Virgilio, 1991; De Virgilio, 1994; and Wiemken, 1990) rather than as a storage compound. Felix *et al.* (1999) showed that this stabilising compound can preserve the function of some cytosolic enzymes subjected to high temperatures (Sola-Penna and Meyer-Fernandes, 1998), and that the protection from damage of the enzyme structure and function depends on the nature of the enzyme (Sola-Penna, *et al.*,1997).

1.5.3. Sugars in plants

The involvement of soluble sugars in desiccation tolerance in plants is suggested by studies in which the presence of specific soluble sugars can be correlated with the acquisition of desiccation tolerance (Leprince *et al.*, 1993). Trehalose is the most effective osmoprotectant sugar in terms of minimum concentration required (Crowe *et al.*, 1992), but it is extremely rare in plants, where sucrose together with other sugars appear to fulfil a comparable role. Although sugar accumulation is not the only way in which plants deal with desiccation (Bohnert, *et al.*, 1995), it is considered an important factor in tolerance.

Many seeds and resurrection plants accumulate soluble sugars during the acquisition of desiccation tolerance (Leprince *et al.*, 1993). Various soluble carbohydrates may be present in fully hydrated tissues, but sucrose usually is present in the dried state. Desiccation of *C. plantagineum* leaves are accompanied by conversion of the C8-sugar 2-octulose (90% of the total sugar in hydrated leaves) into sucrose, which then comprises about 40% of the dry weight (Bianchi, *et al.*, 1991).

Plants can maintain total water potential during mild drought by osmotic adjustment. Sugars may serve as a compatible solute permitting such osmotic adjustment, although many other compounds, usually associated with salt stress, such as proline, glycine, betaine, and pinitol are also active (Ishitani, *et al.*, 1995, McCue and Hanson, 1990, Yoshiba *et al.*, 1995). Increasing sucrose synthesis and sucrose-phosphate synthase activity is not only a drought-response of desiccation-tolerant plants such as *C. plantagineum* but also of plants that cannot withstand extreme drying, such as spinach (Quick *et al.*, 1989).

Sugars may protect a plant cells during severe desiccation by promoting glass formation rather than solute crystallisation, as through the presence of sugars a supersaturated liquid is produced with the mechanical properties of a solid (Koster, 1991). Glass formation has been demonstrated in viable maize seeds and has been associated with their viability (Williams and Leopold, 1989). Differential scanning calorimetry has been used to examine the effect of temperature on glass formation by sugar mixtures; only sugar mixtures equivalent in concentration and composition, rather than just concentration to those of desiccation-tolerant embryos are able to form glass at ambient temperatures (Williams and Leopold, 1989). During desiccation, glass would

fill space, thus preventing cellular collapse, and in restricting the molecular diffusion by chemical reactions would permit a stable quiescent state (Williams and Leopold, 1939).

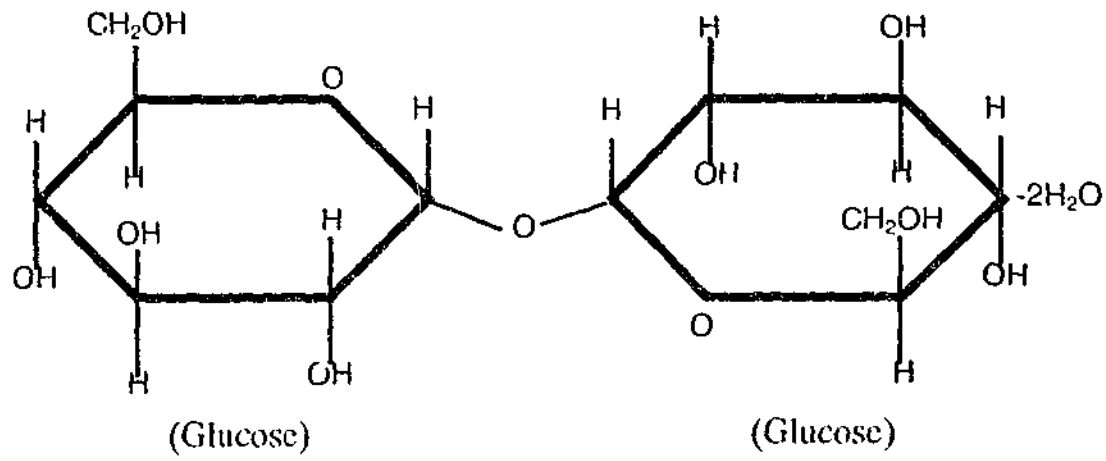


Fig. 1.2. Trehalose structure

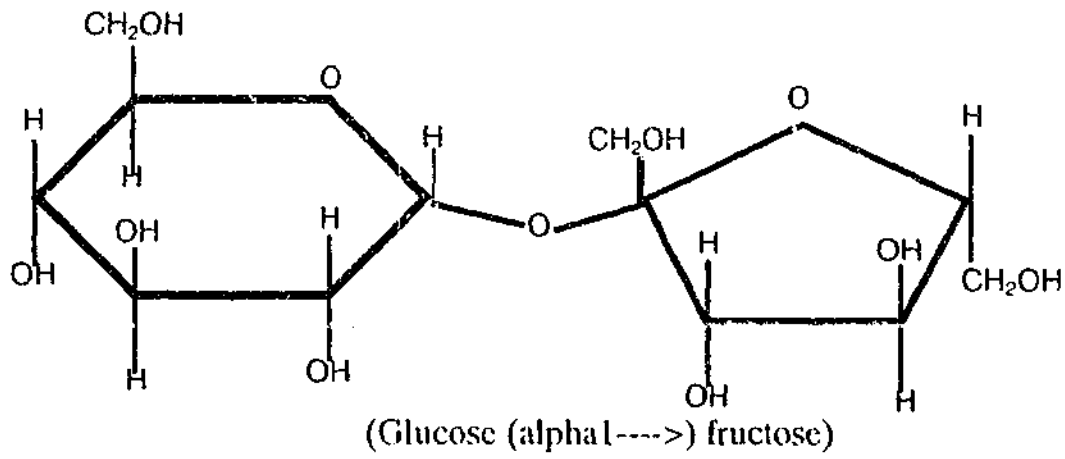


Fig. 1.3. Sucrose structure

1.6. Trehalose in naturally occurring anhydrobiotes

Trehalose (α-D-glucopyranosyl-1, 1-α'-D-glucopyranoside) is a non-reducing disaccharide of glucose which involved in several physiological stress responses, including osmotolerance (DeVirgilio, 1991, 1994, Hottiger, 1994) and anhydrobiosis (Crowe and Madin, 1975). Both of these phenomena are responses to loss of cellular water, either through hypertonicity of the environment or through desiccation. The chief role of trehalose in osmotolerance is as a compatible solute, acting to counterbalance extracellular osmotic pressure (Kempf and Bremer, 1998) and to stabilise biomolecules. In anhydrobiotes trehalose has been proposed as a water replacement molecule (Clegg *et al.*, 1982), with an ability to prevent damaging phase transitions in biomembranes (Crowe *et al.*, 1984) and form glass which will envelop biological molecules in a protective matrix (Franks and Hatley, 1991). Since the early stages of desiccation can involve exposure to a hypertonic environment, trehalose may also play a role as a compatible solute in anhydrobiosis. Concentrations of trehalose in desiccation tolerant plants can reach 35% of the dry weight of the cells (Crowe *et al.*, 1984 and Wiemken, 1990), which in the hydrated cells would correspond to a molar concentration.

1.7. Trehalose in experimentally induced anhydrobiosis

Crowe *et al.* (1983 and 1984) showed that trehalose was superior to the other sugar (sucrose) tested for preserving dry-membranes, and stabilising liposomes during drying. However at a high concentrations, other sugars proved to be equally effective, and the differences between the sugars tend to disappear, making it difficult to establish their relative effectiveness. Nevertheless numerous workers have reported that trehalose seems to have special abilities in preserving biological materials (Beattie, 1997; Hincha, 1989; Hirata, *et al.*, 1992; Israeli, *et al.*, 1993; Mansure, *et al.*, 1994; Roser, 1991, Smorag *et al.*, 1990, Storey *et al.*, 1998). Bacteria freeze-dried in the presence of trehalose show remarkably high survival immediately after freeze-drying (Israeli, *et al.*, 1993), and they also remain viable even after long exposure to moist air. In contrast, when bacteria are freeze-dried with sucrose, they show lower initial survival and when they are exposed to moist air, viability decreases rapidly.

A liposome model showed that exposure to 58% humidity rapidly leaked their contents when they were dried with sucrose, but not when they were dried with trehalose. Measurements of the liposomes showed that they had undergone extensive fusion in the moist air when dried with sucrose, but not with trehalose (Crowe, *et al.*, 1987).

The superior stability of the trehalose system is associated with several properties of the trehalose glass, including low free volume, restricted molecular mobility and the ability to resist phase separation and crystallisation during storage (Sun *et al.*, 1998). Examination of the physical state diagram for trehalose provides an explanation for this effect (Crowe, *et al.*, 1996). The glass transition temperature (Tg) for trehalose is much higher than that for sucrose (Carpenter and Crowe, 1988; Green and Angel, 1989). The addition of a small amount of water to sucrose by adsorption in moist air decreased Tg to below the storage temperature. At the same water content, Tg for trehalose remained above the storage temperature. Degradation does proceed in samples below Tg (+120°C), albeit at slower rate. With trehalose, a sample at +20°C would be nearly 100°C below Tg. By contrast, one dried with sucrose would be only about 45°C below Tg (+65°C). Under these conditions, one would expect the sample dried with sucrose to be degrade more rapidly (Crowe *et al.*, 1996).

Aldous *et al* (1995) have suggested and proved that because the crystalline structure of trehalose is a dihydrate, some of the sugar might, during adsorption of water vapour, be converted to the crystalline dihydrate, thus reducing the likelihood of the remaining trehalose from coming in contact with water keeping Tg for the remaining glassy sugar unexpectedly high.

However the elevated Tg seen in trehalose is not anomalous, as has been claimed. Indeed, trehalose lies at the end of a continuum of sugars that show increasing Tg, although the basis for this effect is not understood (Chen *et al.*, 2000).

1.8. Sugars and water replacement hypothesis

In a pioneering series of studies in the 1950s, Webb found that bacteria could be stabilised during air-drying if the sugar inositol was added (cited in Clegg, 1984). Webb suggested that this sugar might serve a water replacement function around labile macromolecules during drying. Subsequently, other evidence based on stereomodels

provided some indirect evidence that such a mechanism might be found in vivo. However, his important suggestion of water replacement by polar organic compounds (Webb, 1965) remained without much experimental support.

The mechanism of protein stabilisation by sugars is better understood than the situation with the membranes. Infra red spectroscopy indicated that trehalose forms hydrogen bonds between its hydroxyl groups and the polar residues of the proteins (Crowe *et al.*, 1992). Hydrogen bonding between the hydroxyl group of trehalose and the phosphate group of phospholipids can be inferred from comparison changes in the infrared spectrum of the molecules during dehydration.

1.9. Experimental models for stabilising dry cells using trehalose.

Some biological compounds can be stabilised for drying with trehalose (Gribbon, *et al.*, 1996). Trehalose forms an inert glass around the macromolecules, replacing water molecules and preventing oxidation and degradation (Crowe *et al.*, 1993). When *E. coli* is induced to produce significant levels of intracellular trehalose, desiccation tolerance is improved (de Castro *et al.*, 2000b). Furthermore, if such trehalose-induced bacteria are dried in a trehalose solution, such that trehalose is present in both inside and outside the cell, remarkable stability can be obtained with essentially no loss viability after storage at 37°C for 6 weeks (Tunnacliffe *et al.*, 2001).

Crowe *et al* (1983) have shown that, in vitro, drying and rehydration of sarcoplasmic-reticulum usually results in the fusion of vesicles and loss of the ability to transport calcium. However when the sugar trehalose is added at concentrations equivalent to those in desiccation-tolerant organisms, function was preserved. Many other studies show that sugars can protect isolated membranes in vitro (Crowe *et al.*, 1992) and it is suggested that sugars alter the physical properties of dry membranes so that they resemble those of hydrated membranes.

Osmotic stresses cause *E. coli* and *Pseudomonas putida* to produce intracellular trehalose and by drying in trehalose solutions resulted in long-term viability in the dry state (de Castro *et al.*, 2000a)

The growth of an aroA mutant of *Salmonella typhimurium* (SL3261) in minimal medium containing high NaCl concentration (0.5M) resulted in the intracellular

accumulation of 2.2 μmol trehalose/mg total protein. Vacuum drying of these bacteria in the presence of extracellular trehalose allowed recovery of 35% cells following drying. But culture in control medium resulted in the intracellular accumulation of only 0.4 μmol trehalose/mg total protein. Vacuum drying of these bacteria in the presence of extracellular trehalose allowed recovery of only 5% cells following drying (Bullifent *et al.*, 2001).

1.10. Induction of anhydrobiosis in mammalian cells by introducing intracellular trehalose

Mammalian cells are unable to synthesise trehalose, and because trehalose will not cross an intact cell membrane, they cannot easily be loaded with trehalose. Several workers have now introduced trehalose into the cytoplasm of cells that do not normally synthesise trehalose. The tested strategies for trehalose introduction are genetic engineering (Guo *et al.*, 2000), chemical permeabilisation (Wolkers *et al.*, 2001b and Puhlev, 2001) and microinjection (Eroglu *et al.*, 2001). They found that intracellular trehalose was not toxic to mammalian cells and did improve resistance to dehydration.

These works are reviewed in greater detail below.

1.10.1. Genetic engineering/Transgenic approaches

Scientists have sought to improve desiccation tolerance in living organisms by adding genes thought to be important for anhydrobiosis. Human fibroblasts infected with an adenoviral vector transiently expressing the *E. coli otsA* and *otsB* trehalose synthesis genes, accumulated 1-1.5 nmol trehalose per 1 million cells (0.3-0.5 pg/cell), and were reported to have survived complete desiccation for up to 5 days (Guo *et al.*, 2000). These results have been subjected to some debate (Guo, 2000 and de Castro *et al.*, 2000a), since de Castro and Tunnacliffe (2000) found genetically engineered mammalian cells which accumulated up to ~40pg/cell (~80mM) intracellular trehalose had improved resistance to partial dehydration resulting from hypertonic shock, but they are unable to survive complete desiccation.

1.10.2. Membrane cell poration

Evidence that the introduction of low concentrations of intracellular trehalose can greatly improve the survival of mammalian cells during cryopreservation was demonstrated through the use of alpha-hemolysin that was isolated from a genetically engineered mutant of *Staphylococcus aureus* to create pores in the cellular membrane of mammalian cells. The pores were closed by adding Zn (Eroglu *et al.* 2000). The introduction of a low concentration (0.2 M) of trehalose permitted long-term post-thaw survival of more than 80% of 3T3 fibroblasts and 70% of human keratinocytes. These results indicate that poration could introduce trehalose into the cells and that such porated cells could be cryopreserved by using a very simple freezing protocol.

1.10.3. Microinjection

Eroglu *et al* (2001) placed human failed fertilisation oocytes in 0.15 M trehalose in DMEM/F12 with 4 mg/mL BSA to cause shrinkage then injected the oocytes with small amounts of 0.8 M trehalose in 10 mM TRIS buffer (pH 7.4) to generate an intracellular concentration of 0.15 M trehalose to obtain isotonic conditions. The oocytes were then cooled at 1°C/min in medium containing 0.5 M Trehalose in DMEM/F-12. Oocytes with 0.15M intracellula: trehalose concentration survived freezing to -60°C, thawing and culture in DMEM/F-12.

Microinjection worked for the oocytes, but this method is not suitable for the smaller size cells such as fibroblasts.

1.10.4. Incubation in trehalose containing medium.

Guo *et al* (2000), Matsuo (2001), Puhlev *et al* (2001) and Wolkers (*et al*, 2001b) reported that mammalian cells and platelets incubated in trehalose containing medium had some protection against drying. The cells or platelets were incubated in medium containing trehalose for a certain time, then dried with the medium. Human keratinocytes incubated in 0.2 M trehalose for 15 min survived drying for 30 min at room temperature (Matsuo, 2001). Wolkers *et al* (2001b) incubated human platelets in medium containing 0.2 M trehalose for 24 h and showed that those platelets could survive freeze drying. The use of trehalose alone allowed mammalian cells to survive

drying. Glycerol addition can also increase cell viability after drying (Puhlev, *et al.*, 2001).

1.11. Electroporation: Evaluation of alternative permeabilisation strategies.

Several methods of introducing intracellular trehalose have been described in the previous section. The use of toxin (Eroglu *et al.*, 2000), genetic modification (Guo *et al.*, 2000) and microinjection (Eroglu et a., 2001) may result in adverse effects or are difficult to be performed in small sized cells. The methods reviewed above are either complicated or may cause instability in further development such as genetic alteration on genetically engineered cells.

One method to gain access to the cell cytoplasm is electroporation, which transiently creates pores in cell membranes by means of applied electrical fields (Glogauer and McCulloh, 1992).

Electroporation has not previously been used to introduce the sugar trehalose into the cells but it has been used to introduce large-molecular-mass such as, dextrans and proteins as probes of the cytoplasmic compartment in cells or organisms (Baum *et al.*, 1994; Fountain *et al.*, 1988) for direct labelling or manipulation of intracellular molecules and metabolic processes (Lambert *et al.*, 1990; Raptis *et al.*, 1994) and DNA for transgenesis (Klenchin *et al.*, 1991; Sukharev *et al.*, 1992, and Golzio *et al.*, 1998). Proteins of up to 66 kDa can be incorporated at intracellular concentrations of 10-15 mM. Cells electroporated in the presence of RNAse exhibited significant reductions of cellular RNA. Baum *et al.*, (1994) reported that electroporation gave a highly efficient gene transfer (20%-100% of surviving cells) in 19 cell lines, including lymphoid, myeloid, glial, fibroblast and COS cells.

Electroporation offers multiple advantages over the use of gene therapy or pore formation with cholera-toxin, including simplicity, high effectiveness, high specificity, rapid action, availability and usefulness with adherent or non-adherent cells (Baron *et al.*, 2000). Evaluation of cell membrane electropermeabilisation can be done by exposing cells to electric pulses in the presence of propidium-iodide, a fluorescent dye activated by binding to cellular DNA. The fraction of permeabilised cells is then determined using a flow cytometer. This widely established method has only minor

drawbacks: (i) an arbitrary choice of minimum fluorescence intensity for characterisation of permeabilised cells; (ii) the inability to detect cells disintegrated because of intense electropermeabilisation; and (iii) false detection of cellular ghosts devoid of fluorescence because of leakage of DNA caused by electropermeabilisation (Kotnik *et al.*, 2000). The combined use of light and fluorescence microscopy with trypan blue and propidium iodide dyes helps to determine the proportion of cells that are viable and permeable. Unless it is optimised, electroporation can caused cell death or fail to permeabilise the membrane.

The technique of electroporation was therefore chosen to introduce trehalose into cells for the work in this thesis.

1.12. Preservation of biological material for ART

Preservation is an integral part of assisted reproductive technologies (ART). The history of liquid and frozen storage of spermatozoa has been described and reviewed elsewhere (Watson, 1990, Salamon and Maxwell, 1995 a,b). Now research has started to focus on dry storage (Holt, 1997). Wakayama and Yanagimachi (1998) followed by Kusakabe *et al* (2001) showed that freeze dried mouse spermatozoa can fertilise oocytes by intracytoplasmic sperm injection (ICSI) and produce live offspring.

1.13. Preservation of spermatozoa for ART

Semen preservation strategies are of value in artificial insemination (AI), in vitro fertilisation (IVF), and intracytoplasmic sperm injection (ICSI). Sperm preservation allows a sire to fertilise more than one female, independent of distance, or time (Salisbury and Van Demark, 1961). Early attempts sperm preservation allowed spermatozoa to be preserved for several days. Currently sperm preservation allows spermatozoa to be stored in liquid nitrogen for unlimited time without reducing their fertilising ability. The most recent results showed that mouse spermatozoa could be stored in dry state (Wakayama and Yanagimachi, 1998; Kusakabe *et al.*, 2001).

1.14. A brief history on sperm preservation

The history of AI with fresh semen began when an Arabian horse breeder in the early 13th century trapped stallion semen in wool placed in the vagina of a mare and then transferred the wool into the vagina of another mare (Heape, 1898, cited in Maxwell and Salamon, 1993).

Sperm preservation began when Lazzaro Spallanzani (1776 cited in Maxwell and Salamon, 1993) showed that cooling frog, stallion and human semen in snow did not kill all the "spermatic vermicules", but rendered them temporarily immotile and induced "a state of lethargy" from which they could recover when returned to higher temperatures.

Luyet and Hodapp (1938, cited in Mann, 1964) revealed that spermatozoa survive storage in dry ice at –79°C or in liquid air at –192°C, after cooling in the presence of 40% sucrose. Bernstein and Petropavlovsky (1937 cited in Salamon and Maxwell, 1995a) successfully used 9.2% or 1M glycerol solution to preserve mammalian (rabbit, guinea pig, bull, ram, boar and stallion) and avian (fowl and duck) spermatozoa at –21°C. Further work by Polge *et al* (1949) confirmed the important role of glycerol as a cryoprotectant.

Early attempts to freeze-dry spermatozoa had some success (reviewed by Smith, 1961), with some researchers reporting finding motile spermatozoa after freeze-drying (Meryman and Kafig, 1959; and Juschenko, 1959; both were reviewed in Smith, 1961). Jeyendran *et al.* (1981) reported a 29% conception rate with bull semen that had been freeze-dried to 6% of its moisture content after dilution in TEST-Yolk diluent, but no pregnancies with spermatozoa dried down to 2% of their normal moisture content. Most recently Wakayama and Yanagimachi (1998) and Kusakabe *et al.* (2001) reported that freeze dried mouse spermatozoa were not motile but could fertilise mouse oocytes after ICSI into mouse oocytes.

1.14.1. Liquid sperm preservation

During the 19th century, attempts were undertaken to store spermatozoa in various natural and artificial media without reducing the fertility of the semen. Donne (1837, cited in Mann, 1964) investigated the influence of milk, urine, vaginal and

cervical secretions on spermatozoa. In 1856, Koelinker (cited in Mann, 1964) published his observations on spermatozoa of various species in a comprehensive survey, and highlighted the importance of media tonicity. He observed that while spermatozoa were rendered motionless by dilution with water, they remained motile in water containing salt or certain organic substances such as sugars, glycerol and some proteins. Gemmill (1900, cited in Mann, 1964) thought that the life of spermatozoa could be prolonged by extension with beef broth, and he recognised the importance of "artificial nutrition" for spermatozoa survival.

There have been many reports since the turn of the century on diluting media for semen of livestock with much of this work originating in the former Soviet Union (Anderson, 1945). The discovery that egg yolk was a useful additive in increasing the preservation properties of the various media, added further impetus to this work (Phillips, 1939). Storage at 5°C lowered the metabolic rate of spermatozoa, which extended their survival (Salisbury and Van Demark, 1961). Since then, many extenders have been developed for liquid storage of semen.

The basic requirements of a diluting fluid as outlined by Melrose (1962) are to protect spermatozoa against osmotic tension, pH changes with an adequate buffering capacity and low toxicity with a correct balance of electrolytes and non-electrolytes, cations and anions. The diluent should also remain stable after prolonged storage, and contain substrates to support metabolism of the stored sperm without the need of antibiotics. Finally, the diluent should be easily and cheaply prepared and allow clear microscopic observation of spermatozoa after dilution and not to render the cleaning of glassware or semen containers difficult.

Van Demark and Sharma (1957) proposed CO_2 narcosis, Norman *et al.* (1958) suggested lowering the pH and Shannon (1965) proposed N_2 gassing as methods to extend the life of spermatozoa by inhibiting their metabolic activity. Of these nitrogen gassing is the technique that has persisted and is used widely. Storage at 5°C reduces metabolic activity, but not all changes associated with lower temperatures are beneficial to spermatozoa. For example, the activity of the Na^+/K^+ pump decreases with reduced temperatures with the result that the cell is unable to cope with diffusion of ions across the cell membrane at 5°C (Quinn and White, 1967). The consequent increase in the intracellular concentration of Na^+ is detrimental to the survival of the spermatozoa (Makler *et al.*, 1981). It was then postulated that storage at ambient temperatures may

be superior to storage at 5°C, provided that the medium in which the spermatozoa are suspended inhibits those pathways that are detrimental to their survival (Shannon, 1962; 1964; 1965). The temperature of storage is an important consideration. The optimum temperature range is considered to be 18°C to 24°C (Shannon and Curson, 1983). Storage at temperatures above this results in lower fertility (Foote *et al.*, 1960; Bartlett and Van Demark, 1962).

1.14.1.1. Medium

The early media contained egg yolk and phosphate as the buffer (Phillips, 1939). The use of citrate as a buffer enhanced the period of survival of spermatozoa stored at 5°C (Willett and Salisbury, 1942). Citrate then became the salt of choice but homogenised whole milk, fresh or reconstituted skim milk and coconut milk have also been used to preserve the fertility of bovine spermatozoa (Melrose 1962; Norman *et al.*, 1962).

1.14.1.1.1. Egg-Yolk

Historically, semen diluents have included anywhere from 12.5% to 50% egg yolk in the medium (Foote *et al.*, 1960). Egg yolk protects the spermatozoa from the toxic effects of seminal plasma; however, it also provides substrates (aromatic amino acids such as -phenylalanine) for preventing H_2O_2 production by an aromatic amino acid oxidase (AAAO) released from dead cells to the detriment of live spermatozoa (Shannon 1962; 1964). The amount of egg yolk required in diluent to provide protection against seminal plasma toxins is proportional to the dilution (Shannon and Curson, 1972, 1983). Thus, when semen is highly diluted, and the seminal plasma concentration is consequently reduced, there would be some advantage in reducing the egg yolk concentration. This was borne out in a large fertility trial, where a decrease in egg yolk concentration from 20% to 5% had no detrimental effect on fertility. Further decreasing the egg yolk concentration affected fertility of some sires, suggesting that the level of egg yolk was insufficient in some cases to be completely protective.

1.14.1.1.2. Coconut milk

Coconut milk extenders have been equivalent to skim milk-glycerol, CUE or CAPROGEN® in maintaining motility and survival of spermatozoa (Norman and Rao, 1972; Foote, 1978). This extender is quite simple and contains 15% coconut milk boiled for 10 min, 2.2% sodium citrate, antibiotics and 5% egg yolk. The presence of

egg yolk was essential to provide a lipid component to the medium. In some cases, the medium has been supplemented with 0.1% calcium carbonate (Norman *et al.*, 1958). Report on the use of coconut extract and coconut milk on ram semen showed dramatic maintenance of motility over a 48 h period of 30°C but no fertility trials have been reported (Chairussyuhur *et al.*, 1993). There have been no recent reports on significant advances in this area.

1.14.1.1.3. Reduced temperatures.

Salamon and Maxwell (2000) in their review described work by Milovanov in 1940 and 1951 on the problems of storage of ram spermatozoa in a chilled state. In the 1940s, British investigators Chang and Walton and some Soviet workers claimed that 10 to 15°C was the "optimum temperature for liquid storage. Most Soviet investigators reported that survival of ram and bull spermatozoa was better with storage at 0 to5°C.

When semen was stored at a low temperature, care had to be taken to not subject the spermatozoa to cold shock (irreversible changes in sperm cells associated with cooling). The detrimental effects of cold shock were discovered in 1931 and Milovanov (1951) overcame cold shock either by gradual cooling of semen from room temperature, or by addition of lipid to the diluent. The value of lipids from various sources, including egg yolk, testicles, corpus luteum, brain and soy beans in the protection of spermatozoa against cold shock was initially reported by Milovanov and Selivanova in 1932 (cited by Milovanov, 1962). Lardy and Phillips (1939) and Phillips and Lardy (1940) (both are cited in Salamon and Maxwell, 2000) applied these observations in the development of the egg yolk–phosphate diluent for the preservation of bull semen. Another generally accepted storage medium, the egg yolk–citrate diluent, was elaborated later by Salisbury *et al.* (1941) and Willett and Salisbury (1942).

The main methods of storage of semen in a liquid state are storage at reduced 0–5° or 10–15°C and at ambient temperatures (Salamon and Maxwell, 2000). Liquid storage of semen, particularly the effect of lowered temperatures on the physiology of spermatozoa, has received much attention (Mann, 1964, and Maxwell and Salamon, 1993), and can be used in combination with other methods have also been developed to reduce metabolic activity, by reducing spermatozoa motility. Reducing metabolic activity is the most important key to the preservation spermatozoa in the liquid state, because lowered metabolic activity will prolong the fertility and life of the spermatozoa.

1.14.1.2. Carbon dioxide and pH

The first experiments on the inhibitory effect of carbon dioxide on the motility of spermatozoa of different species were performed in 1924 by Krshyshkovsky and Pavlov (cited by Milovanov, 1962).

Carbon dioxide was found to be a very effective inhibitor of spermatozoa motility. Early experiments showed that spermatozoa motility was reversibly inhibited when exposed to CO_2 for short periods, but prolonged exposure to this gas proved toxic to spermatozoa. Van Demark and Sharma (1957) proposed CO_2 narcosis as an effective means of maintaining viability and fertilising ability of bovine spermatozoa for 6 to 7 days at room temperature. The first diluent designed on the basis of CO_2 immobilisation of spermatozoa was the IVT (Illinois Variable Temperature) diluent (Van Demark *et al.*, 1957). The IVT diluent contained a mixture of salts, sugar and antibacterial agents, and was gassed with CO_2 until the pH decreased to 6.35. The final mixture contained 10% egg yolk. Bovine semen extended with this diluent and stored at room temperature retained fertility for over 3 days (Bartlett and Van Demark, 1962). Several diluents were then formulated using this concept to optimise spermatozoa survival and to extend the shelf-life of diluted semen.

Optimising the concentration of bicarbonate (0.1 M), and glucose (0.067 M) in the IVT diluent maintained spermatozoa motility at high levels (>45%) for over 90 days at 5°C. While motility was maintained for extended periods, fertility decreased sharply after 2 days of storage at 5°C, (Bartlett and Van Demark, 1962). This inconsistency between fertility and motility was evident also in other studies where CO_2 narcosis was used to maintain motility and fertility of spermatozoa for extended periods (Dunn and Foote, 1958; McFee *et al.*, 1958; Foote *et al.*, 1960).

An early observation in 1924 by Krshyshkovsky and Pavlov, cited by Norman *et al.* (1958), showed that spermatozoa were immobilised when placed in sealed tubes at room temperature, but subsequent exposure to air produced a resumption of activity. Inhibition of spermatozoa motility was due to the decrease in pH by the accumulation of lactic acid in the medium. Further studies by Norman *et al.* (1958) confirmed the finding and suggested the decrease of pH as an effective method to inhibit metabolic activity of spermatozoa. In this study, conclusive evidence was obtained that lowering the pH substantially reduced metabolic activity measured by O_2 consumption, lactate

production, and motility of live spermatozoa. This effect was reversible, as activity resumed when the old diluting medium was replaced after 150 h incubation with fresh medium at neutral pH. Altering the pH from 5.76 to 7.45, could alter motility of spermatozoa from relative immobility to rapid movement (Norman *et al.*, 1958). The effect of acidic conditions on spermatozoa metabolism was identified by Koelliker (1856) and Guenther (1907) (both were cited by Norman *et al.* 1958), who concluded that activity of the spermatozoa is a function of hydrogen ion concentration. A decrease in pH down to 5.5 was well tolerated by spermatozoa, and the effect could be reversed by alkaline conditions, but a pH below 5.5 was spermicidal and caused irreversible enzyme denaturation.

Another diluent, also developed for storage of bull semen at ambient temperature, the Cornell University Extender (CUE) is a self-gassing medium in which the carbon dioxide is derived from the reaction of citric acid and sodium bicarbonate (Foote *et al.*, 1958). Both IVT and CUE have been modified for use with ram semen; they were saturated with carbon dioxide by gassing to pH 6.3 for about 10 min before use, or were "self-carbonating" as a result of their carbonate or bicarbonate and acid content. As an alternative to gassing, diluent containing volatile organic oxalic and acetic acids were also examined Habibulin (1963, cited in Salamon and Maxwell, 2000).

The main difference between the IVT diluent and the CUE was that CUE was self carbonating and relied on the action of citric acid on bicarbonate to release CO2 with no major effect on the pH of the medium. The CUE was a substantial improvement on the IVT diluent in this regard. Numerous field trials confirmed that CUE was the most successful liquid semen diluent utilising CO_2 (Shannon, 1964; Foote, 1978).

1.14.1.3. Nitrogen gassing

Shannon (1964 and 1965) developed the CAPROGEN® diluent for bovine semen, which replaced the self-carbonating concept with a method to reduce the dissolved O_2 levels in the medium with N_2 gas. This had no effect on the pH, but substantially reduced the metabolic activity of spermatozoa. An additional effect of N_2 gassing was that it nullified the adverse effect of high dilution on spermatozoa survival.

Nitrogen saturation halted the decline in incubation life of stored spermatozoa. This effect was more noticeable when spermatozoa were stored in a dilute form (12.5 million/mL), compared to storage in a concentrated state (over 200 million/mL). At the same time, inclusion of volatile saturated fatty acids (Shannon, 1962) and catalase (Shannon 1972; 1973. Macmillan *et al.*, 1972) significantly improved the length of time that diluted bovine semen could be stored. The diluent was originally developed for use at 5°C, but proved superior for semen stored at temperatures between 15°C and 27°C (Shannon and Curson, 1982, 1983). The conception rates with semen stored in CAPROGEN® were 0.9% better at the elevated temperatures on the day of collection and 2.3% higher on the following day compared to semen stored at 5°C (Shannon, 1971).

1.14.2. Prospects for liquid stored semen

Liquid semen technology would make major gains if the decline in fertility upon storage at ambient temperature could be halted or reduced. As outlined above the physiological processes that contribute to aging of spermatozoa upon storage in vitro are partially understood. Reactive oxygen species, which are more likely to be generated in an ambient temperature system have to be contained in the media in which the spermatozoa are suspended. Intracellular activity could also contribute to the production of these free radicals and the slowing down of respiratory activity could be beneficial.

The use of liquid stored semen allows high utilisation of individual sires. This is possible because of the low spermatozoa numbers required for good fertility in combination with the extended shelf-life of up to 4 days. This allows the effects of semen ageing to be partially overcome. The high efficiency and utilisation of liquid stored semen has made non-frozen AI very popular in places such as New Zealand (Vishwanath and Shannon; 2000).

While it may be possible to further improve liquid semen storage other storage protocols such as cryopreservation and drying are potentially valuable.

1.15. Sperm cryopreservation

The principles and practice of semen cryopreservation have been reviewed elsewhere (Bwanga, 1991; Hammerstedt and Graham, 1992; Hammerstedt *et al.*, 1990; Hofmo and Almlid, 1991; Holt, 2000, Salamon and Maxwell, 1995a,b; Watson, 1979, 1990, 1995; Wolf and Patton, 1989).

Although cryopreserved semen is routinely used for dairy cattle breeding, critical examination reveals that the proportion of fully functional spermatozoa in frozen thawed semen is dramatically reduced. Shannon (1968) showed that 10 times the number of cryopreserved spermatozoa were required to obtain a comparable pregnancy outcome to fresh spermatozoa. Vishwanath *et al.* (1996) showed that 10 to 15 million bull spermatozoa are required per insemination straw using frozen thawed spermatozoa compared to 1-1.5 million spermatozoa required for AI using fresh spermatozoa. Leibo and Bradley (2000) showed that almost 50% of mammalian spermatozoa were dead after freezing and thawing. This fertility reduction is acceptable for frozen bull sperm used in AI.

1.15.1. Basic principles of semen cryopreservation and cryoinjury

When cells are frozen, they are subjected to stresses resulting from the water-solute interactions that arise during ice crystallisation. Exposure of cells to the hyperosmotic, yet unfrozen solution between the ice crystals causes withdrawal of intracellular water resulting in cell shrinkage and possible influx of ions (Mazur, 1984). Thawing involves a reversal of these effects, and the consequent inward water flux may cause cell membrane disruption. If cooling is too slow damage results from the prolonged exposure of cells to hyperosmotic conditions or ''solution effects''; if it is too rapid there is intracellular ice crystallisation (Mazur, 1977). Cytoplasmic disruption through intracellular ice formation may be further compounded by the growth of ice crystals (recrystallisation) during thawing. It has been suggested that a freezing rates exist where the damaging effects of these two different sources of cryoinjury can be minimised (Watson, 1990).

Early cryomicroscopic observations showed that slowly frozen erythrocytes became sequestered in narrow channels between plates of ice (Nei, 1970). Subsequent observations with fluorescence cryomicroscopy have shown that as spermatozoa can

also lie across the channels, single spermatozoon could be exposed to high and low salt concentrations simultaneously (Holt, 1997).

The incorporation of micro-architectural considerations into a hypotheses of cryoinjury may help to explain why some spermatozoa survive the cryopreservation process intact, while others suffer acrosomal disruption, plasma membrane damage and loss of motility. If cell survival were dependent upon spatial orientation in relation to regions of heterogeneously distributed solute concentrations then different types of microstructure would alter the random likelihood of achieving optimal orientations. Hypotheses of spermatozoa cryoinjury must account for the known thermodynamic and structural properties of the spermatozoa plasma membrane. It is well known that the spermatozoa plasma membrane contains an unusual array of lipids (Lin *et al.*, 1993; Parks *et al.*, 1987) and that the plasma membrane is organised into different domains (Friend, 1984, Holt and North, 1984). The phospholipids typically adopt unusual configurations, with a high proportion of plasmalogens that contain ether-linked fatty acids instead of the more usual ester linkages. Phospholipids account for 65–70% of the total and a large proportion of these contain a docosahexaenoic acid side chain, which may confer membrane fluidity and instability. Possibly to counteract these destabilising effects, spermatozoa plasma membranes contain variable amounts of sterols. The spermatozoa plasma membrane lipids respond to temperature changes by alterations in their physical phase state. Although regions of fluid and gel phase lipids coexist at physiological temperatures, reductions of temperature favour fluid to gel transitions; the presence of sterols is thought to inhibit these phase changes. As spermatozoa are not adapted to undergo the temperature changes involved in cryopreservation, they cannot modify their lipid content to suit the environmental conditions. The consequence is a phase transition in the plasma membrane.

Within the temperature range of 17–36°C, spermatozoa undergo a typical membrane lipid phase transition (Drobnis *et al.*, 1993, Holt and North, 1984; 1985, Parks and Lynch, 1992). Their changes are species dependent, which could go some way towards explaining the variations in cryopreservation sensitivity seen in spermatozoa from different species. It is also likely that during a typical freeze–thaw cycle, the spermatozoa membranes must undergo phase transitions during both cooling and rewarming. Damage to the spermatozoa membrane and also possibly specific

protein channels lead to membrane leakiness that increases membrane permeability after cooling (Robertson and Watson, 1986).

Spermatozoa membrane transitions occurred when spermatozoa were cooled to each of a series of low temperatures 5°C, -10°C or -20°C and then rewarmed to 30°C (Drobnis *et al.*, 1993; Holt *et al.*, 1992). They found that the membranes became physically damaged and more permeable or leaky, and showed that warming can also alter the membrane composition.

Lipid phase transition effects cause non-linear kinetic responses in some enzymes, including some of the membrane ATPases whose activity depends upon the physical state of annular lipids (Kimelberg, 1977). This may explain the loss of control of intracellular calcium concentration which is evident at temperatures below about 17°C (Bailey *et al.*, 1994; Robertson and Watson, 1986). The addition of ethylenediaminetetra-acetic acid (EDTA) and citrate in some semen diluents would chelate calcium and diminish the concentration gradient across the spermatozoa plasma membrane. Intracellular calcium concentrations (0.1 μM) are four orders of magnitude lower than those in the external milieu. EDTA chelates other metallic ions and might also act by inhibiting lipid peroxidation. Cytoskeletal elements are also temperature-sensitive and cooling results in depolymerisation of actin filaments (Saunders and Parks, 1999).

Addition and removal of cryoprotectant causes an osmotic stress on spermatozoa plasma membranes, but it depends on cryoprotectant permeability (Gao *et al.*, 1993). Osmotic stress can be reduced by stepwise addition and dilution of the cryoprotectant. Some cryoprotectants are toxic to spermatozoa, even though they may work for other types of cells (Storey, *et al.*, 1998).

Overall the most important principle of cryopreservation is to reduce damage caused by intracellular ice formation. This can be achieved by dehydrating the cell before or during cooling.

1.15.2. Freezing medium

The basic components of the freezing medium for semen are essentially the same as those used for ambient temperature storage. The minimal requirements are: ionic or non-ionic substances to maintain the osmolarity and to buffer the medium.

Other commonly used ingredients include a source of lipoprotein or a high molecular weight material, such as egg yolk or milk to prevent cold shock; cryoprotectants (glycerol, propanediol or DMSO), an energy source (glucose or fructose), sugars (disaccharide and polysaccharides) and, other additives such as enzymes and antibiotics. Among those components, cryoprotectants are usually the most important element that are required to maintain spermatozoa viability after freezing and thawing.

1.15.3. Cryoprotectants

Cryoprotectants help to protect spermatozoa from damage caused by freezing and warming. Permeable cryoprotectants such as glycerol, together with substances such as methanol, ethylene glycol, 1,2-propanediol, butanediol, acetamide and DMSO permeate into the cellular cytoplasm. Most semen preservation protocols still favour glycerol in the cryoprotective media, following the example set by Polge *et al.* (1949), however some other cryoprotectants have been explored (Jeyendran and Graham, 1980; Jones, 1973; Molinia *et al.*, 1994, Storey, 1998; Songsasen and Leibo, 1997).

1.15.3.1. Glycerol

Glycerol permeates the cellular cytoplasm. Lovelock and Polge (1954) proposed that the protective effects of glycerol were mediated by its colligative properties; depression of freezing point and the consequent lowering of electrolyte concentrations in the unfrozen fraction at any given temperature as these would help to counter the harmful ''solution effects'' imposed during the freezing process. Unfortunately, it is also evident that glycerol is somewhat cytotoxic to spermatozoa. Species differences in ability to withstand glycerol exposure therefore interact in a complex fashion with the freezing rates used, and the degree of cryoprotection conferred (Holt, 2000).

Hammerstedt and Graham (1992) reviewed the actions of glycerol on poultry spermatozoa. While exposure to glycerol abolishes the fertilising ability, its removal restores fertility. They pointed out that since glycerol reaches the interior of the cell it probably affects cytoplasmic viscosity, thereby changing rates of all diffusion limited processes. Previous evidence that cytoplasmic viscosity differs between species Hammerstedt *et al.* (1978) suggests that glycerol could have species-specific effects on spermatozoa.

Hammerstedt and Graham (1992) suggested that exposure of cells to 0.5M glycerol in cryoprotective media would yield an intramembranous concentration of about 1 mM. This might contribute to the alteration of cell membrane properties by inducing changes in lipid packing structure and hence the stability and water permeability of the cell membrane would be altered. Membrane fusogenicity and the responses of signal transduction pathways could also be affected by such changes, thus contributing to the possibility that post-thaw spermatozoa longevity is reduced through accelerated capacitation (Watson, 1995).

Glycerol itself is known to be metabolised by ram, bull, boar and goat spermatozoa (Aalbers *et al.*, 1961; Jones *et al.*, 1992) the metabolic pathway differs from that operating in tissues such as the liver where glycerol is phosphorylated by a kinase, being recruited instead by an NADP-dependent dehydrogenase (Jones *et al.*,1992). Hammerstedt *et al.* (1990) argued in some detail that in its capacity as a substrate, glycerol would alter the bioenergetic status of spermatozoa, perhaps interfering with the balance between ATP synthesis and utilisation. If an ATP deficit occurred, for example during cooling, metabolic control over ion-dependent cellular processes might be compromised, leading to inappropriate activation of phospholipases and proteases and irreversible cell damage. These authors did not believe that a mismatch between ATP synthesis and utilisation occurred in bull or ram spermatozoa, but considered that the concept may be valid for other species.

Recent studies of marsupial spermatozoa have indicated that they have an unusually high tolerance of glycerol (Johnston *et al.*, 1993; Molinia and Rodger, 1996; Rodger *et al.*, 1991; Taggart *et al.*, 1996). Indeed, it seems that unless the glycerol concentration exceeds ~10%, and approaches 20%, there is little recovery of motility although the fertility of these spermatozoa has yet to be investigated. In contrast to these requirements for high glycerol concentrations, boar spermatozoa suffer loss of fertility if the concentration of glycerol exceeds 3% (Johnson, 1985) an effect associated with the increase in acrosomal damage. Mouse spermatozoa, whose preservation has also been investigated recently seem unable to withstand more than about 1.75% glycerol (Penfold and Moore, 1993, Tada *et al.*, 1990). Some investigators have avoided glycerol completely for mouse spermatozoa, preferring instead a diluent based upon the non-permeating sugar raffinose (Nakagata and Takeshima, 1993). Despite the

possible perturbing actions of glycerol discussed above, there is at present no plausible explanation for these interspecific differences in cryoprotectant sensitivity.

While comparative studies of semen cryopreservation are likely to be valuable in providing explanations for the observed variations in cryosensitivity, there is some indication that between-strain studies of inbred mice might also be useful. Nakagata and Takeshima (1993) collected and cryopreserved spermatozoa from caudae epididymides of eight different strains of mice and then used them for in vitro fertilisation assays. Post-thaw motility ranged from 23% to 62%, and fertility in vitro ranged from 25.5% to 88.9%. The strain mostly used for transgenic work, C57BL/6N, consistently showed the worst post-thaw motility and fertility rates. Although it is premature to attribute these differences to biochemical properties of the cell, rather than to simpler explanations such as spermatozoa head shape or flagellar efficiency, the intriguing possibility should be considered that genetically determined differences in membrane properties are involved. The gross differences between species are undoubtedly under genetic control, but the subtle use of a within-species experimental model, where membrane organisation and function is likely to be consistent, may permit more detailed investigations of for example membrane permeability and ion transport, to be performed. Willoughby *et al.* (1996) included comparisons between two mouse strains outbred ICR and inbred B6C3F1 in their determinations of mouse spermatozoa membrane properties, and failed to observe statistically significant inter-strain differences in the recovery of spermatozoa motility after exposure to a range of an-isosmotic conditions. Despite this formal result, their data seems to show that spermatozoa from the inbred strain had lower tolerance to extremes of osmolarity (Willoughby *et al.*, 1996). A significant difference between strains was also observed in the maintenance and recovery of mitochondrial integrity after exposure to the same range of an-isosmotic conditions. It would be interesting to confirm these results, identify the source of such variability if it really exists, and then correlate such differences across, rather than within, species.

1.15.3.2. Other cryoprotectants

Besides glycerol and the other penetrating cryoprotectants, non permeable compounds including sugars such as raffinose and lactose, polymers such as polyvinyl pyrollidone (PVP) and the amphipathic compounds glycine betaine, glutamine and

proline have been identified as potentially cryoprotective. Raffinose has been used, with and without glycerol, for the preservation of mouse spermatozoa. A mixture of 11% lactose with glycerol has been found useful in combination with pellet freezing methods, where it has been used for carnivore e.g. ferret, (Howard *et al.*, 1991) Giant panda (Moore *et al.*, 1984) as well as for ram and boar spermatozoa (Salamon and Lightfoot, 1969; Wilmut and Polge, 1977). Sugars are thought to act by increasing the percentage of unfrozen water at any given temperature or reducing the concentration of salts in the unfrozen aqueous solution. Glycine betaine, proline and trehalose are thought to interact directly with membrane lipids and proteins, altering their phase transition behaviour and hydration state (Rudolph, *et al.*, 1986). Experimentally, however, these amphiphatic substances have only proved effective in the presence of glycerol and egg yolk when tested with ram (Sanchez-Partida *et al.*, 1992) and stallion spermatozoa (Koskinen *et al.*, 1989).

1.15.3.3. Egg Yolk

Egg yolk is routinely included in cryopreservation protocols for semen from domestic animals and many exotic species. Egg yolk is regarded as protecting against cold-shock, a lipid-phase transition effect (Drobnis *et al.*, 1993). Watson (1976) showed that the active component of egg yolk is a low-density lipoprotein, but direct evidence for its mode of action has remained elusive. Evidence from cryomicroscopic studies of ram spermatozoa showed that egg yolk protected against membrane damage and loss of motility induced below the extracellular freezing point at -20°C (Holt *et al.*, 1992). In that study, the onset of membrane damage during thawing was detected by the loss of intracellular fluorescein where, in contrast to unprotected cells where fluorescein was lost soon after thawing, egg yolk inhibited fluorescein loss until the cells were rewarmed above +20°C. In an earlier cryomicroscopic study of cold-shock effects in ram spermatozoa, it was evident that egg yolk prevented the spermatozoa tail from bending into a rigid ''bow-like'' configuration during cooling (Holt and North, 1986). Furthermore, egg yolk abolished the tendency for these tails to undergo sudden, irreversible, midpiece bending through 180° when the temperature was lowered to about 12–14°C. As the sudden bending phenomenon could not be induced by detergent-mediated membrane permeabilisation, it was suggested that a localised membrane lesion permitted influx of ions that activated the axonemal mechanism in a highly specific region. The direct modulation of spermatozoa plasma membrane lipid phase

transition behaviour by interaction with egg yolk is an attractive idea, but one which has little evidence in its favour.

1.15.3.4. Other components

In addition to the choice of cryoprotectant and various potential additives, semen must be diluted in an aqueous medium. Some commonly used formulations, especially those with high sugar content, do not contain a pH buffer even though components such as egg yolk can affect the solution pH. Many media include sodium citrate, Tris (tris hydroxymethyl aminomethane) or zwitterionic buffers such as TES (N-tris hydroxymethyl methyl-2-aminoethane sulphonic acid). Tris titrated with TES (TEST) media have proved a particularly successful choice for wild species owing to its wide applicability, especially as background data on spermatozoa responses to diluent is frequently non-existent. BF5, a widely used freezing diluent for routine boar semen freezing (Pursel and Johnson, 1975) contains the TEST combination (Graham *et al.*, 1972). The comparative merits of buffer systems were discussed in some detail by Watson (1990) and Salamon and Maxwell (1995a,b).

1.15.4. Frozen semen packaging

The current methods for semen packaging are largely based on the French mini-straw, the 0.25-mL or 0.5-mL pailette (Cassou, 1964). The straws have been in use for many years and have generally worked well for packaging frozen and liquid semen in many countries (Chupin and Schuh, 1993). The straw allows essential details of the sire to be recorded and this has become mandatory for semen traded internationally. The packaging method for liquid semen in New Zealand is the Minitub straw sealed at both ends with glass beads Minitub, Germany (Vishwanath and Shannon, 2000)

The other method is by freezing of semen in pellets, that was first described by Japanese workers (Nagase and Graham, 1964) and is still used in some countries for storing semen from young sires and for local use. This procedure is not widely practiced because details of the sire cannot be recorded on a pellet. However, pellet storage remains the best method for ram semen preservation (Salamon and Maxwell, 2000).

1.16. Attempts to preserve spermatozoa in dry state

Early attempts to freeze-dry spermatozoa had some success (reviewed by Smith, 1961), with some researchers reporting finding motile spermatozoa after freeze-drying (Juschenko, 1959 and Meryman and Kafig, 1959; both were reviewed in Smith, 1961). Jeyendran *et al.* (1981) reported a 29% conception rate with bull semen that had been freeze-dried to 6% of its moisture content after dilution in TEST-Yolk diluent, but no pregnancies when spermatozoa were dried down to 2% of their normal moisture content. Most recently Wakayama and Yanagimachi (1998) and Kusakabe *et al.* (2001) reported that freeze dried mouse spermatozoa were not motile but could fertilise mouse oocytes after ICSI into mouse oocytes.

Wakayama and Yanagimachi (1998) freeze dried mouse spermatozoa in CZB (Chatot-Ziomek-Bavister) medium without any excipient. The results was improved by Kusakabe *et al* (2001) by using medium containing 50mM EGTA, which protected the sperm chromatin integrity against damage during the freeze drying.

1.17. The use of somatic cells for ART

Somatic cell nuclear transfer is a revolution in ART. Somatic cells are being exploited for uses in agriculture, medicine and conservation of endangered animals, but may also be value of cell based therapies, xenotransplantation, and disease (Wilmut *et al.*, 1998). This technology makes it possible to generate normal or transgenic live young from somatic cells (embryos or adult origin) either by creating chimeras with whole cells or by nuclear transfer or using somatic cells as an alternative source of DNA for gametes (Tsai, *et al.*, 2000; Lacham-Kaplan, 2002).

Nuclear transplantation of embryonic or somatic cells into enucleated oocytes have allowed successful embryo reconstitution and the birth of live young (Campbell *et al.*, 1993; Czolowska *et al.*, 1984; Stice and Robl, 1988; Willadsen, 1986; Wilmut et el., 1997). The insertion of a donor nucleus or whole cells into enucleated oocytes (Campbell *et al.* 1993, 1996) have been applied in sheep (Willadsen, 1986), in cattle (Prather, *et al.*, 1987), and in pig (Prather, *et al.*, 1989).

Groups of genetically identical animals could be used in research to control genetic variation and to allow transfer of cells between individuals. Offspring derived

from cells from the same donor are genetically identical allow us to separate genetic and environmental effects or produce of larger numbers of identical offspring.

In medicine, these techniques will enhance existing transgenic programs for the production of proteins needed to treat disease and the supply of organs such as hearts, livers and kidneys from pigs (Wilmut *et al.*, 1998). Cloning is also likely to be important for conservation of endangered animal (Wells *et al.*, 1998).

These technologies will ultimately require storage and transport of a large number and varieties of somatic cell lines.

1.18. Fibroblast

Fibroblasts are somatic cells representative of a structural class of cells that exist in distinct functional forms (races) in different tissues. Fibroblasts or connective tissue cells have been extensively used for a wide range of cellular and molecular studies. They often have an oval nucleus with two or more nucleoli. Active fibroblasts are spindle shaped (fusiform) and contain well-developed rough endoplasmic reticulum and many Golgi complexes. Synthetically active fibroblasts produce procollagen and other components of the extracellular matrix. Quiescent fibroblasts are small, flattened cells containing little rough endoplasmic reticulum. Although synthetically inactive, they may revert to the active state if stimulated (e.g. during wound healing).

Fibroblasts were successfully cultured before other cell types. Since the late 1800s, a variety of growth media were considered for in vitro culture of mammalian cells, tissues, and organs; ultimately, serum was found to be a valuable source of undefined factors that were required to support cell growth (Eagle, 1955). Fibroblast cells have been the most studied mammalian cell type over the past 40 years, and much of their value is due to their well-characterised ability to propagate in culture. They are durable and amenable to a wide variety of manipulations ranging from studies employing gene transfection to microinjection. They have been used for many cell structure studies, especially those employing immunofluorescence. Their growth patterns are affected by density limitation, and in general they have long but finite life span of about 50 generations in culture (Freshney, 1994).

1.18.1. Fibroblast cryopreservation

The first culture collection was established in Prague in 1889 by Frantisek Kral. Since then culture collections have developed an important role as a service and support activity for industry and research (Stacey and Doyle, 2000). Before a culture collection can be used for the long-term storage of an organism, appropriate preservation procedures must be in place. Frozen cells serve as a backup for loss cells in laboratory accidents or failures, and frozen stocks provide cells that have not undergone genetic drift.

The propagation, storage and transport of a large number and variety of somatic cell lines is significantly adding to the cost of somatic cell cloning.

Slow cooling has become a standard fibroblast preservation method (Liu *et al.*, 2000; Murakawa *et al.*, 2000). Fibroblasts can be successfully held in liquid nitrogen (-196°C); or in liquid nitrogen vapour at -175°C to -135°C and for many months to years at -80°C (Freshney, 1994), following cooling at a rate of between 0.5° and 3°C per minute (Freshney, 1994 and Teasdale *et al*, 1993). The storage ampoules are most commonly thawed rapidly in a waterbath at 37°C (Freshney, 1994).

The freezing medium usually consists of growth medium or serum supplemented with cryoprotectant. The addition of cryoprotectants is important to protect cell from any disruption during the freezing and thawing process. Teasdale *et al.* (1993) showed that DMSO led to the highest cell viability after freezing and thawing as compared to glycerol or ethanediol (ethylene glycol). Nevertheless, DMSO does have toxic properties and cells should be washed immediately after thawing.

Eroglu *et al.* (2000) reported that the introduction of low concentrations of intracellular trehalose in the absence of other cryoprotectant could protect mammalian cells (fibroblasts and keratinocytes) from cryopreservation (snap freezing). They used a genetically engineered mutant of *Staphylococcus aureus* alpha-hemolysin to create pores in the cellular membrane in the presence of trehalose and loaded trehalose into cells. Low concentrations (0.2 M) of trehalose permitted long-term post-thaw survival of more than 80% of 3T3 fibroblasts and 70% of human keratinocytes. These results indicated that cryoprotectants such as DMSO which can transform the cells can be replaced by significantly less toxic alternative cryoprotectant.

1.18.2. Attempts to store fibroblasts in dry state

Cell drying leads to massive damage to cell membranes and protein except in anhydrobiotic organisms (Crowe *et al.*, 1992). Recently several attempts had been made to modify mammalian cells to allow them to survive drying (Table 1.2). Most of them rely on trehalose to protect mammalian cells from drying. Trehalose is an effective excipient for preserving biological materials, however it is still not known whether trehalose alone allows viable cells survive drying and storage (Crowe *et al.*, 2001).

Table 1.2. Some attempts to preserve mammalian cells in the dry state.

Cell type	Drying method	Excipient	Results	References
Rat red blood cells	Freeze drying	PVP	Recovered	Meryman (1966)
Red blood cells	Freeze drying		Recover enzymatic function	Goodrich *et al.* (1992); Soewemimo-Coker *et al.* (1993)
		HES	Recover enzymatic function	Rindler *et al* (1999 a,b)
Fibroblast	Air drying	Trehalose	Viable and proliferate cells	Guo *et al* (2000)
Corneal epithelial	Air drying	Trehalose	Viable cells	Matsuo (2001)
Fibroblast	Air drying	Trehalose	Viable cells	Puhlev *et al* (2001)
Human platelets	Freeze drying	Trehalose	Recover enzymatic function	Wolkers *et al.* (2001b)
Human mesenchyme stem cells	Air dry and vacuum dry	Trehalose	Recover viable cells but the number was reduced by time	Gordon *et al* (2001)

Guo *et al.* (2000) claimed successful drying of mammalian fibroblast by introducing *ots-A* and *ots-B* genes from *E. coli* to induce intracellular trehalose synthesis. The use of adenovirus vectors to bring *otS-A* and *otS-B* into the cells can however result in significant toxicity after high multiple infection (Puhlev, 2001). Other successes have also reported. Matsuo (2001) showed that corneal epithelial cells incubated in 200mM trehalose in PBS for 15 min protected the cells against partial

desiccation (30-min drying at room temperature). Puhlev *et al* (2001) showed that cells incubated in 50 mM trehalose for 24 hr then dried under vacuum at 76 cm Hg to remove 90% of the air and leave an O_2 content of approximately 2% and stored at room temperature under vacuum in the dark showed higher desiccation tolerance. Cells incubated in less than or more than 50 mM trehalose were less desiccation tolerant. Wolkers *et al* (2001b) freeze dried human platelets. The platelets were incubated in 50 mM trehalose at 37°C for 4 h to load trehalose into the cells. Platelets were frozen from 22 to -40°C at -5 and -2°C/min, then transferred to a -80°C freezer for half an hour, then lyophilised with a condenser temperature of -45°C. To prevent any meltback while the chamber was evacuated, the flasks were held in liquid nitrogen for approximately 1 h until vacuum had reached minimal levels (about 20 mTorr) they then increased the temperature to -35°C and held that temperature during primary drying. During secondary drying the sample temperature increased to ambient in an uncontrolled manner. Samples were kept in the lyophilizer for at least 24 h at room temperature and directly used for experiments. The water content after freeze-drying was typically between 3 and 5%. Dried platelets were either directly rehydrated or prehydrated (between 0-3h) in a closed box with moisture-saturated air at 37°C, prior to rehydration. Prehydration prevents imbibitional damage of dried cells or tissues upon rehydration (Crowe *et al*., 1989). The results showed 90% functional platelets upon rehydration.

Gordon *et al* (2001) incubated human mesenchymal stem cells (hMSCs) with 50 mM trehalose and 3% glycerol and then dried them using air-drying, air-drying with storage under vacuum or vacuum only. The hMSCs were rehydrated with 3 ml of hMSC medium and were able to regain their spindle-shaped morphology and adhesive capability. In addition, they maintained high viability and proliferation capacity immediately after rehydration, but in the extended culture, the proportion of viable cells was dramatically reduced.

Despite the reports of successes, there are some controversies in this field. De Castro *et al* (2000a), de Castro and Tunnacliffe (2000), and Tunnacliffe *et al* (2001) showed that trehalose protects mammalian cells against hyperosmotic shock but not against drying. de Castro and Tunnacliffe (2000) reported that they did not obtain any viable cells after a similar transfection. While it may be true that the transfected cells do not survive complete drying, it is also possible that differences in the methods or storage protocols had some effect on the outcome. Puhlev *et al* (2001); Wokers *et al*. (2001b,

and 2002) and Gordon *et al* (2001) stored their specimens under vacuum. Puhlev *et al* (2001) and Gordon *et al* (2001) used a vacuum pack while Wolkers *et al*. (2001b) kept the platelets in the flask attached to lyophiliser for 24 hr.

Chapter 2. General materials and methods

2.1. Animals

2.1.1. Mice

Mice used in this study were obtained from Monash Central Animal Services (Monash University). Four week old female mice were used as the oocyte donors, 6 to 8 week old male mice were used as the spermatozoa donors and female mice aged 8 weeks or more were used as the embryo recipients (surrogate mothers).

2.1.1.1. Strains

Two different strains of mice were used, they were C57BL/6 x CBA F1 hybrids and inbred Balb-c. The F1 mice were used as the oocyte and sperm donors and surrogate mothers. Balb-c mice were only used in one trial as the recipients (surrogate mothers) for F2 embryos.

2.1.1.2. Husbandry (female, male, pregnant, and newborn)

All mice were kept in Monash Medical Center Block B and Block E Animal Houses with 16 h light/day (6 am-8 pm). Mice were either kept in standard mouse boxes (up to 5 mice) or large boxes (up to 25 mice), of the same sex and age in each box. Food and drinking water were given *ad libidtum* (unlimited).

Surrogate (foster) mothers were housed in large cages in groups of about 25 mice before being placed with vasectomised males (1-3 females per male). Females which had vaginal plugs on examination the next morning were removed from the males and placed in a standard cage with other plugged females until surgery for embryo transfer (section 2.5 this chapter). Following transfer the pregnant surrogate (foster) mothers were housed in groups of 1 to 3 animals. Females were culled either 14 days after surgery to evaluate fetal development, or left to go to term. Newborn pups remained with their surrogate (foster) mother until weaning (19 days old).

2.1.1.3. Vasectomy procedure

The males were anaesthetised with an intraperitoneal (I.P.) injection of 0.6mg/mouse of Rompun (Troy Laboratories, Australia) and 3mg/ mouse of Ketamine

(Parnell Laboratories, Australia). The abdominal skin was swabbed with 70% alcohol. An incision was made through the skin and peritoneum across the abdomen, in front of the subcutaneous fat pad overlying the pelvis. With the use of fine forceps, the intra-abdominal fat pad on one side (partially surrounding the testis, the epididymis, and the vas deferens), was gently pulled up and exposed through the incision. The hair pin loop of the vas deferens was dissected away from the fat pad, and a small segment removed by cauteri- ation (using a pair of forceps heated over a bunsen burner to cut through the vas and seal any cut blood vessels at the same time). The testis, vas deferens and fat pad were gently returned to their normal anatomical position. This procedure was repeated on the other side of the animal so that both the left and right vas deferens were transected. The peritoneum was closed by four interrupted sutures using 4/0 chromic gut T-5 absorbable suture. The skin was closed with 3 or more interrupted sutures using 3/0 non-absorbable suture or michelle clips and the wound irrigated with Bupivacaine. The animals were kept in a warm cage until they recovered from the anaesthetic. They were monitored daily to ensure that the wounds healed and that there were no signs of illness or distress. The males were then separated into individual cage and fertility tested by mating them to normal and superovulated females, and once proven sterile were used for inducing pseudopregnancy for up to 1 year after surgery. Entire males and vasectomised males were caged individually.

2.2. Spermatozoa

2.2.1. Spermatozoa collection

Adult male mice were killed by cervical dislocation. Spermatozoa were obtained by carefully isolating the epididymis from the testis. Both epididymides were punctured using sharp scissors in several locations and placed in prewarmed (37°C) media (media volume and type differed depending on the experiment) in 1.5 mL microcentrifuge tubes (Interpath, Melbourne). The tube was left in a test tube warmer (LEC Instruments Melbourne-Australia) at 37°C for 15 min to allow the motile spermatozoa to swim up.

2.2.1.1. Spermatozoa motility

Spermatozoa motility was assessed by placing a drop of sperm suspension on a glass slide (Menzel-glass, Germany) which was covering with a coverslip (Menzel-glass, Germany), and examined under the light microscope (Olympus CH30, Olympus-Australia) at 100x magnification. Motility was subjectively assessed as the percentage of spermatozoa moving progressively forward.

2.2.1.2. Spermatozoa concentration

Spermatozoa concentration was assessed with an improved Neubauer Haemocytometer chamber. A drop of 10 μL sperm suspension was placed on the haemocytometer under the coverslip. Cell concentration was counted based on the number of cells found in the 5 diagonal large squares. The total number of cells found were multiplied by 10000 to obtain the cell concentration per mL (WHO, 1987).

2.2.1.3. Live and dead

Assessment of the proportion of live and dead spermatozoa was done using 2% Eosin-Y (E-4382, Sigma) in Calcium-Magnesium free Phosphate Buffered Saline (PBS, Cat. No. 14190-144, Gibco-Invitrogen). This stain measures the integrity of the cell membrane. A 10 μL drop of sperm suspension was mixed with a 10μL drop of 2% Eosin-Y then smeared over a glass-slide using one edge of a glass slide. Viable spermatozoa exclude the stain thus making the spermatozoa head appear transparent. Dead spermatozoa cannot exclude the stain and the head appear dark pink. Observations were made under a light microscope (Olympus CH30, Olympus Australia) at 100x magnification. The number of spermatozoa in each field were counted to obtain the proportion of live and dead spermatozoa.

2.2.1.4. Spermatozoa drying and storage

Spermatozoa were dried in each different method as described in each experimental chapter (chapters 3 and 4). Following drying the spermatozoa were stored as described in each chapter.

2.2.1.5. Spermatozoa rehydration

Prior to use for ICSI spermatozoa were rehydrated as described in each experimental chapter (chapters 3 and 4).

2.3. Oocytes

2.3.1.1. Superovulation

Four week old female C57BLxCBA/F1 mice were injected intraperitoneally with 5 IU PMSG (Folligon, Intervet-Australia), followed by 5 IU hCG (Chorulon, Intervet-Australia) 48 h later.

2.3.1.2. Oocyte collection and cumulus cells removal.

Female mice were killed by cervical dislocation, 13-15 h after the hCG injection. Oviducts were removed and each one placed in a 50 µL drop of prewarmed (37°C) handling media containing 30 IU/ml Hyaluronidase Type I-S from bovine testes (H-3506, Sigma, Australia) under mineral oil (M-8410, Sigma-Australia). The ampullae were punctured to release the oocytes into the hyaluronidase solution. Oocytes were incubated in the hyaluronidase drop for 5 min to remove the cumulus cells, and then collected using a pulled pasteur pipette (Red-Leaf, Canada) and washed 3 times in three 50 µL drops of handling medium (KSOM-Hepes-AA-BSA, made by replacing 25 mM $NaHCO_3$ with 20 mM Hepes and 5 mM Na HCO_3). The remaining cumulus cells were removed by repeated aspiration into the pulled pasteur pipette. Oocytes were given a final wash in a drop of 50µL culture medium (KSOM-AA-BSA, Specialty Media, Cat. MR-041-D) before being placed in the same culture medium at 37°C in 5%CO_2 with humidified air until used for ICSI.

2.4. Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed using Leitz (Leitz-Germany) micromanipulators attached to a Leica inverted microscope (Leica DM IRB). There were two ICSI methods, the first was manual ICSI and the second was Piezo-drill ICSI.

2.4.1. Microinjection pipette

Microinjection pipettes were made from thin walled borosilicate glass capillary tubes Cat. No. GC-100T-15 (Harvard Apparatus, UK) with an outer diameter of 1.00 mm and an inner diameter of 0.78 mm. The capillary was pulled in a Sutter Pipette puller P-87 (Sutter Medical instruments, USA). The settings for heat time and velocity were adjusted when the filament was replaced. The tips of the pipettes were bent on a de Fonbrune microforge (CIT, Alcatel, France), by approximately 30°, 2 mm from the end of the tip to allow the tip to reach the bottom of the dish to capture the spermatozoa during ICSI.

The tip of the injection pipette for *manual* ICSI was broken by twisting the tip in the opening of the holding pipette once it was installed in the micromanipulator, to make a sharp tip with a diameter of approximately 10 µm.

The tip of the injection pipette for *Piezo* ICSI was broken to give a diameter of approximately 10 µm. This was achieved by placing the pipette near the filament glass bead of the Microforge (De Fonbrune, CIT-Alcatel-France) and heating the filament (set at 2.5 according to the setting on the knob) with the blower on. The tip was cut by briefly heating the bead. During heating (on) the bead was moved toward the tip until the heated bead stuck to the tip. When the power was then turned off, the filament was cooled and the tip was cut off. The aim was to get a flat ended tip approximately 10 µm in diameter. The tip of the pipettes were bent on a de Fonbrune microforge (CIT, Alcatel, France), by approximately 30°, 2 mm from the end of the tip to allow the tip to reach the bottom of the dish to capture the spermatozoa during ICSI.

2.4.2. Holding pipette

Holding pipettes were made from thick walled borosilicate glass capillary tubes Cat. No. GC-100-15 (Harvard Apparatus, UK) with an outer diameter of 1.00 mm and an inner diameter of 0.58 mm. The capillary was pulled after heating over a natural gas bunsen. The tip was cut and made flat at the end. The end of the tip was polished on a de Fonbrune microforge. The holding pipettes had an outer diameter of 60-80μm and an inner diameter of 20-30μm. The tips of the pipettes were bent on a de Fonbrune microforge, approximately 30°, 2 mm from the end of the tips to allow the tip reach the bottom of the dish to hold the oocyte during ICSI.

2.5. Spermatozoa preparation

2.5.1. ICSI with fresh spermatozoa

Fresh spermatozoa were prepared as described in section 2.2.1 in this chapter. If necessary motile spermatozoa for ICSI were diluted in media to reduce their concentration. Spermatozoa were selected from a 30 μL drop of sperm suspension on the lid of a 10 cm culture dish (Falcon 35-3003, Becton Dickinson-Australia) covered by mineral oil (M-8410, Sigma-Australia).

2.5.2. ICSI with dried spermatozoa

Prior to ICSI the dried spermatozoa were rehydrated as detailed in each chapter. If required the rehydrated spermatozoa were diluted further to reduce their concentration. A 30 μL drop of rehydrated spermatozoa suspension was placed on the lid of 10 cm culture dish (Falcon 35-3003, Becton-Dickinson) covered by mineral oil (M-8410, Sigma-Australia).

2.5.3. Intracytoplasmic spermatozoa injection procedures.

Three 30 μL drops of handling media (M2 or KSOM-Hepes) were made on a lid of a 10 cm culture dish (Falcon 3003, Becton Dickinson) under mineral oil (Sigma M-8410, Sigma-Australia). The first drop contained spermatozoa, the second drop was a wash drop and the last drop was for the oocytes (up to ten oocytes). After ICSI, all

oocytes, were transferred into culture medium (KSOM, Specialty media, or prepared by G. Thouas in CEHD-MIRD) in an incubator 37°C, 5%CO_2, in a humidified atmosphere. Some embryos were cultured for 5 days to the blastocyst stage. The embryos for embryo transfer were cultured to the 2 cell stage.

2.5.3.1. Manual ICSI

Manual ICSI was performed using Leitz micromanipulators attached to a Leica DM-IRB inverted microscope at 100 to 400x magnification using Nomarski optics. The spermatozoa from the sperm drop were aspirated into the injection pipette and then positioned near the tip of the injection pipette. The injection pipette was then moved to the ICSI drop, passing through the wash drop to remove any spermatozoa attached to the outside of the pipette. In the ICSI drop the oocyte was held by the holding pipette, and the injection pipette was moved towards the oocyte to penetrate the zona and then pushed against the membrane. The membrane was slowly aspirated into the injection pipette until the membrane ruptured. The spermatozoon was then slowly injected into the cytoplasm and the pipette withdrawn.

2.5.3.2. Piezo ICSI

The lumen of the injection pipette for piezo ICSI was filled with Mercury (83559 Fluka-Sigma) (to approximately 1 mm of the tip) to assist the piezo pulse. Five percent PVP 40,000 (PVP40-Sigma) or 8 mg/ml BSA (Albumax, Gibco-Invitrogen) was used in the sperm drop. Piezo ICSI was performed using a Burleigh Piezo drill (Burleigh Instruments, USA) attached to the Leitz micromanipulator on a Leica DM-IRB inverted microscope at 100 to 400x magnification using Nomarski optics. The spermatozoa head was separated from the tail by aspirating the whole spermatozoa into the pipette and applying a pulse to cut the tail. Up to 5 sperm heads were aspirated into an injection pipette and then lined near the tip of the injection pipette.

To obtain a good result the piezo was adjusted for its amplitude, pulse duration and frequency. The first series of piezo pulses (Pulse A), was designed for zona pellucida penetration but also used to separate the sperm head and tail. Another series of pulses (Pulse B) was applied to penetrate the membrane to penetrate membrane. The injection pipette was moved through the wash drop to remove any spermatozoa and PVP that attached to the outside wall of the pipette before entering the ICSI drop. In the

ICSI drop the oocyte was held by the holding pipette, and the injection pipette was moved towards it. After penetrating the membrane, a single sperm head was slowly released into the cytoplasm before withdrawing the pipette.

2.5.4. In vitro embryo culture

Embryo culture medium, KSOM-AA-BSA was purchased from Specialty Media, USA or made from stocks stored at 4°C and used within two weeks of preparation. Four to six drops of 40 µL of embryo culture medium (KSOM-AA-BSA) were prepared in 30 mm culture dishes (Falcon 35-3001, Becton Dickinson-Australia) under mineral oil (M-8410, Sigma). The drops were equilibrated for at least 3 hr in an incubator at 37°C, with 5% CO_2 in humidified air prior to use.

Following ICSI, embryos were cultured in drops with up to 10 embryos per drop. Four to six hour later, the embryos were selected. The embryos that survived ICSI were separated from the lysed embryos. The embryos that survived ICSI were grouped in same culture drop until the 2- cell stage and then transferred into psudopregnant fosters (recipients) or cultured to the blastocyst stage (day 5).

2.5.5. Embryo transfer

Embryos were transferred into day one pseudopregnant recipient mice 24 to 48 h after ICSI.

2.5.6. Preparing pseudopregnant fosters/recipients.

Females (6-8 weeks) were placed with vasectomised males (1-3 females per male) of the same strain to detect oestrus. Females were examined for evidence of a vaginal plug each morning. On the day a mating plug was present the females were used as the embryo transfer recipient.

2.5.7. Embryo transfer

Recipient fosters were anaesthetised using 0.6 mg/mouse of Rompun (Troy Laboratories, Australia) and 3 mg/ mouse of Ketamine (Parnell Laboratories, Australia) by intraperitoneal injection. The depth of the anaesthesia was indicated by the absence

of eye blink and footpad reflex. Embryos were transferred into the fimbria of the oviduct. Surgery was performed using a mid dorsal incision as described in Shaw and Kasai (2001).

After surgery the incision was closed using Michelle clips, and the mouse was injected with 0.01 ml strength Reverzine (Parnell Laboratories, Australia) intravenously to assist with the recovery from anaesthetic. The mice were placed in a warm cage and monitored every 10 to 30 min until they had recovered from the anaesthetic, they were then returned to the animal house and monitored daily to ensure that the wounds healed and that there were no sign of illness or distress.

2.5.8. Evaluation of foetal development

In experiments to evaluate foetal development the surrogate mothers were killed by cervical dislocation on day 15 of pregnancy. The uterus was removed from the abdominal cavity and the foetuses with their placentae were removed carefully from the uterus. Foetuses were removed from the placenta, blotted with a tissue paper and then weighed.

2.5.9. Evaluation of offspring

In some experiments pregnant surrogate mothers were allowed to deliver their offspring (approximately between day 19-21 after embryo transfer). The offspring were sexed and weaned at 19 days of age.

The fertility was evaluated for some offspring by mating the F_2 siblings. Their pregnancies and live born F_3 offspring were monitored to indicate fertility.

2.6. Fibroblast Cell

In this thesis fibroblast cells from mice were used as a model for somatic cells.

2.6.1. Cell type

Mouse STO fibroblasts were used. This type of fibroblast were originally derived from mouse foetuses, and usually used as a feeder layer to maintain teratocarcinoma stem cells in undifferentiated state and for culturing embryonic stem

cells. The STO cells used in this experiment were provided by Assoc. Prof. Martin Pera.

2.6.2. Culture conditions

The STO cells were cultured in vitro in 25 cm^2 culture flasks (Falcon Cat 35-3082, Becton-Dickinson) in DMEM (Trace-Elements, USA), supplemented with 10% Fetal Bovine Serum (FBS, Gibco-Invitrogen) and 2 mM Glutamine (Gibco-Invitrogen), 625µL Penicillin-Streptomycin (Gibco-Invitrogen) in an incubator at 37°C with 5% CO_2 in a humidified atmosphere.

The culture media was replaced every 2 or 3 days. The stock cells were passaged when they reached confluency. Cells were used for experiments during the growth phase before they became confluent.

Smaller containers (4 well-NUNC or 96-well dishes, Falcon-Becton Dickinson) were used to culture the cells following an experiment.

2.6.3. Cell harvesting

Cells were harvested from the culture flask by removing the culture medium and then adding 1 ml prewarmed (37°C) 0.25% Trypsin, 1 mM EDTA in Ca-Mg free HBSS (Gibco-Invitrogen) for 5 min, followed by gently shaking the flask to detach the adherent cells. Trypsin activity was stopped by adding 5 ml DMEM with 10% Fetal Bovine Serum.

The suspension of detached cells was then placed into a 15 ml conical tube (Falcon Cat No. 35-2096, Becton-Dickinson, USA) and centrifuged at 1000 rpm (Kubota-Japan) for 5 min at room temperature to obtain a pellet. After centrifugation, the supernatant was removed and the pellet was resuspended with 1 ml fresh culture medium. A drop (10 µl) of cell suspension was put on an Improved Neubauer counting chamber to determine the cell concentration. The method was similar to that in section 2.2.2.2.

2.6.4. Freezing and thawing stock cells

Cells were frozen to maintain a supply of STO fibroblasts. Freezing was performed by suspending a STO fibroblast pellet in 10% DMSO (D-5879, Sigma-Australia) in Fetal Bovine Serum (FBS, Gibco-Invitrogen) at a concentration of 20 million cells/mL, then 500 µL aliquots of cell suspension were frozen in 2 mL cryovials (Greiner, Interpath Melbourne) at 1°C/min using a cooling chamber ("Mr. Frosty", Nalgene) filled with 250mL Isopropyl Alcohol (I-0398, Sigma, Australia) placed in a -80°C freezer overnight, and then stored in liquid-nitrogen for subsequent use.

Stock cells were thawed by warming the cryovials in water at 37°C until the contents melted. The cell suspension was expelled and cultured in 30 ml of DMEM 10%FBS in a 25 cm^2 TC-Flask (Falcon Cat No. 35-3082, Becton-Dickinson). The cells were then used as described in 4.3 after they reached confluency.

2.6.5. Cell electroporation

Electroporation was used to make transient pores through the cell membrane. Electroporation was performed on cells suspended in medium (varied depending on the experiment) using a BioRad Gene Pulser (BioRad, England). Cell suspensions (400 µL at a concentration of 10-30 million cell/ml) were loaded into 2 mm gap Cuvettes (BioRad). Each cuvette was loaded into the electroporator and pulsed with the required voltage (50-350 Volts) and capacitance 500µF. Electroporation was performed at room temperature. Cells were either used as soon as possible after electroporation or at specific time intervals after electroporation.

2.6.6. Cell drying

Cells were dried in several ways including air drying, vacuum drying, nitrogen drying and freeze drying, as a suspension or as adherent cells. Details of the cell drying methods are described in the experimental chapters.

2.6.7. Assays for cell viability and permeability

Cell viability was determined using 0.4% Trypan-Blue (T-6146, Sigma-Australia) in Ca/Mg free PBS (Gibco-Invitrogen). A drop (10 µL) of cell suspension

was mixed with a drop (10μL) of 4% Trypan-Blue in Ca/Mg free PBS (Gibco-Invitrogen), then immediately transferred into an improved Neubauer Counting Chamber or glass slide. The dead cells took up the dye (blue) because their membranes were permeable, while the live cells did not take up the dye (clear). Observations were made under 200x magnification.

Cell permeability was determined by adding a100μg/mL of Propidium iodide (P-4170, Sigma) to the electroporation, freezing or incubation medium. The events of cell permeabilisation were indicated by the presence of PI inside the cells after observation under epifluorescent microscope with a texas-red filter. Viable permeabilised cells (and dead cells) take up the PI dye (PI+) only while the pores are open. Once the pores close the cell recover the capacity to exclude vital dyes such as PI or Trypan Blue.

A drop of stained cells was immediately placed on a glass slide then placed on a Leica microscope equipped with a Leica digital camera and connected to a PC. Observations were made under 200x magnification. The images were captured using Leica-DC Viewer software (Leica-Germany) and analysed using Adobe Photoshop ver.5.0 (Adobe) to determine the number and proportion of live and dead cells. Cells that had taken up vital die from the electroporation medium (PI+) but could subsequently exclude a second vital dye (TB) were classified as viable and transiently permeabilised (details in Chapter 5).

Chapter 3. Evaluation of evaporative drying for spermatozoa.

3.1. Introduction.

Preservation by drying is of enormous practical importance and widely used for biological products in clinical medicine, for food stuffs, pharmaceuticals and in agriculture. Drying is particularly valuable, as the storage costs are very low. In nature a range of animals and plants tolerate desiccation, often for prolonged periods of time. However the majority of living organisms cannot tolerate desiccation, in particular at ambient temperatures.

Early attempts to dry spermatozoa were partially successful (reviewed by Smith, 1961), with some researchers reporting finding motile spermatozoa after freeze-drying (Meryman and Kafig, 1959; and Juschenko, 1959; both were reviewed in Smith, 1961). Jeyendran *et al.* (1981) reported a 29% conception rate with bull semen that had been freeze-dried to 6% of its moisture content after dilution in TEST-Yolk diluents, but no pregnancies with spermatozoa dried down to 2% of their normal moisture content. This need for minimum residual water is likely to be the result of biophysical changes induced by complete removal of water.

Katayose *et al.* (1992) showed that sperm nuclei were fairly resistant to dehydration as freeze-dried mature hamster and human spermatozoa heads microinjected into hamster oocytes remained capable of developing into pronuclei after 12 months of storage at 4°C. The level of DNA synthetic activity in the spermatozoa (male) pronucleus was comparable to that in the oocyte (female) pronucleus. Sperm nuclei stored in 100% ethanol, 100% methanol, a chlorotorm-methanol (2:1) mixture for 20 days or dehydrated (fixed) in Carnoy's fluid could also develop into normal pronuclei. However, the level of DNA synthesis in the pronuclei derived from these chemically dehydrated nuclei was generally lower than that in the female pronuclei.

Wakayama and Yanagimachi (1998) conclusively demonstrated that mouse spermatozoa could be dried and stored at room temperature. The spermatozoa were snap frozen and then freeze- dried in CZB medium the absence of any compounds that may have provided protection from desiccation injury, then stored in sealed glass ampoules under Argon. Although the spermatozoa remained immotile following re-

hydration they produced viable offspring after microinjection into oocytes. More recently they have improved their technique (Kusakabe *et al.* 2001) by using solutions containing 50mM EGTA to maintain sperm-chromosome integrity and storing the freeze-dried samples under vacuum. The materials proved to be stable, as they could be stored for 3 month at room temperature. Although they obtained excellent results, the equipment that they used was very costly, and renewed investigation aimed at finding cheaper and simpler protocols seems warranted.

Wakayama and Yanagimachi (1998) and Kusakabe *et al.* (2001) both used oxygen free conditions, but it remains unclear from their work whether the use of oxygen free conditions was fundamental to their success. Puhlev *et al* (2001) also showed that a high vacuum was optimal for preserving cellular viability

Oxygen is vital to life, but it is often lethal to dried organisms and cells (Brawn and Fridovich, 1981). Oxygen is thought to be detrimental to biological material because it forms oxygen radicals that lead to oxidative damage to a range of cellular components including DNA (Aitken, 1999; Irvine *et al.*, 2000; Lopes *et al.*, 1998; Oliver *et al.*, 2002; Shen and Ong, 2000; Twigg *et al.*, 1998). Dried tissues are susceptible to radical damage to their phospolipids, DNA and protein (Oliver *et al.*, 2002) with lipid peroxidation and phospolipid deesterification as the main symptoms.

Nitrogen is the most abundant component of air. At room temperature and atmospheric pressure, nitrogen is a colorless, odorless, nontoxic, nonflammable gas. It constitutes 78% by volume of the atmosphere. Gaseous nitrogen consists of diamers (2 nitrogens bonded together), which are very stable, allowing it to be used as an inert gas in a range of applications including electrical systems (e.g. incandescent lamps), and in the chemical industry. By using nitrogen to displace oxygen an oxygen free environment which prevents oxidation can be maintained. Dry nitrogen gas is therefore widely used in the food industries for drying and storage.

Nitrogen can also reduce contamination from pathogenic organisms. Fresh meat stored under nitrogen developed fewer pathological microorganisms and retains the red color longer as compared to meat stored under CO_2 or O_2 (Kakouri and Nychas1994; and Saucier, *et al.*,2000). Since some microbes require either oxygen (aerobes) or CO_2, nitrogen is valuable in that it replaces both O_2 and CO_2.

To determine the effect of evaporative drying on spermatozoa, it was important to consider gross, visible, changes such as altered motility and morphology, as well as changes in spermatozoa function. On entering an oocyte a normal spermatozoa will initiate embryo development (Johnson and Everitt, 2000). Normal events in fertilisation and embryo development are reviewed elsewhere (Aitken, 1997; Capco, 2001; Cuasnicu *et al.*, 2001; Green, 1993; Kupker *et al.*, 1998; Moore, 2001; Mori *et al.*, 2000; Sutovsky and Schatten, 1998; Swann, 1996; Topfer-Petersen *et al.*, 2000; Wassarman, 1987; Wassarman, 1999; Wassarman *et al.*, 2001; Wassarman and Litscher, 2001). Briefly normal fertilisation requires a series of coordinated changes in both the oocyte and the spermatozoa. After penetrating the zona pellucida, spermatozoa fuse with the plasma membrane by interacting with receptors and initiate embryo development. Upon spermatozoa-oocyte fusion, a sperm cytosolic factor is released into the oocyte which interacts with unknown cytosolic targets and generates Ca^{2+} influx oscillations (Fissore *et al.*, 1998). It induces exocytosis of cortical granules to block polyspermy, stimulates oocyte activation which leads to the completion of the second meiotic division, and permits extrusion of the second polar body (Moomjy *et al*, 1999). The sperm nuclear membrane breaks down followed by chromatin decondesation which releases sperm chromatin into cytoplasm and replace sperm DNA protamine with histones. Each haploid chromosome set forms two pronuclei. Both (male and female) pronuclei replicate their DNA (at syngamy) as the final stage of fertilisation and form a zygote. Spermatozoa of some species also contribute their centrioles which govern chromosomal movements and embryo development (Johnson and Everitt, 2000).

Normal spermatozoa possess an intact sperm DNA matrix which contributes to normal development after fertilisation (Kishikawa, *et al.*, 1999; Ward *et al.*, 1999 and 2000). Ward *et al.* (2000) showed evidence that the structural integrity of mouse sperm nuclear matrix may be necessary for the proper unpacking of sperm DNA for participation in embryogenesis. It is likely that the sperm nuclear matrix contributes to the organisation of the sperm DNA and its disturbance can seriously damage the paternal genome or its expression. Fully functional mature spermatozoa possess the ability to activate an oocyte. This ability is acquired during spermatozoa maturation (Chang, 1984; Flesch and Gadella, 2000; Fraser, 1992; Gadella *et al.*, 1999), but is lost through processes such as fixation and some, but not all, drying procedures (Katayose *et al.* 1992; Kusakabe *et al.* 2001). The drying protocols developed by Wakayama and

Yanagamachi 1998, and Kusakabe *et al* 2001 were highly effective in initiating embryo development but were not ideal. They utilised highly sophisticated (costly) drying equipment and as the spermatozoa were not motile following rehydration fertilization had to be achieved by microinjection, which also requires costly equipment. For drying to become the future means of storing spermatozoa, drying protocols which permit the recovery of motile spermatozoa, and/or cheap and effective drying protocols and equipment need to be developed.

These experiments aimed to develop improved drying protocols by establishing whether mouse spermatozoa dried and stored under different types of gas with and without oxygen could retain their viability and ability to fertilise mouse oocytes. In the event that the drying procedures failed to initiate oocyte development, this study aimed to induce activation artificially by a 5-minutes exposure to 8% ethanol in M2 at 37°C.

3.2. Materials and methods

3.2.1. Mouse spermatozoa drying

Mouse spermatozoa were obtained from the epididymides of adult male mice (Chapter 2, General Materials and Methods). Cauda epididymides were isolated. The cauda epididymides were cut in two places with scissors and suspended in 1 ml warmed (37°C) handling media to release the spermatozoa.

3.2.1.1. Handling media

Three handling media were tested namely, M2; Ca-Mg free phosphate buffer saline (Ca-Mg free-PBS) and Modified Hepes Basal Salt (mHBS).

3.2.1.2. Drying procedures

Sperm suspensions were dried by blowing a continuous flow of gas on the surface of sperm suspension drop or layer.

3.2.1.3. The gases

Several different gas compositions and sources were used to dry the spermatozoa.

Table 3.1. Gas type/composition and source

Gas Type	Source	Package	Control
Compressed air	Medical gas supply	NA	Tap
Dry 5%CO_2, 5%O_2 and 90%N_2	BOC Gases Australia	G-Size	2-stage regulator
Dry CO_2	BOC Gases Australia	G-Size	2-stage regulator
Dry N_2	BOC Gases Australia	G-Size	2-stage regulator

3.2.1.3.1. Containers

Sperm suspensions were dried onto either dishes or the inner surface of insemination straws. The protocol for drying in dishes was as follows. A sperm drop (100µL) was placed in a 35 mm tissue culture dish (Becton Dickinson 35-3001) and spread. The dish was then placed in a modular incubator chamber (Billups-Rothenberg, USA) which was then sealed to make it gas tight. Air or gas was blown through a 0.22 µM syringe filter into the inlet port of the incubator at 12.5 Psi pressure. The outlet port was left open for approximately 4 hours until the drops had dried. After drying the modular incubator was opened and lids placed on the dishes. The dishes and lids were then wrapped with aluminium foil to protect them from light and stored at -20°C (freezer); 4°C (refrigerator) or at room temperature (20-25°C) for 1 week. Drying using either medical air or filtered atmospheric air left a dry blot on the surface of TC-dish.

The protocol for in-straw drying was as follows, a 10µL sperm drop was aspirated into a 250µL straw (IMV, France). This drop was aspirated further along the straw to form a thin layer along the inner wall of the straw. A steady flow of nitrogen gas (2-4 L/min) was then blown through the straw for five minutes before sealing it inside an outer 500µl straw (IMV, France) to trap the nitrogen inside both the straws. Straws were then stored in a modular incubator filled with nitrogen gas or in an aluminium foil pouch (West's Packaging Ltd.) filled with nitrogen and heat sealed to trap nitrogen inside the pouch and maintain an oxygen free environment inside the pouch.

3.2.1.4. Storage procedures

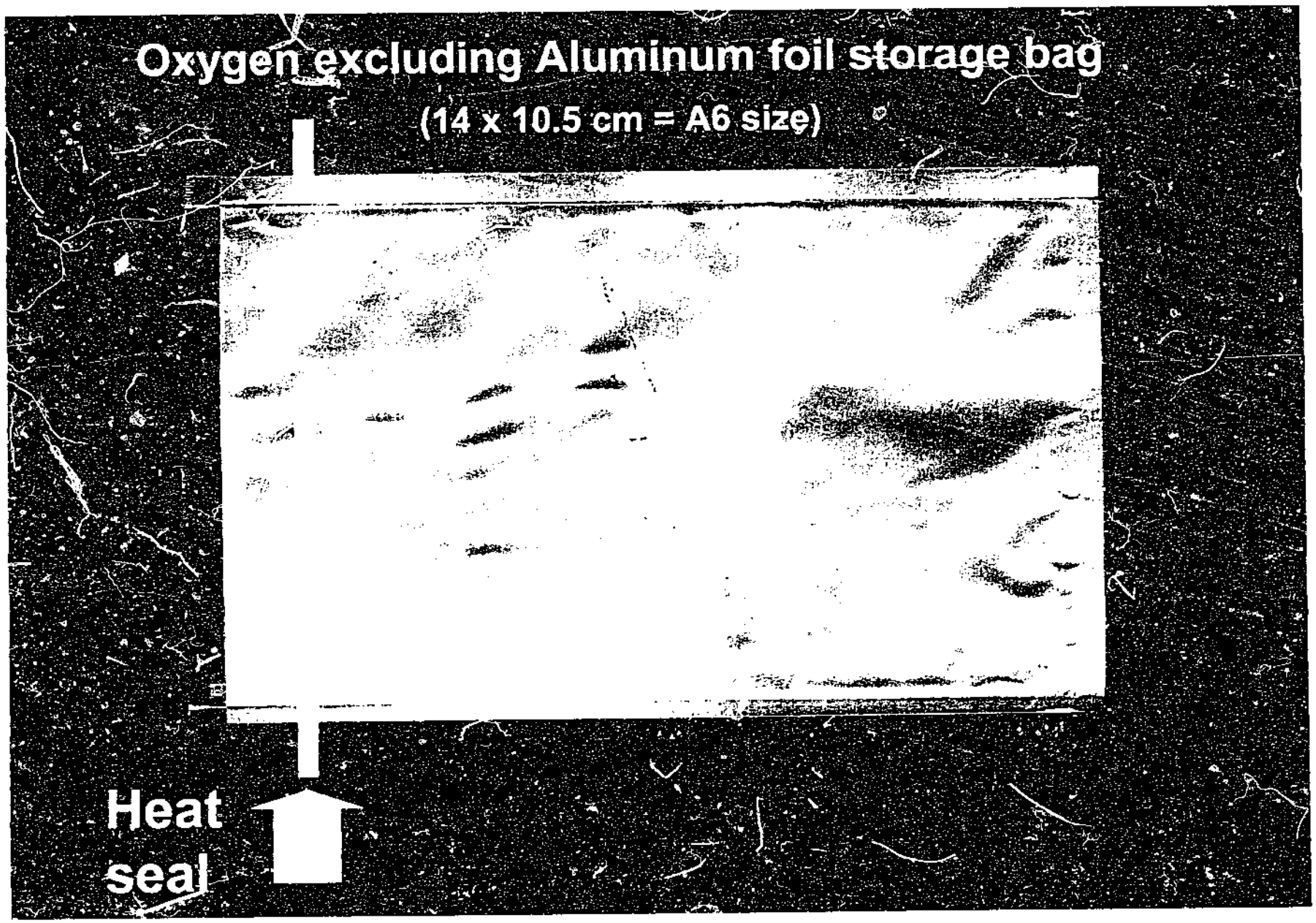
Several storage conditions were evaluated including storage within plastic insemination straws, storage in gassed gas proof aluminium foil bags, and storage in gas tight modular incubator chambers (Billups-Rothenberg, USA) .

3.2.1.4.1. Modular incubator

Modular incubators (Billups-Rothenberg, USA) were used to store straws containing dried spermatozoa. The chamber was filled with N_2 gas after a continuous flow 10 min to remove all remaining gases. The both inlet and outlet pipes were sealed using tube clamps. The modular incubator is normally used for in vitro oocyte maturation or embryo culture.

3.2.1.4.2. Bags

Aluminium foil bags (14x10.5 cm; A6 paper size) with one open end were purchased from West's Packaging (Australia). The inner surface of the bags come lined with plastic to allow them to be sealed with a standard heat sealer (Fig. 3.2). After inserting the straw the bags were sealed and filled with dry nitrogen gas through a 19G needle then sealed again to keep Nitrogen inside the bag.


Oxygen excluding Aluminum foil storage bag
(14 x 10.5 cm = A6 size)
Heat
seal

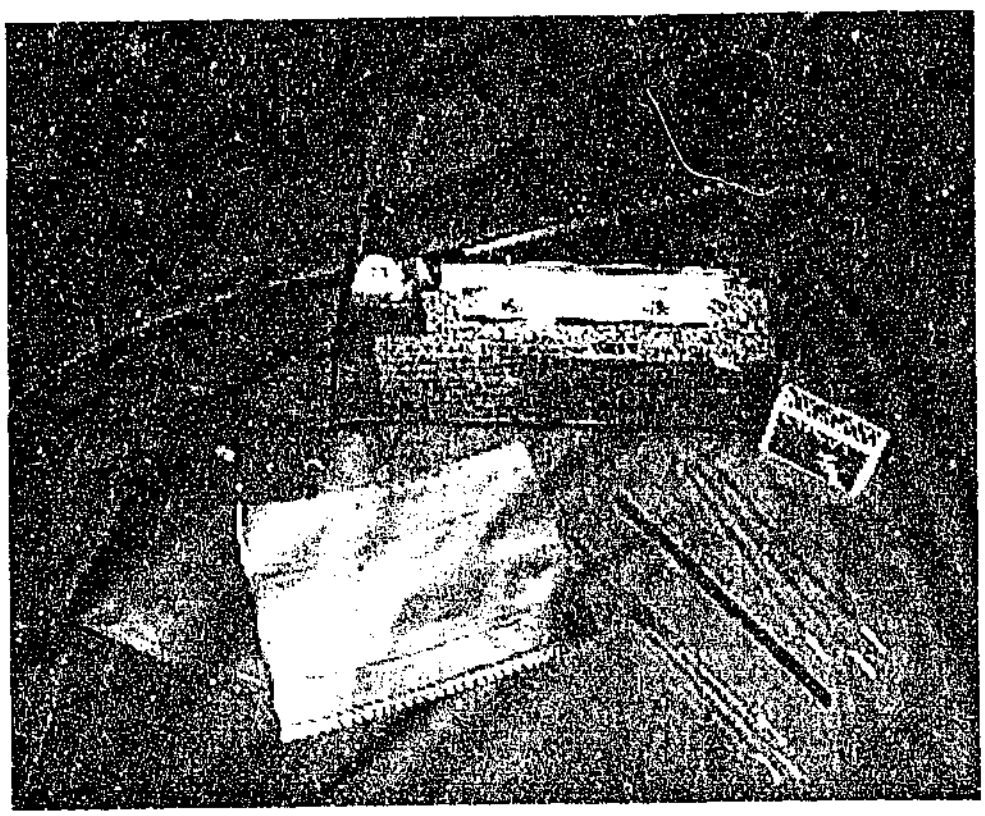


Fig. 3.2. The heat sealer, foil bags and straws. (the matchbox is a size indicator)

3.2.2. Assessment of spermatozoa morphology

Observations on spermatozoa quality were done with a light microscope (Olympus) with 100× magnification to assess spermatozoa motility and morphology as well as the uptake of vital dye (2‰ Eosin-Y in PBS) (WHO, 1987). The spermatozoa "integrity" was judged by looking at several criteria, whether the spermatozoa was intact (head and tail) with a normal tail, complete but with a hook on the tail (bent tail) or incomplete (a sperm head without a tail). Observations were made on at least 200 spermatozoa from several different fields.

Differential staining using 2‰ Eosin-Y in PBS is a traditional staining method with reliable results. The head of dead spermatozoa will take up the dye and appear red, while the live spermatozoa will not take up the dye and look transparent.

3.2.3. Assessment of spermatozoa function

Spermatozoa function was assessed using the following parameters, spermatozoa motility, pronuclear formation, polar body extrusion, cleavage, implantation, fetus formation, the birth and weaning and reproductive capacity of any resulting offspring.

3.2.4. Protocol for Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed manually or with Piezo driven micromanipulators as described in Chapter 2 (General Materials and Methods). All ICSI procedures were performed using a Leitz Micromanipulator attached to a Leica DM-IRB inverted microscope, on a lid of a 10 cm TC-Dish (Becton-Dickinson 35-3003), in drops of M2 under oil. Following ICSI, the oocytes were cultured in vitro in KSOM (Specialty medium, USA) in a 40 μL drop under oil in an incubator with 5% CO_2 in air at 37°C.

3.2.5. Activation of oocytes

In some experiments oocytes were activated experimentally. Activation was induced artificially by a 5 minute exposure to 8% ethanol in M2 on a warm stage (37°C), followed by 3 washes in M2 and 2 washes in fresh embryo culture medium (KSOM).

3.2.6. Embryo transfers

Embryos for embryo transfer were transferred at the 2-cell stage into pseudopregnant recipients after 24 h in culture. Recipients were either examined on day 15 of gestation (these were all F1 hybrids, C57x CBA), or left to go to term (these were either pseudopregnant C57x CBA F1 hybrids mated to vasectomised males or pregnant inbred Balb/C mice mated to fertile Balb/c males). The latter were used to prove that the pups were derived from the transferred embryos (pups derived from the F2 embryos would be Black or Agouti while the Balb/C fosters own offspring would be white).

3.2.7. Experimental design

3.2.7.1. Evaluation of the gas permeability of plastic insemination straws.

DMEM (100μL) with a neutral pH (containing Phenol Red as a pH indicator) was inserted into a 250 μL straw and the straws were sealed by three different methods. The first group was heat-sealed at one end with a cotton plug at the other end, the second group was heat-sealed at both ends and the third group was heat sealed at both ends and stored inside a 500μL straw (double straw) which was also heat-sealed at both ends. Those straws were stored in an incubator at 37°C with 5% CO_2; or within aluminum foil bags or a modular incubator filled with 100% CO_2 gas,100% N_2 gas or air. Straws were observed daily to determine how long it took for the medium to change color (pH) from pink to yellowish or yellow. The color changes indicated that CO_2 had penetrated the straw.

3.2.8. Assessment of the gas permeability of aluminum foil bags.

Aluminium foil pouches used for embryo culture were adapted for storage of dried materials. The supplier stated that the bags permeability to oxygen was $0.02cm^3/m^2/24hr$ at 25°C. To determine the permeability of the bags as used in this study, bags were filled with gas and then sealed. The composition of the gas was determined by Dr. B. Dumsday (Dept. of Zoology, The University of Melbourne) using mass spectrometry on gas bags on the day of filling, and for gas in bags stored for 5 weeks before analysis.

3.2.9. Spermatozoa dried in gases containing oxygen

The caudae were left in the handling media for 15 – 30 min to allow the motile spermatozoa to swim out into the solution. A drop of 100 μL sperm suspension was dropped on to a 35 mm tissue culture dish (Becton Dickinson 35-3001), then dried using different sources of atmospheric air (dry medical air; filtered atmospheric air approximate composition 21% O_2, 78% N_2, 0.2% CO_2) and gas mixtures (5%CO_2, 5%O_2 and 90%N_2; and 5% CO_2) as described in the materials and methods section.

Rehydration was done by adding 100μL TC-grade water (CSL-Laboratory Melbourne) to reconstitute the spermatozoa. Observations were made on their motility,

viability (live and dead using 2% Eosin-Y), morphology, and fertilising ability after ICSI.

All spermatozoa were injected by manual ICSI and the resulting embryos were cultured in KSOM (Specialty Media, Cat. MR-041-D) at 37°C in 5%CO_2 with humidified air for 72 hour.

3.2.10. Spermatozoa dried in Carbon dioxide

The preparation drying and rehydration procedures for spermatozoa dried with CO_2 (BOC Gases, Australia) was as described above for spermatozoa dried with oxygen containing gasses. The CO_2 gas was obtained from a G size cylinder with a 2 stage CO_2 regulator. The gas was passed through a 0.22µm filter before use as described above in the materials and methods section. Spermatozoa were resuspended in three different medium PBS, M2 or mHBS and dried in 100% CO_2 then stored for 1 week at three different temperatures (RT; 4°C or refrigerator and -20°C or freezer). Observations were made on their motility, viability (live and dead using 2% Eosin-Y), morphology, and fertilising ability after manual ICSI.

3.2.11. Spermatozoa dried in Nitrogen

Several spermatozoa drying experiments were carried out using nitrogen gas. The three groups of experiments included;

A) Spermatozoa dried with nitrogen and stored under oxygen. Spermatozoa prepared and dried as described above for oxygen containing gases;

B) Spermatozoa dried with nitrogen and stored under nitrogen. Spermatozoa prepared as described above for oxygen containing gases but dried in straws and stored in aluminium bag/pouch (Fig. 3.2) or modular incubator.

C) Spermatozoa prepared, dried and stored under nitrogen or totally oxygen free conditions. The N_2 gas was obtained from G size cylinders with a 2-stages N_2 regulator. The gas was passed through a 0.22µm filter before use.

3.2.11.1. Detailed preparation for each group of experiments.

3.2.11.1.1. Preparation protocol for group A

Nitrogen was used to dry a drop of sperm suspension on a 35 mm TC dish. Dishes with the dry sperm blot were stored at room temperature. The dried spermatozoa were rehydrated by adding 100µL of TC water.

3.2.11.1.2. Preparation protocol for group B (drying in straw)

A 10µl drop of the sperm suspension was placed in a conventional 250µl plastic insemination straw (IMV, France). This drop was aspirated into the straw to form a thin layer along the inner wall of the straw. This was then dried with a steady flow of nitrogen gas blown through the straw for five minutes, after which the straw was inserted into an outer 500µl straw (IMV, France) and then both were sealed to trap the nitrogen inside both the straws. Straws were then stored in a modular incubator filled with nitrogen gas or in an aluminium foil pouch (West's Packaging Ltd.) filled with nitrogen and heat-sealed.

The spermatozoa dried in straws were rehydrated by aspirating 50-100µL of handling medium (KSOM-HEPES) into the straw to detach the dried spermatozoa from the inner wall of the straw and expelled again.

3.2.11.1.3. Preparation for group C (oxygen free preparation).

Modified HBS was degassed by placing 30mL of that medium in a 50mL Falcon tube inside a vacuum dryer flask attached to the vacuum pump until bubbles ceased to form. Dry nitrogen gas was then bubbled through the medium for 5 min to eliminate the remaining oxygen and saturated with nitrogen. The medium was stored and frozen in a -20°C freezer. Prior to use the medium was thawed and further equilibrated in a modular incubator filled with nitrogen.

Spermatozoa were dried with the technique used in group B, in a modified hood under constant nitrogen flow to prevent oxygen contamination. The dried spermatozoa were stored in an aluminium bag filled with nitrogen. No motile spermatozoa were found in the medium after swim up. This result was different as compared to the previous group (up to 90% of motile spermatozoa were found when spermatozoa were swim up in normal mHBS). This indicated that oxygen is required to obtain motile spermatozoa during swim up.

Prior to ICSI the oxygen free spermatozoa were rehydrated with oxygen free mHBS and placed the drop directly on ICSI dish under oil. ICSI was performed manually. Following ICSI the oocytes were activated with 8% Ethanol (16μL ethanol in 184μL M2 or KSOM Handling medium) for 4 min at RT, then washed 3 times in handling medium and 2 times in fresh in vitro culture medium (KSOM-AA-BSA) before subsequent culture. The surviving embryos were cultured for 30 hr, then the cleaved embryos were transferred into recipients.

Dehydration and Storage

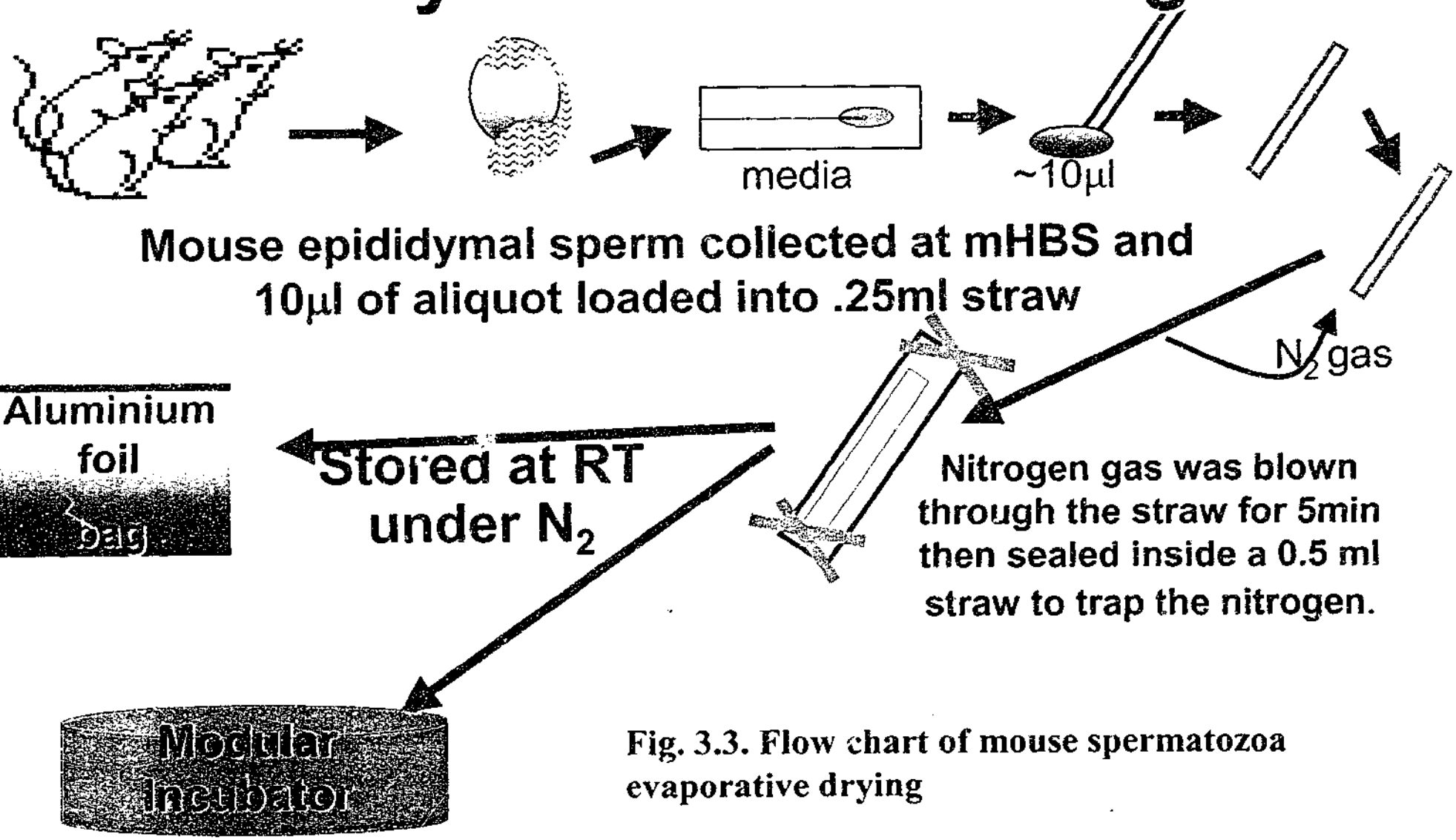


Fig. 3.3. Flow chart of mouse spermatozoa evaporative drying

3.2.11.2. ICSI using spermatozoa dried in Nitrogen

Spermatozoa dried in group A, B and C were injected into mouse oocytes and cultured in vitro. In group B, on each day for 8 days following drying one straw was flushed with 300µl of handling medium to expel the spermatozoa. Single spermatozoon was injected into C57xCBA F1 oocytes and the resulting embryos were cultured in KSOM (Specialty Media, Cat. MR-041-D) at 37°C in 5%CO_2 with humidified air for 72 hour.

3.2.11.3. Effect of different sources of BSA for spermatozoa dried in nitrogen

Two different brands of Bovine serum albumin (fraction V BSA, (Sigma Australia) and Albumax (Gibco, Invitrogen)) were compared. Spermatozoa were prepared as in group B in different media. Spermatozoa were dried in three different media (Na-EGTA, mHBS, and KSOM) with or without BSA using dry nitrogen in a 250µL straw. Straws with dried spermatozoa were stored in aluminum foil bags filled with nitrogen at room temperature. Spermatozoa were used for ICSI using both manual and Piezo drill.

Mouse sperm collection, drying and storage were similar to those in group B.

3.3. Results

3.3.1. Assessment of gas permeability of plastic insemination straws.

Straws filled with media at neutral pH containing phenol red which were placed in an atmosphere of 5% CO_2 or 100% CO_2 (which acts as an acid) changed colour (pH) over time (Table 3.2). This showed that CO_2 could penetrate through the straw into the test medium (DMEM-culture) and change its colour (pH). Sealing and packaging methods influenced the rate of colour change from pink to yellow.

The colour of DMEM did not change in straws held in air (<0.2% CO_2) or 100% N_2 (data not shown).

Table 3.2. Time taken for media held within insemination straws to change pH (colour change from pink to yellow) when placed in an atmosphere containing CO_2 gas (a weak acid).

Sealing method *)	5% CO_2 Incubator	100% CO_2 Bags	100% CO_2 Modular Incubator
1	1-2 h	0.5-1 h	1 h
2	7-12 h	4-16 h	4-15 h
3	12-20 h	6-26 h	5-23 h

1)Straws were heat-sealed at one end with the cotton plug at the other end.

2)Straws were heat-sealed at both ends or,

3)Straws were heat-sealed at both ends and also stored inside a 500µL straw (double straw) heat-sealed at both ends.

Oxygen molecules are smaller than CO_2. Results in table 3.2 showed that CO_2 could penetrate the straw wall and affect the medium pH (indicated by color changes). That means O_2 which has a smaller molecule size could also penetrate the straw. This indicated that straws had to be maintained in bags or a gas tight modular incubator to control gas exchange (prevent oxygen contamination).

3.3.2. Assessment of gas permeability into aluminum foil bags.

The results (Table 3.3) show that the composition of gas held within the aluminium foil pouches used for storage of dried materials changed only slightly (n.s) over the 5-week test period. This means the pouches were a reliable barrier to prevent oxygen penetration.

Table 3.3. Changes in nitrogen content in foil pouch after 5 weeks storage

Gas type	Storage time	Nitrogen (%)	Change
Pure Nitrogen	Specification label from BOC*)	99.5 +/- 0.17	-0.20% (n.s)
	After 5 weeks in the pouch	99.3 +/- 0.64	
Mixed 5%O2:5%CO2:90%N2	Specification label from BOC	89.8	-0.50% (n.s)
	After 5 weeks in the pouch	89.3 +/- 0.18	

*) Specification label from manufacturer (BOC gases) attached to the gas bottle.

3.3.3. Spermatozoa morphology

3.3.3.1. Spermatozoa dried in gases containing oxygen

Drying onto tissue culture plates. All gasses effectively dried the 100µl sperm drop down to a dry spot on the dish surface. By adding 100 µL TC-grade water, the blot of sperm suspension was rehydrated and allowing the spermatozoa to re-expand. Further observations were done on drops (10µL) of rehydrated spermatozoa suspension on a glass slide observed under microscope.

The results show that most dried spermatozoa remained intact after rehydration (Table 3.4). The morphological integrity, (whole intact spermatozoa, spermatozoa with bent tails and sperm heads without a tail) was assessed for at least 200 spermatozoa from several different fields. The proportions of each type of spermatozoa are described in table 3.4.

3.3.3.2. Differential staining.

Following differential staining with 2% Eosin-Y in PBS, all spermatozoa took up the dye, indicating that no spermatozoa had an intact membrane.

Table 3.4. Effect of storage temperature for spermatozoa dried using compressed medical air and filtered air (spermatozoa morphology expressed as % of all spermatozoa) [1]

Storage Temp[a].	Spermatozoa Morphology[b]	Medical air			Filtered atmosphere air		
		M2	PBS	mHBS	M2	PBS	mHBS
RT	Intact[2]	70	81	76	61	73	78
	Bent Tail [3]	12	10	8	27	9	13
	Head[4]	18	9	16	12	18	9
4°C	Intact	73	76	73	76	73	76
	Bent Tail	20	10	20	14	15	12
	Head	7	14	7	10	12	12
-20°C	Intact	71	71	76	70	61	65
	Bent Tail	24	23	17	14	29	19
	Head	5	6	7	16	10	16

n.s between temperature and type of air

P<0.01 between proportion of each spermatozoa morphology.

1) Based on observation on at least 200 spermatozoa from several different fields.

2) Spermatozoa with intact head, neck and tail

3) Spermatozoa with tail hooked to the neck

4) Only sperm head without tail (Detached head)

The dried spermatozoa had been stored for 1 week. Spermatozoa morphology was recorded within 1 hr of rehydration.

Table 3.5. Effect of drying and storage protocol on spermatozoa morphology (%) after drying in 5% O_2, 5% CO_2, 90 N_2. Proportion (%) of spermatozoa with intact, and spermatozoa with bent tail or detached heads.

Medium	Storage Temperature	Morphology (%)[*]		
		Normal	Bent tail	Head
M2	RT	61	29	10
	4°C	72	12	16
	-20°C	61	34	5
PBS	RT	73	18	9
	4°C	76	12	12
	-20°C	60	22	18
mHBS	RT	71	15	13
	4°C	69	24	7
	-20°C	71	14	15

Spermatozoa were rehydrated after 1 week of storage

[*] Classification as in table 3.4.

Table 3.6. Effect of collection media and storage conditions on spermatozoa morphology after drying in 100% CO_2. Proportion (%) of spermatozoa with intact, and spermatozoa with bent tail or detached heads.

Medium	Storage Temperature	Morphology (%)*)		
		Normal	Bent tail	Head
M2	RT	83	7	10
	4°C	69	25	6
	-20°C	73	15	12
PBS	RT	68	18	14
	4°C	80	10	10
	-20°C	72	18	10
mHBS	RT	69	13	18
	4°C	78	10	12
	-20°C	70	23	7

Spermatozoa were rehydrated after 1 week of storage

*) Classification as in table 3.4.

Table 3.7. Effect of collection media and storage conditions on spermatozoa morphology after drying in 5% CO_2 in air. Proportion (%) of spermatozoa with intact, and spermatozoa with bent tail or detached heads

Medium	Storage Temperature	Morphology (%)*)		
		Normal	Bent tail	Head
M2	RT	76	7	17
	4°C	66	24	10
	-20°C	77	7	15
PBS	RT	80	9	11
	4°C	65	15	20
	-20°C	60	25	15
mHBS	RT	71	9	20
	4°C	83	15	2
	-20°C	80	3	17

Spermatozoa were rehydrated after 1 week

*) Classification as in table 3.4.

3.3.4. Spermatozoa Functions

3.3.4.1. Membrane integrity.

Following differential staining with 2% Eosin-Y in PBS all spermatozoa took up the dye, indicating the membrane lost their integrity and became permeable.

3.3.4.2. Motility

Following rehydration, no motile spermatozoa were found in any sample. This indicated that spermatozoa experience severe damage to their mid-piece and membranes preventing spermatozoa motility.

3.3.4.3. Fertilising capacity

Since none of the dried spermatozoa were motile fertility had to be assessed by ICSI. Following ICSI none of the oocytes developed pronuclei, and extended culture did not show any evidence of embryo development (Table 3.8). This means the spermatozoa lost their ability to fertilise the oocyte.

Table 3.8. Oocyte development following ICSI using spermatozoa dried in gases containing oxygen.

Gas source	No. oocytes injected	No. oocytes survived (%)	In vitro development	No. Replicates
Medical air (tap)	62	24	Nil	3
Compressed air (bottle)	67	26	Nil	3
5%O_2; 5% CO_2; 90%N_2	64	22	Nil	3
100% CO_2	69	23	Nil	3
5% CO2 in air	65	26	Nil	3

3.3.5. The effect of activation on embryo development

To determine whether embryo development could be enhanced by artificial oocyte activation, oocytes were injected with sperm dried in different types of gases. Following manual ICSI, some oocytes were then activated using 8% ethanol in M2 at 37°C for 5 min.

Table 3.9. Oocyte development following ICSI with dried and fresh spermatozoa and activation in 8% ethanol in M2 at 37°C for 5 min.

Spermatozoa dried	Activated					Not-Activated				
	Inject N	Viable N	2PN N	2-cell N	Blast N	Inject N	Viable N	2PN N	2-cell N	Blast N
5% O_2	60	24	14	20	4	53	18	0	0	0
20%O_2	54	20	9	15	4	56	21	0	0	0
Medical air	43	16	N.A.	14	4	56	16	0	0	0
N_2	58	17	N.A.	14	6	60	21	17	13	6
Fresh sperm	28	11	11	10	9	32	12	8	8	6
Sham**)	48	N.A.	N.A.	N.A.	40	38	35	N.A.	N.A.	0
Not Injected	38	N.A.	N.A.	N.A.	34	30	N.A.	N.A.	N.A.	0

*)5% O2 spermatozoa dried in mixed gas contains 5% O2, 5% CO2 and 90% N2; 20% O2 spermatozoa dried in mixed gas contains 20% O2 in air.
**) Sham oocytes were punctured without spermatozoa injection
N.A: Not assessed.

Results in table 3.9 showed that spermatozoa dried in gas contain oxygen lost their ability to fertilise and activate oocytes and develop into normal embryos. Activation did allow some oocytes injected with mixed gas containing oxygen to develop to blastocysts. Spermatozoa dried in nitrogen were able to fertilise oocytes and develop into blastocyst, similar to those in oocytes injected with fresh spermatozoa even though the proportion of dried spermatozoa ICSI embryos were slightly lower.

Manual spermatozoa injection itself contributed to the reduction in oocyte survival. The data in table 3.9 showed that most of the sham and the non injected oocytes were becoming blastocysts (40 of 48 and 30 of 38). In the sham oocytes the zona and membranes were punctured. Manual ICSI involves puncturing the zona and membrane and then expelling the spermatozoa into the cytoplasm, and causes a significant reduction in oocytes survival after ICSI.

3.3.6. Spermatozoa dried and stored in Nitrogen

3.3.6.1. Swim up

In both group A and B, epididymides were placed in warmed handling media to swim up the spermatozoa. Normal motile spermatozoa were obtained following swim up in the medium.

Spermatozoa in group C did not swim up. As no motile spermatozoa were found after placing epididymides in the oxygen free medium the spermatozoa had to be obtained by gently shaking the suspension to release spermatozoa from epididymides.

3.3.6.2. General appearance.

Group A treatment formed a milky blot in the TC-dish on drying as a result of the sperm suspension leaving cells and salts behind.

In group B and C, following drying using dry nitrogen gas, the sperm suspension coated the inner wall of the straw formed a milky coloured layer in the inner surface of the straw as a result of salt sedimentation similar to those group A.

3.3.6.3. Morphology.

Following after rehydration of spermatozoa from group A, we found high the proportion spermatozoa with physical damage such as bent and broken tail. Staining using 2% Eosin-Y in PBS showed that all spermatozoa heads took up the dye.

Group B, as in the previous section in this chapter, following rehydration most of the dried spermatozoa showed a normal morphology. However the number of spermatozoa recovered from each straw was dramatically reduced compared to the original concentration. Some times no spermatozoa were recovered. There was some loss due to the spillage during drying by blowing nitrogen gas through the straw (Fig. 3.3). Some abnormal spermatozoa were observed including spermatozoa with a broken tail or without a tail. No motile spermatozoa were found, and staining using 2% Eosin-Y in PBS showed that all spermatozoa heads took up the dye.

Group C spermatozoa were handled and dried in a modified, dry nitrogen gassed, hood to provide a oxygen free environment. As in group B, the dried spermatozoa in group C were immotile and took up eosin stain.

3.3.6.4. ICSI with nitrogen dried spermatozoa

3.3.6.4.1. Group A,

Individual heads were ICSI'd into mouse oocytes, but none of the injected oocytes developed into embryos after in vitro culture. This result was similar to spermatozoa that were dried in gases containing oxygen.

3.3.6.4.2. Group B,

Individual heads were ICSI'd into mouse oocytes. In vitro culture showed that the oocytes formed embryos and developed in vitro into blastocysts (Table 3.10 and 3.11). Transfer of 2-cell stage embryos resulted in the birth of normal offspring.

Tables 3.10 and 3.11 show that the proportion of oocytes that developed into 2 cells following ICSI using spermatozoa dried in mHBS (N=344) or M2 (N=167) were 20.35% and 17.96% respectively. The proportion of oocytes developing into blastocysts per injected oocyte was 7.85% and 7.19% respectively. These results are comparable to the results obtained after ICSI with fresh spermatozoa as the control for which the proportion of oocytes that fertilised (2PN) and developed into blastocysts after ICSI was 30% and 3% (N=93), respectively.

Table 3.10. ICSI with spermatozoa dried using dry nitrogen in mHBS (Group B)

	Injected	Survived	%	2 PN	%	2 Cell	%	Blastocyst	%
Day 1	70	67	95.7	13	18.6	18	25.7	6	8.6
Day 2	35	30	85.7	5	14.3	3	8.6	1	2.9
Day 3	40	40	100.0	7	17.5	12	30.0	4	10.0
Day 4	46	36	78.3	8	17.4	8	17.4	1	2.2
Day 5	60	44	73.3	8	13.3	8	13.3	5	8.3
Day 6	41	35	85.4	8	19.5	8	19.5	4	9.8
Day 7	20	12	60.0	6	30.0	6	30.0	2	10.0
Day 8	32	29	90.6	7	21.9	7	21.9	4	12.5
Total	**344**	**293**	**85.2**	**62**	**18.0**	**70**	**20.4**	**27**	**7.9**

Table 3.11. ICSI with spermatozoa dried using dry nitrogen in M2 medium (Group B).

	Injected	Survived	%	2 PN	%	2 Cell	%	Blastocyst	%
Day 1	24	6	25.0	5	20.8	8	33.3	2	8.3
Day 2	28	12	42.8	9	32.1	6	21.4	2	7.1
Day 3	26	9	34.6	6	23.1	8	30.8	4	15.4
Day 4	20	4	20.0	0	0.0	0	0.0	0	0.0
Day 6	18	6	33.3	3	16.7	2	11.1	0	0.0
Day 7	10	6	60.0	3	30.0	1	10.0	1	10.0
Day 10	30	10	33.3	3	10.0	2	6. 7	2	6. 7
Day 11	11	4	36.3	1	9.1	1	9.1	1	9.1
Total	**167**	**57**	**34.1**	**30**	**17.9**	**28**	**16.8**	**12**	**7.2**

3.3.6.4.3. Group C

Oxygen is detrimental particularly for dry biological material (Brawn and Fridovich, 1981) because it stimulates free radical damage. The uses of oxygen free media and environments for swim up did not result in motile spermatozoa. ICSI using spermatozoa from group C produced viable embryos (Table 3.12).

On day 15 after ET there were no implantation sites on either uteri of the foster. This indicated that the embryos did not grow in vivo. There is a possibility that this problem arose because the female was day 3 rather than day 1 pseudopregnant at the time of transfer.

Table 3.12. Results of ICSI using oxygen free medium.

	Oxygen free medium	Control (dried in oxygen containing medium)
No oocytes injected	46	31
Survived (N)	17	12
2PN (N)	5	5
ET (N 2 cells 30 hr later)*)	5	5

*) Embryos were transferred into 1 foster, but no implantation occurred possibly because the foster was not day 1 pseudopregnant at the time of transfer.

3.3.6.5. Foetal development and offspring

Only oocytes injected with spermatozoa from group B developed in vivo after ET. There was no ET from group A. None of oocytes from group C developed after embryo transfer (Table 3.12).

Embryo transfer of oocytes from group B showed 3 of 5 (60%) embryos ICSI'd with fresh spermatozoa developed into fetuses with 2 resorption sites. Embryo transfer after ICSI with dried spermatozoa showed 4 of 5 (80%) embryos developed into fetus with one resorption site.

Table 3.13. Foetal development after ICSI using spermatozoa dried using dry nitrogen.

Treatments	Injected	Survived (% of injected)	2-cell (% of injected)	E.T (% of injected)	Day 15 fetus (% of ET) *	Implantation sites without a fetus
Fresh	16	6 (37.5)	5 (31.3)	5 (31.3)	3 (60.0)	2
Dried	20	5 (25.0)	5 (25.0)	5 (25.0)	4 (80.0)	1

n.s between fresh and dried spermatozoa.

The fetuses derived from dried spermatozoa had developed normally. The fetus weight at day 15 were 30.3±0.43 grams for fresh and 30.4±0.34 grams for dry spermatozoa and no different (P<0.05) to fetuses derived from fresh (control) spermatozoa. All fetuses had normal extremities (indicated by the number and stage development of the digits in each extremity, and in their general morphology in their body and head.

Further experiments therefore concentrated on the use of spermatozoa dried as in group B, by allowing the pregnancy to term.

Table 3.14. Offspring produced after ICSI using spermatozoa dried using dry nitrogen.

Injected	Survive	2-cells (% of survive)	ET (% of survive)	Offspring (% of ET)	Foster (n)
29	13 (44.8%)	7 (24.1%)	7 (24.1%)	5 (71.4%)	F1 (1)
96	31 (32.3%)	18 (18.8%)	18 (18.8%)	5 (27.8%)	Balb-C (4)

3.3.6.6. Fertility of F-2 mice derived from dried spermatozoa in group B

To evaluate whether the fertility of offspring derived from dried spermatozoa was normal, the first generation of F2 pups derived from dried spermatozoa, 4 females and 1 male, were sibling mated , the results are given in table 3.15.

Table 3.15. Fertility of the offspring produced from embryos generated with using spermatozoa dried using dry nitrogen.

Female no.*)	No. pups (F3)
1	4
2	6
3	5
4	11

All F2 females delivered normal offspring after mating to the F2 male. The number of pups per litter varied but all F3 pups were normal.

3.3.7. Repetition of experiment 3.3.6 (Group B) with spermatozoa dried and stored in nitrogen

3.3.7.1. Different sources of BSA

Several attempts were made to repeat and improve on the results of nitrogen drying using protocol B. However none those additional attempts produced viable offspring. In retrospect it appeared possible that different sources of BSA or the media could have influenced the results (the original batch ran out). Two different sources BSA (Invitrogen and Sigma) and 3 different m[illegible]a were therefore tested. After swim up there were more than 90% progressive moti[illegible] spermatozoa in mHBS and KSOM. However no progressively motile spermatozoa were found in Na. EGTA, only vibrating spermatozoa were found. Spermatozoa were dried for all groups in straws using Nitrogen followed by storage in aluminium bags filled with nitrogen at room temperature as described earlier.

All these dried spermatozoa were used for ICSI by using manual ICSI by Maria Diamente or Piezo drill ICSI by Dr. Orly Lacham-Kaplan

Table 3.16. Repeating Nitrogen dried spermatozoa (Group B) using manual ICSI with different source of BSA.

Medium	BSA source	No. oocytes	2-PN (%)	2-Cells (%)	ET (2-cells)	No. repeat/s
Na-EGTA	Albumax	28	2 (7.1)	0 (0)	0	1
	Sigma	32	4 (12.5)	0 (0)	0	1
	Without BSA	26	2 (7.7)	0 (0)	0	1
mHBS	Albumax	69	31 (44.9)	26 (37.7)	26x2-cells	3
	Sigma	21	3 (14.3)	0 (0)	0	2
	Without BSA	26	10 (38.5)	7 (26.9)	7x2cells	1
KSOM	Albumax	67	15 (22.4)	12 (17.9)	12x 2-cells	2
	Sigma	38	12 (31.6)	8 (21.1)	8x2-cells	2
	Without BSA	28	8 (28.6)	6 (21.4)	6x2-cells	1

The results showed that spermatozoa dried in mHBS and KSOM had a higher 2-PN development compared to Na-EGTA ($P<0.01$). Spermatozoa dried in medium containing Albumax BSA had a higher in vitro development compared to Sigma BSA and without BSA ($P<0.01$). There was no pregnancy obtained after transfer those embryos into recipient foster.

Table 3.17. Repeating Nitrogen dried spermatozoa (Group B) using Piezo ICSI with different source of BSA (Data from one experiment).

Medium	BSA source	No. oocytes	2-PN (%)	2-cell (%)	E.T (2-cells)
Na-EGTA	Albumax	34	16 (47.1)	12 (35.3)	12x2cells
	Without BSA	28	10 (35.7)	9 (32.1)	9x2cells
mHBS	Albumax	74	25 (33.8)	22 (29.7)	22x2cells
	Without BSA	62	23 (37.1)	20 (32.3)	20x2cells

None of the transferred embryos developed into offspring and the uterine horns of the fosters did not reveal any implantation sites. This indicated that none of the transferred embryos developed in vivo.

3.4. Discussion

The results showed that even though the spermatozoa looked intact, they failed to recover motility after drying and were permeable to Eosin Y. A proportion of these spermatozoa were however capable of fertilizing oocytes and producing viable embryos. This is in agreement with other studies which show that abnormal or even incomplete spermatozoa can fertilise and produce embryos. Ohsumi *et al.* (1988) showed that nuclei from human spermatozoa could develop into pronuclei in Xenopus oocytes. When lysolecithin (LC)-and dithiothreitol (DTT)-treated spermatozoa were incubated with egg extract prepared with an extraction medium containing 10 mM EGTA, sperm nuclei decondensed and formed into chromosomes, bypassing the pronuclear stage. On the contrary, LC-DTT-treated spermatozoa incubated in egg extract without EGTA mimicked LC-permeabilised Xenopus spermatozoa in that they underwent pronucleus formation accompanied by DNA synthesis and subsequent chromosome condensation in the correct chronological order.

The fertilising ability of damaged spermatozoa at various levels of disintegration (cellular and molecular) has been investigated in homologous (mouse) and heterologous (human spermatozoon, hamster oocyte) models. Live pups were produced after destruction of spermatozoa at various cellular and molecular levels followed by injection into oocytes (Ahmadi and Ng, 1999b). These studies demonstrated that with

damaged spermatozoa, the key point in the fertilization process is the activation of the oocyte by injection of cytosolic sperm factor. A similar fertilization rate as that using live intact spermatozoa can be achieved following activation. However, the integrity of the genetic material influenced in-vitro development of the embryos and live fetuses.

There are several possibilities why only some of the dried spermatozoa studied in this chapter formed pronuclei and embryos. Oxygen is one possible cause of the problem. The influence of oxygen during drying and storage may have an adverse effect on the sperm DNA and destroyed their capacity to develop into normal embryos after ICSI. Reactive oxygen metabolites are known to disrupt sperm-oocyte fusion, spermatozoa movement, and DNA integrity; however, the relative sensitivities of these elements to oxidative stress are unknown (Aitken, *et al*, 1998 and Lopes, *et al*., 1998). Oxygen is also well known to attack dry membranes, producing free radicals that are extremely damaging (Crowe and Crowe, 1988).

Although many investigators (Aitken, *et al*, 1998 and Lopes, *et al*., 1998, Crowe and Crowe, 1988) have observed the lethal effect of air on dried cells and measured inactivation rates, few have reported quantitative data on the effect of intermediate oxygen concentrations. The relationship between loss of viability and oxygen is not linear. Since a small amount of oxygen can have a great effect on viability of dried cells, it is important to seal samples in glass ampoules under high vacuum to obtain maximum survival of sensitive cells.

Medical air and atmospheric air, both are similar in their composition; they mainly comprise of 21% oxygen, .2 % carbon dioxide and 78% nitrogen. The high oxygen concentration in the air may lead to an adverse effect on spermatozoa ability to develop into normal embryos. Similar to those in medical air, mixed gas also posses some oxygen molecules in their composition.

Air-drying processes are associated with the risk of oxidative and thermal damage (Lavelli, *et al*., 1999).

The failure of the spermatozoa dried in air or mixed gas to fertilise oocytes after ICSI showed that morphological integrity is different to fertilising ability. Similarly non intact spermatozoa from bull (Goto, *et al*., 1990) or mouse (Wakayama, *et al*., 1998) those were frozen thawed without cryoprotectant or freeze dried mouse spermatozoa (Wakayama and Yanagimachi, 1998, Kusakabe *et al*., 2001) could fertilise

oocytes and produce live offspring. In those experiments, spermatozoa were stored under oxygen free condition, such as, liquid nitrogen (Goto *et al.*, 1990, Wakayama *et al.*, 1998), argon (Wakayama and Yanagimachi, 1998) or vacuum (Kusakabe *et al.*, 2001).

Spermatozoa were dried and stored in dry nitrogen gas. Dry nitrogen gas for drying and storage has been used in food industries. Nitrogen displaces oxygen, and maintaining an oxygen free environment will prevent oxidation. This condition has been used to preserve wine and prevent it from oxidation.

Results in this study (Table 3.8 and 3.9) showed that spermatozoa dried and stored in oxygen or dried with nitrogen but stored under oxygen could not fertilise oocytes.

Yanagimachi's team showed that freeze dried and morphologically damaged hamster, human and mouse spermatozoa could fertilise oocytes and develop into embryos and offspring following ICSI (Katayose, *et al*, 1992; Ogura *et al.*, 1996; Tateno *et al.*, 1998; Wakayama, *et al.*, 1998; Wakayama and Yanagimachi, 1998 and Kusakabe, 2001). Those spermatozoa were immotile and considered to be dead, but storage under oxygen free environment retained the mouse spermatozoa's ability to fertilise the oocytes. These results all indicated that live and motile spermatozoa are not required to fertilise oocytes.

Despite difficulties that have been encountered in maintaining strict anaerobes, they may not be more sensitive to oxygen in the dried state than aerobic organisms (Phillips, *et al.*, 1975). By maintaining absolute oxygen free conditions, during growth and reconstitution, biological material can be preserved well by lyophilisation (Phillips, *et al.*, 1975). Although their dried cultures were not deliberately exposed to oxygen for a prolonged period, they were exposed to air for about 10 minutes between the primary drying and re-evacuation before sealing the ampoules. Therefore previous failures to preserve anaerobic cultures by lyophilisation possibly were not due to the effect of oxygen on dry cell, but rather to the effect of oxygen in rehydration media (Heckly, 1978).

Without oxygen the injury might be repairable if the cell were given proper conditions, such as rehydration with oxygen free water. But if oxygen is admitted, it very rapidly makes the injury permanent and the cells that are no longer able to form a

colony. Israeli *et al.* (1975) concluded that drying damage is often reversible and that repair required protein synthesis. They also demonstrated that after exposure to oxygen the injury become irreversible and the bacterium can no longer form a colony.

Similar to those in Wakayama and Yanagimachi (1998) and Kusakabe *et al.* (2001), spermatozoa dried and stored in dry nitrogen gas produced viable embryos and offspring. Unfortunately subsequent attempts failed to repeat that result. Despite those spermatozoa that had been stored under oxygen free environment, developed into two cell embryos, the subsequent experiments failed to produce normal offspring after ET those embryo into recipient foster. There were some undefined technical problems that still need to be explored and solved.

Spermatozoa in the oxygen free medium were not motile. This indicated that oxygen is required to obtain motile spermatozoa. Spermatozoa dried in the absence of oxygen in the medium, atmosphere and storage system showed no pregnancy after ICSI and embryo transfer.

Those results indicated that factors other than the albumin, medium and oxygen contributed to the failure in repeating the first result. Ability to maintain chromatin stability may play an important role in fertilisation and foetal development.

Intact sperm DNA matrix is required to allow normal fertilisation (Kasai, *et al.*, 1999; Kishikawa, *et al.*, 1999; Ward *et al.*, 1999 and 2000). Ward *et al.* (2000) showed evidence that the structural integrity of mouse sperm nuclear matrix may be necessary for the proper unpacking of sperm DNA for participation in embryogenesis. It is likely that the sperm nuclear matrix contributes to the organisation of the sperm DNA and its disturbance can seriously damage the paternal genome or its expression.

Ward *et al.* (1990; 2000) further suggest that even very subtle changes in the sperm nuclear structure may have a significant impact on embryo development.

3.5. Conclusion

Spermatozoa dried with air or mixed gases that contain oxygen failed to develop following ICSI into mouse oocytes. This was attributed to damage by oxygen.

In one experiment mouse spermatozoa that were dried and stored under nitrogen retained functional integrity as 7.85% (mHBS) and 7.19% (M2) oocytes developed into

blastocysts, and formed viable fertile offspring after ICSI. The low proportion of oocytes that developed into blastocysts from either fresh or dried spermatozoa was mostly due to a low proportion of oocytes surviving ICSI. That problem was mainly due to the manual injection, which gives lower survival rates than Piezo injection (Huang *et al.*, 1996).

Subsequent experiments with nitrogen dried and stored spermatozoa resulted in 2-PN and 2-cells embryos, but no further offspring were born. The reason for this variability could not be established.

Chapter 4. Vacuum Drying of Mouse spermatozoa

4.1. Introduction

The evaporative drying procedures tested on mouse spermatozoa in chapter 3 gave inconsistent results. This chapter therefore evaluated vacuum drying as an alternative method for drying spermatozoa.

Vacuum drying is an integral part of freeze drying protocols, but can be used on liquid, non-frozen, samples. Freeze drying commonly includes cooling to -40°C to transform the water into ice, then applying a vacuum to remove the ice by sublimation. Mouse spermatozoa are both fragile and chill sensitive, with the result that freezing to -40°C is detrimental to mouse spermatozoa resulting in some loss of cellular viability and function (Korber, et al., 1991)

Following fusion of a sperm with the egg membrane, the spermatozoa undergo decondensation to unpack its DNA. Mammalian sperm DNA is the most tightly compacted eukaryotic DNA (Balhorn, 1982; Ward and Coffrey, 1991), being at least six-fold more highly condensed than the DNA in mitotic chromosomes. To achieve this high degree of packaging, sperm DNA interacts with protamines (Maier et al., 1990) to form linear, side-by-side arrays of chromatin. This differs markedly from the bulkier DNA packaging of somatic cell nuclei and mitotic chromosomes, in which the DNA is coiled around histone octamers to form nucleosomes (Balhorn, 1982; Ward and Coffrey, 1991).

Protamines are small, highly basic proteins that replace histone and testicular basic proteins during the development of mature spermatozoa during spermatogenesis (Lee and Cho, 1999). Destruction of protamine in human sperm inhibits sperm binding to and penetration of the zona-free hamster oocyte (Ahmadi and Ng, 1999), but increases sperm head decondensation and male pronuclear formation in the hamster-ICSI assay pronuclei. (Yanagida et al., 1991). During decondensation in egg cytoplasm, sperm nuclei protamine is replaced by histone.

Heat stress causes spermatozoal DNA denaturation by decreasing protamine disulfide bonding at the spermatogenic cell stage and reflected during ejaculation (Love and Kenney, 1999). Watson (2000) showed that freezing alters functional status of

spermatozoa (e.g., membrane stability, oxidative damage, membrane receptor integrity, nuclear structure).

To circumvent the problems associated with freezing, this study evaluates whether vacuum drying of non-frozen specimens would provide an effective alternative to evaporative drying and freeze-drying. This was considered to be particularly important the chromosomal damage reported by Kusakabe et al (2001) may be due to damage incurred during freezing rather than the vacuum drying itself.

4.2. Materials and methods

Mouse spermatozoa were obtained from the cauda epididymis of 8 week old male C57xCBA F1 mice. The cauda was cut and placed into pre-warmed (37°C) handling medium, M2 (Sigma, St. Louis MO) to allow spermatozoa to swim out into that medium at room temperature. Approximately 50 µl of the spermatozoa suspension was placed into 2 ml polypropylene vials (Quantum, Australia). The vials were then placed in a speedy-vac (Dynavac) at 1 mTorr pressure at room temperature for 8 hours to remove water. After 8 h, the vials were removed and their lids closed before being individually wrapped with aluminium foil and stored at 4°C. Prior to use, the dried spermatozoa were rehydrated by adding 50µl of tissue culture water (CSL, Melbourne) into the vial at RT. Spermatozoa were individually aspirated into an intracytoplasmic injection pipette and injected into C57xCBA F1 metaphase II oocytes. The injected eggs were cultured in M16 (Sigma) at 37°C in 5%CO_2 with humidified air for 72 hours.

4.2.1. Confocal microscopy study on sperm decondensation following ICSI

To assess the rate of sperm head decondensation groups of oocytes were fixed 0, 15 min, 30 min, 1 hr, 2 hr and 3 hr after ICSI. The eggs were fixed using 3% Glutaraldehyde in 1 M Na-Cacodylate buffer, then stained with 100µg/mL propidium iodide (PI) then stored at 4°C in the dark.

The fixed and stained eggs were placed on a cover slip attached to a glass slide. Observations were made on a Bio-Rad confocal microscope with 548 nM to excite PI. Pictures were taken using Z-series with Kalman filter with 6 times scanning. Pictures were manipulated using Confocal Assistant ver 4.02.

4.2.2. Experimental design

4.2.2.1. Embryo development

To determine the effect of different storage times on the ability of vacuum dried mouse spermatozoa to form embryos, oocytes were injected with spermatozoa that had been stored up to 28 days after drying. The resulting embryos were then in vitro cultured until blastocysts. These results were compared to the ability of fresh spermatozoa to form embryos.

4.2.2.2. Sperm decondensation

To determine whether vacuum dried spermatozoa undergo normal sperm decondensation some eggs were fixed in 3% Glutaraldehyde in 1 M Na-Cacodylate buffer at 0, 15, 30 min, 1 hr, 2 hr and 3 hr after ICSI. In this study vacuum dried spermatozoa compared to the fresh spermatozoa.

4.3. Results

4.3.1. Fresh Sperm

The fresh spermatozoa prepared by swim up in M2 from the epididymis were highly motile (more than 90% motile). The sperm concentration after swim-up was more than $5X10^7$/ml. Control (fresh) spermatozoa showed similar proportion of motile spermatozoa after swim up. Any spermatozoa showing agglutination were not used.

4.3.2. Vacuum drying spermatozoa

4.3.2.1. Macroscopic appearance

Following vacuum drying, the spermatozoa suspension formed a thin yellowish powder like residue on the bottom of the vial. The colour of the powder was attributable the ingredients of the M2 medium. After rehydration with 50µL of MQ-water, the powder immediately dissolved and formed a yellowish clear suspension and no crystals or sedimentation were observed.

4.3.2.2. Spermatozoa morphology

Light microscopic observation using an Olympus microscope at 100x magnification showed that following rehydration, most spermatozoa were morphologically normal. However some abnormal spermatozoa were found with hooked tail or with damaged (broken) tail. The proportions were similar to those seen in chapter 3. No motile spermatozoa were found, and staining using 2% Eosin-Y in PBS showed that all spermatozoa heads took up the dye.

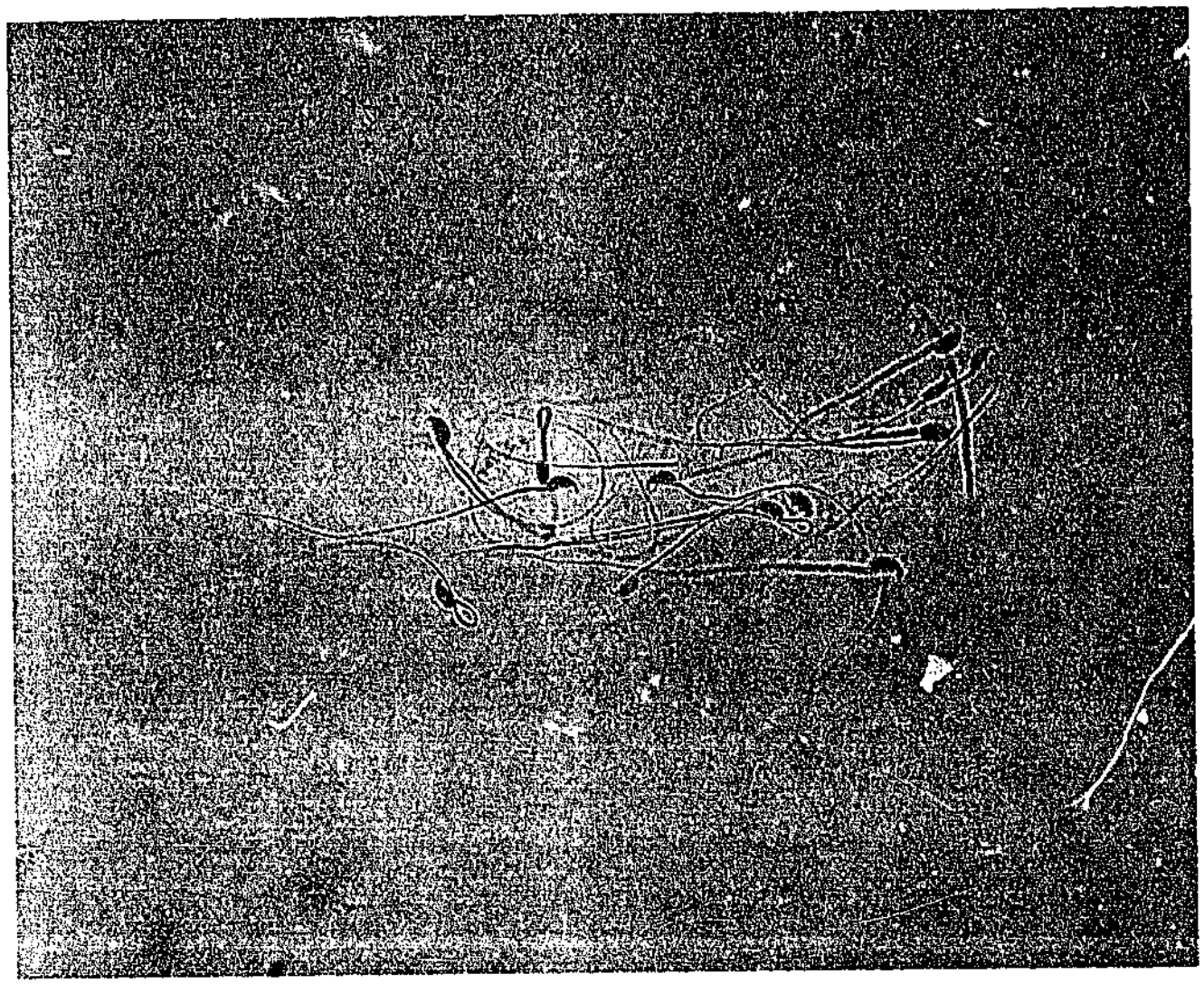


Fig. 4.1. Morphology of dried spermatozoa stained with Eosin-Y.

4.3.2.3. Intracytoplasmic sperm injection

Since there was no motility following rehydration, the spermatozoa were individually injected into eggs. The results are shown in table 4.1.

Table 4.1. ICSI using vacuum dried sperm.

	Injected	Intact		2-cells		Blastocysts	
		N	%*	N	%*	N	%*
Day 1	35	12	34	6	17	5	14
Day 2	13	5	38	3	23	1	8
Day 3	37	12	32	10	27	4	11
Day 4	15	8	53	3	20	2	13
Day 5	12	4	33	4	33	1	8
Day 6	38	27	71	12	31	5	13
Day 7	36	13	36	10	28	2	6
Day 14	24	8	33	5	21	2	8
Day 21	38	12	32	9	24	4	11
Day 28	41	19	46	11	27	2	5
Total for Vac-dry	289	121	42	73	25	28	9
Total for Fresh (control)	293	124	42	80	27.	39	13

No significant difference was observed between ICSI using control (fresh) and vacuum-dried spermatozoa.

* % of injected eggs.

Following ICSI the number of surviving oocytes was 42.32% with fresh (N=293) and 41.52% with dried (N=289) spermatozoa (P<0.2, NS). The cleavage rate (2-cells) was 27.30%, and 25.26%, and the proportion forming blastocysts was 13.31%, and 9.69% for fresh and dry spermatozoa respectively. Among the dried groups the lowest proportion forming 2-cells was 20% for sperm stored for 2 weeks, and the lowest proportion forming blastocysts was 5% for sperm stored for 4 weeks (Table 4.1)

Statistical analysis using Two Factor ANOVA showed no differences (P>0.05) between storage time or between fresh and vacuum dried spermatozoa. However, there is a significant reduction (P<0.01) in the development from 2-cells to blastocyst in the oocytes fertilised with dried spermatozoa compared with the controls.

4.3.3. Confocal microscopy study on sperm decondensation after ICSI

Evaluation by confocal microscopy showed a significant delay in sperm head decondensation following ICSI of dried sperm as compared to fresh sperm. Observation under the confocal microscope showed that the sperm head of vacuum dried spermatozoa remained intact, while the head of fresh spermatozoa swelled within 15 minutes (Table 4.2). The swelling heads indicated sperm head decondensation that associated with the event when sperm DNA are unpacked. Unpacked sperm DNA dispersed in the egg cytoplasm and formed male pronuclei. Vacuum dried spermatozoa began to decondense 3 h after ICSI (Table 4.2).

Table 4.2. Sperm h l decondensation after ICSI.

Time	0 min	15 min	30 min	1 hr	2 hr	3 hr
Vacuum dried	Intact	Intact	Intact	Intact	Intact	Decondense
Fresh	Intact	Decondense	Disappear	Disappear	Disappear	Disappear

The delay in sperm decondensation was also reflected on subsequent embryo development such as the pronuclear formation, cleavage and blastocyst formation. However the delay did not affect embryo quality judged from its morphological appearance.

Table 4.3. Delay in embryo development after ICSI using vacuum dried sperm.

	Stage	2 PN	2-Cell	4-Cell	Morula	Blastocyst
Fresh*)	Time (h)	8.71	11.14	25.71	72.00	93.43
	SEM	0.29	0.34	1.71	0.00	1.21
Vacuum dried*)	Time (h)	13.28	26.57	48.00	84.00	116.57
	SEM	0.89	1.21	0.00	0.00	2.21
Delay**)	h	4.57	15.43	22.29	12.00	23.14

*) Significant difference ($P<0.01$) fresh vs. vacuum dried spermatozoa.
**) Compared to the fresh

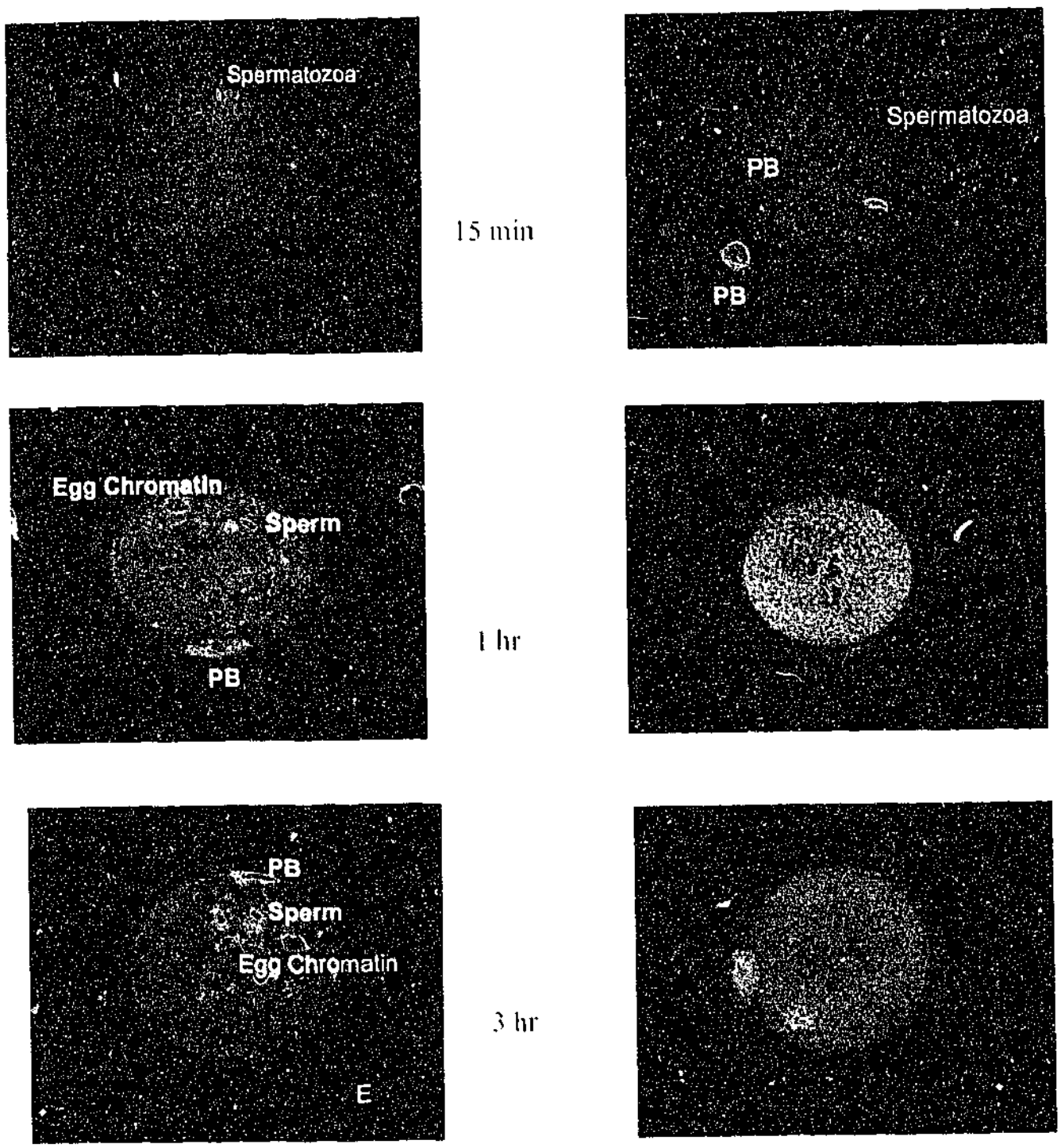


Fig. 4.2. Sperm decondensation events at 15 min (Top), 1 hr (middle) and 3 hr (bottom) after ICSI using vacuum dried (left) and fresh (right) spermatozoa. Vacuum dried sperm remain intact, while fresh sperm head had decondense at 15 min disappear at 1 and 2 hr after ICSI.

Statistical analysis showed no difference ($P>0.05$) in the proportion of eggs which developed into blastocysts after ICSI using vacuum dried spermatozoa. However there was a significant ($P<0.01$) delay in embryo development following ICSI using vacuum dried spermatozoa as compared to fresh spermatozoa.

4.4. Discussion

Vacuum drying is an integral part of freeze drying. Vacuum drying would have maintained a low partial pressure of oxygen during drying, but this machine did not allow the vials to be sealed under oxygen free conditions and it is likely that oxygen infiltrated the samples during their removal from the flask and the sealing of the vial. Sperm evaporative drying using gas containing oxygen did not result in embryos developing after ICSI, but this effect was not apparent for the vacuum dried spermatozoa, even though they too would have been exposed to oxygen. This indicated that oxygen might be most disruptive to sperm integrity while moisture is present. This may be explained by the results from other publications (Aitken, 1995; Kim and Parthasarathy, 1998; Shen and Ong, 2000; Twigg et al., 1988) who showed that oxidative damages can be occurred in membrane or DNA.

Neither vacuum nor evaporative dried spermatozoa with or without oxygen produced any motile spermatozoa. This indicated that severe damage had taken place. It is however not known whether it was the membrane, or other components of the sperm e.g. the mid-piece that contains elements that are important to maintain sperm motility that caused this effect (Cerolini et al., 2001; Watson, 2000)

In this study, ICSI of vacuum-dried mouse spermatozoa into mouse eggs allowed 9.69% of the eggs to develop into blastocysts. This finding showed that vacuum drying might not disrupt the intactness of the sperm nuclear matrix. Since Ward et al., (1999; 2000) showed that structural integrity of the mouse sperm nucleus with stable nuclear matrix may be necessary for the proper unpackaging of sperm DNA for participation in embryogenesis. However it needs to be established whether the delays in embryo development, were due to time taken to repair DNA or to decondense and unwind the DNA, or slower activation.

The sperm nuclear matrix contributes to the organisation of the sperm DNA and its disturbance can seriously damage the paternal genome or its expression, hence even very subtle changes in the sperm nuclear structure may have a significant impact on embryo development (Ward, et al., 1999 and 2000).

Protamine cross-linking by disulfide (-SS-) bonds is the main factor responsible for the stability of the chromatin structure in mammalian spermatozoa (Andreetta, et al., 1995). Decreasing disulfide bonding is associated with an increased susceptibility of spermatozoa-DNA to denaturation in the absence of protamine changes (Love and Kenney, 1999). Drying in a vacuum, and storage in a dry state in air containing oxygen therefore do not alone prevent sperm from forming embryos capable of developing into blastocysts. Thus further research is required to explore what caused the delay in embryo development, and to establish better drying and storage protocols.

4.5. Conclusion

The results show that mouse spermatozoa subjected to vacuum drying and storage at 4°C for up to 28 days were able to fertilise mouse oocytes and allow those oocytes developed into blastocysts. There was a delay in the sperm decondensation and embryo development processes after ICSI using vacuum dried spermatozoa, but the proportion of embryo development was comparable to ICSI with fresh spermatozoa.

Chapter 5. Freezing and drying of cells following electroporation in the presence or absence of trehalose.

5.1. Introduction

5.1.1. Cell preservation

Cooling biomaterials to -196°C (the temperature of liquid nitrogen) is probably the most effective procedure of preserving their biochemical and morphological parameters, providing that the adverse effects of cooling can be controlled (Muldrew and McGann, 1994).

The damaging effect of cooling to these temperatures is caused mainly by the formation of intracellular ice. Traditional cryopreservation procedures help reduce damage by intracellular ice formation either by intracellular water loss during slow cooling or by adding high concentrations of glass-promoting solute(s) which maintain a vitrified state during cooling (Mazur *et al.*, 1993). Most of these freezing procedures rely on protection provided by penetrating cryoprotectants.

Most cryoprotectants are toxic, but this is only a problem if cells are very sensitive, or if it is to be combined with drying. The most commonly used cryoprotectants (e.g. DMSO, glycerol) are less volatile than water, with the result that cells which are dried or freeze dried in the presence of cryoprotectant will ultimately, once the water is removed, be exposed to 100% cryoprotectant which is toxic. Alternative, non-toxic, cryoprotectants may be essential for more effective drying and freeze drying protocols.

The non-reducing disaccharide trehalose (α-D-glucopyranosil 1-1,1-α'-D-glucopyranoside) is a cryoprotectant which provides positive protection to the plasma membrane of mouse spermatozoa (Storey *et al.*, 1998). Trehalose partially preserves inner mitochondrial membrane integrity after freeze-thawing (Tsvetkov *et al.*, 1985) and has been added as a cryoprotectant to preserve lung tissue (Bull *et al.*, 2000; Fukuse *et al.*, 1999), yeast (Diniz-Mendes *et al.*, 1999), endothelial cells (Isowa *et al.*, 1996), fibroblasts (Eroglu *et al.*, 2000) and human oocytes (Eroglu *et al.*, 2001). However only Eroglu *et al.* (2000 and 2001) has shown that intracellular trehalose alone protects mammalian cells against freezing and storage in liquid nitrogen.

5.1.2. Introduction of intracellular trehalose

Introduction of foreign material into the cytoplasm of cells is now a routinely used tool in cell biology and biotechnology. A range of techniques allow normally non-permeable foreign materials to enter a living cell including chemical, viral, physical (microinjection) or transgenic approaches.

Three published studies demonstrate that the introduction of trehalose into mammalian cells and human oocytes can protect them against drying or freezing. One study used a genetic engineering approach. Human primary fibroblasts were genetically engineered to express the *ots*A and *ots*B genes of *Escherichia coli*, which encode trehalose biosynthetic enzymes to induce intracellular trehalose production (Guo *et al*, 2000; Puhlev *et al.*, 2001). The second approach used alpha-hemolysin to create reclosable-pores in the cellular membranes. Using this method, Eroglu *et al* (2000) were able to load trehalose into cells. Low concentrations (0.2 M) of trehalose permitted long-term post-thaw survival of more than 80% of 3T3 fibroblasts and 70% of human keratinocytes.

Eroglu *et al.* (2001) introduced trehalose into human oocytes by microinjection. They found that small amounts of intracellular trehalose in the absence of any other cryoprotectant protected 63%, 53% and 66 % of human oocytes against cooling at 1°C/min to -15°C, -30°C and -60°C, respectively. Only 13% of control (non trehalose) and 22% of oocytes surrounded by extracellular trehalose alone survived cooling to -15°C, and none survived below that temperature. This proves that intracellular trehalose provided significant protection against freeze-associated stresses. However, the complexity of both of these methods would prevent them from becoming routine tools for cell drying.

This chapter investigates whether there are other simpler strategies that may let trehalose enter the intracellular compartment of mammalian fibroblasts.

5.1.3. Electroporation

In the present study, electroporation was examined as a method to introduce exogenous molecules into living cells. As outlined in the General Introduction (Chapter 1) membrane electroporation is a powerful technique that uses electric field pulses to render cell membranes permeable to otherwise impermeable substances.

Electroporation is affected by medium composition and conductivity (Djuzenova, *et al.*, 1996). Normal culture medium is high in sodium and calcium and low in potassium, while intracellular liquids are high in potassium and low in sodium and calcium. Since electropermeabilisation allows exogenous material into the cells, the electroporation medium may cross into the cell and affect further cell development.

While much is known about electroporation media, little is known about electroporation in the presence of trehalose. This chapter therefore aimed to ascertain whether trehalose incorporation during electroporation differs in three different electroporation media (DMEM, modified HBS, and Cytomix). These differ in ionic composition. DMEM is the normal cell culture medium and is high in sodium (110 mM) and low in potassium (5 mM) and calcium. Modified HBS (mHBS, or ITS) is rich in potassium (120 mM KCl) and low in sodium (10 mM NaCl) and calcium free (Eroglu, *et al.*, 2000). Cytomix (van den Hoff, *et al.*, 1992, 1995) is a modified electroporation medium which is rich in potassium (120mM KCl) and sodium free.

To ascertain that electropermeabilisation has occurred, cells can be electroporated in the presence of propidium iodide, a fluorescent dye activated by binding to cellular DNA (Weaver *et al.*, 1988; Bartoletti, *et al.*, 1989). Dead cells have a very bright red fluorescence as they are rapidly equilibrated with PI present in the pulse medium (Rutter *et al.*, 1992). Permeabilised cells that survive electroporation retain this PI (Djuzenova *et al.*, 1996). Viable -non permeabilised cells will not contain PI.

In this study mouse STO fibroblasts were electropermeabilised in three different electroporation media, in the presence or absence of trehalose and using propidium iodide (PI) as a reporter for the transient high-permeability state of the membrane. To differentiate between viable-permeable cells and non-viable cells, trypan blue dye was added into cell suspension after the pores had reclosed (approximately 30 min after electroporation).

5.2. Materials and Methods

5.2.1. Media

The culture medium, electroporation medium, Trypan Blue and Propidium Iodide were made and used as described in Chapter 2, (General Materials and Methods).

5.2.2. Cells

Mouse fibroblasts (STO cell line) were used in these experiments as described in chapter 2 (General Materials and Methods). Cells were in vitro cultured in DMEM supplemented with 10% FBS, at 37°C with 5% CO2 in air. Sub-confluent cultures were detached from the culture flask using 0.25% Trypsin-1mM EDTA in HBS (Gibco-Invitrogen). The cells were washed with DMEM 10%FBS then centrifuged at 1000 rpm for 5 min to obtain a pellet. The supernatant was withdrawn and the pellet was washed and resuspended in electroporation media.

5.2.3. Electroporation

5.2.3.1. Preliminary experiment

To obtain information about suitable electroporation settings and sugar types the cells were electroporated in each of three different sugars (trehalose, mannitol and sucrose) dissolved in Milli-Q water or media (DMEM and mHBS) at 0.2 and 0.3 M concentration, using methods described in sub section 5.2.3.2.

Cell viability was determined by taking a 10 μL drop and observed in a Neubauer haemocytometer chamber (as described in chapter 2) with trypan blue dye.

5.2.3.2. Cell electroporation

The cells were electroporated in three different media supplemented with 0; 0.1; 0.2; 0.3 or 0.4 M trehalose (Donation from British Sugar, MW, 378.3) and 100μg/mL Propidium Iodide (Sigma, P-4170, Sigma-Australia, MW 668.4). Prior to electroporation the viability of an aliquot of the cell suspension in each medium was tested using Trypan-Blue (Sigma, T-6146, Sigma-Australia).

Electroporation was performed in a 2 mm gap cuvette (Bio-Rad, UK) on a Bio-Rad Gene Pulser (Bio-Rad, UK) at voltages between 50 and 350 V with 50 volt increments and 500 capacitance with an exponential decaying pulse. A 400μL aliquot of STO cell fibroblast suspension at a concentration of 2-10 million cells/mL was loaded into a cuvette immediately prior to electroporation. A single electroporation pulse was applied to each cuvette (experiment 1). In experiment 2, the pulses were repeated 5 and 10 times with pulse strength between 50 to 250V in 0.4 M trehalose in Cytomix with 100 μg/mL PI.

Following electroporation, the cell suspensions were left in the cuvettes for 30 minutes at room temperature before adding Trypan-Blue (Sigma, T-6146, Australia) to assess viability. Immediate assessment using Trypan Blue may not be accurate since the pores take time to reclose and the pores would let Trypan Blue to across the membrane.

Observations were made on a fluorescent microscope (Leica-Germany) using Texas-Red filter and bright field. The images were captured using a digital camera (Leica DC-200-Germany), and analysed using Photoshop ver. 5.0. (Adobe). Images from the brightfield were selected, copied then pasted over the image from the fluorescence microscopy from the same observation field. Those images were blended using their luminosity to differentiate the dye excitation on each cell.

The viable and transiently permeabilised cells were PI positive but TB negative. The non-viable (dead) cells were both PI positive and TB positive. The viable but non-permeable cells were clear and not containing any dye. The classification is summarised in Table 5.1.Cells were then cultured in vitro at 37°C with 5% CO2 in air in 4-well NUNC dishes to determine their ability to grow in vitro.

Table 5.1. Classification of the cells following electroporation and staining.

	PI -	PI +
TB -	PI- and TB- Viable but not permeabilised	PI+ and TB- Viable and permeabilised
TB +	PI- and TB+ None observed (not possible)	PI+ and TB+ Not viable (dead)

Following a 24 hour recovery period in vitro culture, the cells were re-evaluated to ascertain how many plated cells contained intracellular PI (assessed under a

fluorescent microscope as described previously). The culture medium/supernatant (containing non-plated/non-viable cells) was then decanted from the culture dish, leaving only the viable (plated) cells behind. These plated cells were observed under epifluorescent microscopy with a Texas Red filter. Cells which took up PI during the electroporation step and plated had a red fluorescent cytoplasm while the non-permeabilised cells had a clear cytoplasm.

5.2.4. Freezing

Cells were frozen in electroporation medium with and without added cryoprotectants and with and without trehalose at two different time points (immediately and 30 min after electroporation) using two different freezing protocols.

5.2.4.1. Addition of permeable cryoprotectant

Cryoprotectants were added to the cell suspension immediately after electroporation. Two different cryoprotectants (Glycerol and DMSO) were used in this experiment. Both were used at the concentration normally used for conventional slow cooling of cells (8% Glycerol; or 10% DMSO), and at one-tenth the normal concentration (0.8% Glycerol; 1% DMSO) used for cell freezing.

To achieve the required cryoprotectant concentration, 100µL of cryoprotectant at 5 times the final concentration (4% or 40% Glycerol, or 5% or 50% DMSO) in Cytomix with or without 0.3 M Trehalose, was added into 400µL of cell suspension in the electroporation cuvette immediately after electroporation. Cryoprotectant was mixed in cell suspension by aspirating/releasing using micropipette (Gilson, France). These mixtures made a final suspension containing 0.8% or 8% Glycerol, or 1% or 10% DMSO, in Cytomix with 0 or 0.3 M trehalose.

5.2.4.2. Slow cooling (1 ℃/min) in a "Mr. Frosty".

An aliquot (50µL) of cell suspension was loaded into each 2 mL cryovial (Greiner, Interpath-Australia) at room temperature. The tubes were then placed in a "Mr. Frosty " chamber at room temperature and then cooled at 1°C/min to -80°C by, placing the chamber in a -80°C freezer (Revco, ULT-1090-5-W14, Revco-USA) overnight. The following morning the tubes were plunged into liquid nitrogen.

5.2.4.3. Snap freezing.

Cryovials (Greiner, Interpath-Australia) were pre-cooled in liquid nitrogen for 15 min. An aliquot (50μL) of cell suspension was then dropped into the pre-cooled tubes and the lids were closed. The tubes were then stored in liquid nitrogen.

5.2.5. Thawing

Cryovials containing the frozen cell suspension were taken from liquid nitrogen and thawed by holding them in air for 10 sec followed by immersion in warm water (37°C) in a waterbath until the suspension had melted. An aliquot of the cell suspension (20μL) was used for viability assay using trypan blue and one for in vitro culture in 200μL DMEM culture medium in a 96-well TC dish (Falcon 35-3072, Becton Dickinson).

5.2.6. Drying

Following electroporation at 250 V in Cytomix in the presence or absence of trehalose with or without cryoprotectant, cells were dried as a suspension or as adherent (plated) cells in several ways:

1. Air drying
2. Evaporative drying using dry nitrogen gas.
3. Vacuum drying, at different temperatures
4. Freeze- drying

5.2.6.1. Drying cell suspensions

Following electroporation the cell suspensions were left at room temperature for 30 min and then dried.

5.2.6.1.1. Air drying

An aliquot (100μL) of cell suspension was dropped and spread in a culture dish (35mm Falcon, 353001, Becton Dickinson) and kept in a modular incubator with a

constant flow of atmospheric air filtered through a 0.22μM syringe filter (Millipore) and controlled by a laboratory suction pump, for 8 hr.

5.2.6.1.2. Evaporative drying

An aliquot (100μL) of cell suspension was placed and spread in a culture dish (35mm Falcon, 353001, Becton Dickinson) and kept in a modular incubator with a constant flow of filtered (0.22μM Millipore) dry Nitrogen gas (from a gas cylinder) at 12.5 psi pressure.

5.2.6.1.3. Vacuum drying

An aliquot (100μL) of cell suspension was inserted into a cryovial (Greiner, Interpath) and placed in a vacuum dryer flask attached to a FD-6 (Dynavac) freeze drying machine, for 4 h at room temperature.

5.2.6.1.4. Freeze drying

An aliquot (100μL) of cell suspension in a 2mL cryovial (Greiner, Interpath) was slowly cooled at 1°C/min to -80°C and then transferred into a freeze drying machine (Christ, Germany) and dried at 8.5mBar pressure for 18 hr.

5.2.6.2. Drying of adherent/plated cells

Following electroporation, 100 μL of the cell suspensions (approximately 1×10^6 cells) were cultured in vitro in 4-well NUNC dishes with 600 μL DMEM and 10% FBS at 37°C 5% CO_2 for 4 hr.

After 4 hr of culture the medium was decanted to leave only the plated cells on the culture dish. These plated cells were then dried.

5.2.6.2.1. Air drying

The culture dishes (4-well NUNC) were placed in a modular incubator attached to a laboratory suction pump to give a constant flow of atmospheric air, for 8 hr. The air was sterilised by filtration through a 0.22μM filter.

5.2.6.2.2. Evaporative drying

The culture dishes (4-well NUNC) were placed in a modular incubator with a constant flow of dry Nitrogen gas (from a gas cylinder) filtered through a 0.22μM syringe filter at 25 psi pressure.

5.2.6.2.3. Vacuum drying

The culture dishes (4-well NUNC) were placed in a vacuum dryer flask attached to a FD-6 (Dynavac) freeze drying machine, for 4 h at room temperature.

5.2.6.3. Weight loss after vacuum and/or freeze drying.

Samples which were vacuum dried or freeze dried as a suspension were weighed before and after drying at room temperature. First, the empty tube was weighed then the tube with the cell suspension to obtain the sample weight. After removal from the vacuum or freeze drying machine, the tubes with the remaining sample were placed in a desiccator with silica-gel to keep the samples/tubes dry during warming to RT and then weighed again. The final weight was subtracted from the tube weight to obtain the weight of the residue.

Samples that were vacuum dried at -79°C or -196°C which still contained ice when they were recovered from the dryer were allowed to melt before weighing.

5.2.7. Rehydration

The dried cells were rehydrated within the drying vessels by the addition of culture medium DMEM 10% FBS. Cell suspensions dried in a 35mm TC-dish were rehydrated with 2 mL culture medium. Adherent cells dried in 4-well NUNC dishes were rehydrated by adding 700 µL culture medium (DMEM 10% FBS). The cells were then either tested for viability using trypan blue or cultured in vitro (as described in Chapter 2).

5.2.8. Experimental design

5.2.8.1. Experiment 1. Single pulse studies

This experiment compared three different electroporation media (DMEM, mHBS and Cytomix), seven pulse strengths (0, 100, 150, 200, 250, 300, and 350 V) and five trehalose concentrations (0, 0.1, 0.2, 0.3 and 0.4 M) in each medium.

5.2.8.2. Experiment 2. Multiple pulse studies

This experiment compared different pulse numbers (5 and 10 pulses) at 3 different pulse intervals (5 sec, 3 min and 6 min) with decay pulses at pulse strengths of 50, 100, 150, 200 and 250V.

The aim was to maximise PI internalisation into viable cells. The results would provide the basis for the subsequent experiments on fibroblast cell freezing and drying.

5.2.9. Freezing

This experiment compared the efficiency of different cryoprotectants (trehalose, glycerol and DMSO). The two permeable cryoprotectants were used at the normal (8% Glycerol; or 10% DMSO), and at one-tenth of the normal concentration (0.8% Glycerol; 1% DMSO) used for cell freezing. It compared two freezing methods (slow cooling at 1°C/min and snap freezing) and two time points after electroporation (ASAP and 30 min later) on mouse STO fibroblasts. The efficiency was indicated by the viability test (Trypan-blue) and the number of plated cells in vitro culture in DMEM with 10% FBS in 96-well TC dishes at 37°C 5% CO_2 in humidified air for 24 h.

In one freeze-drying experiment Methanol was evaluated as a cryoprotectant as it is more volatile than either DMSO or glycerol.

5.2.10. Drying

This experiment compared different cell preparations (suspension or plated/adherent), cryoprotectants (the presence or absence), drying methods and drying temperatures for their outcome on cell viability and plating.

In each experiment data was obtained from three replicates and analysed using two factor ANOVA using GraphPad Prism version 3.00 for Windows (GraphPad software, San Diego, California, USA, www.graphpad.com).

5.3. Results

5.3.1. Cell permeabilisation.

The viability of the cell suspensions prior to electroporation was more than 99% viable cells and none of these viable cells were permeable (i.e. PI negative in PI containing solutions).

5.3.1.1. Preliminary data

Preliminary observation compared the efficacy of sugars,

capacitance and media on cell viability after electroporation. The result showed that trehalose gave the higher proportion of viable cells after electroporation in water (Table 5.2), DMEM or mHBS (Table 5.3) at 500 and 975 μF capacitance.

Sugar dissolved in Milli-Q water gave the lower proportion of viable cells as compared to DMEM and mHBS. More viable cells were obtained after electroporation at 500 μF as compared to 975 μF.

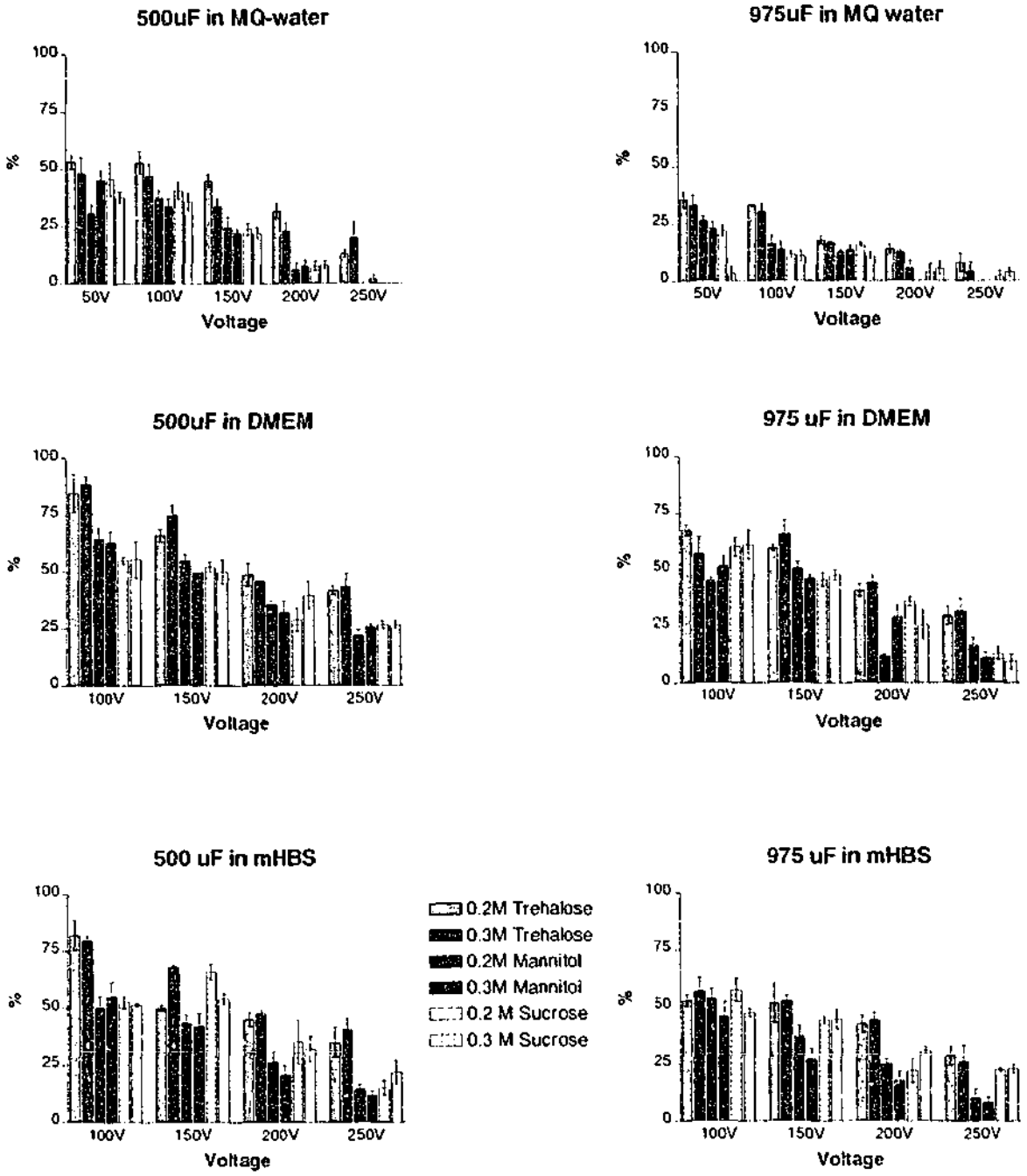


Fig. 5.1. Viable cells after electroporation in different sugars dissolved in Milli-Q water and media at 500 and 975 μF capacitance. Data in appendix 5A and 5B.

Those data suggested that optimal electroporation result can be obtained by electroporating cells in trehalose dissolved salts solution (DMEM or mHBS) at 500 μF capacitance.

5.3.1.2. Experiment 1. Single pulse studies

5.3.1.2.1. Cell permeability

Following electroporation, cells were assessed for their viability and permeability to PI and TB. The results for experiment 1 are in Fig. 5.2. Under the fluorescent microscope with a Texas Red filter, the cells with intracellular PI are red (Fig. 5.5). Observations using light microscopy showed that the viable cells did not take up trypan blue (Fig. 5.5), while the non-viable cells did take up trypan blue (Fig. 5.5). The use of both epifluorescent and bright-field observation and the combination of both images using computer graphic software (PhotoShop ver 5.0, Adobe) allowed the cells to be classified into viable-non permeabilised (clear, did not take up both PI and Trypan Blue), viable-permeabilised (red, PI positive only, and did not take up Trypan blue) and non-viable cells (red and blue, PI and TB positive), as shown in Figs. 5.2 and 5.3.

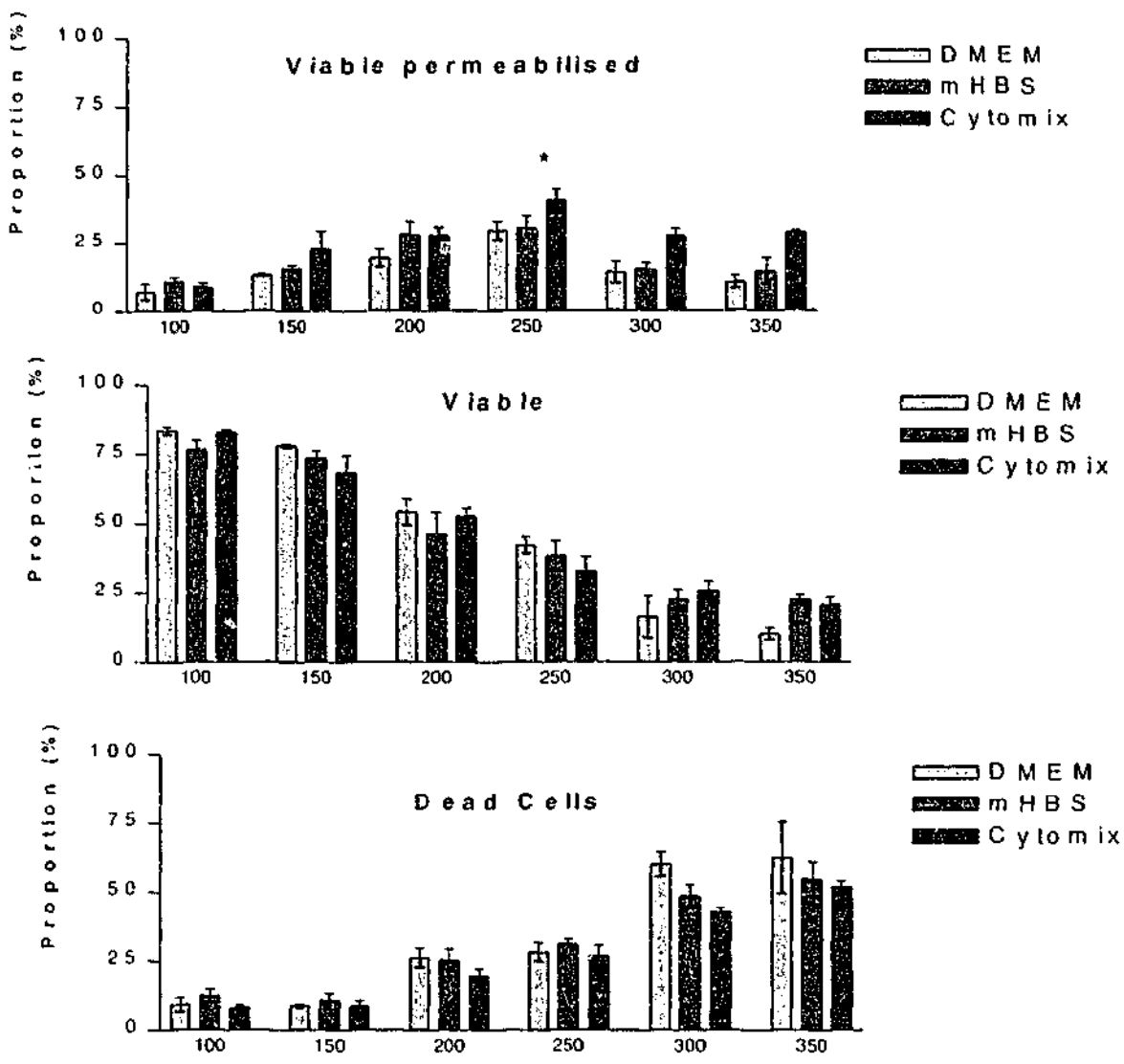


Fig. 5.2. Proportion of viable transiently permeabilised (upper graph), viable (middle graph) and dead (lower graph) cells after single pulse in solution without trehalose (Data in appendix 5C)

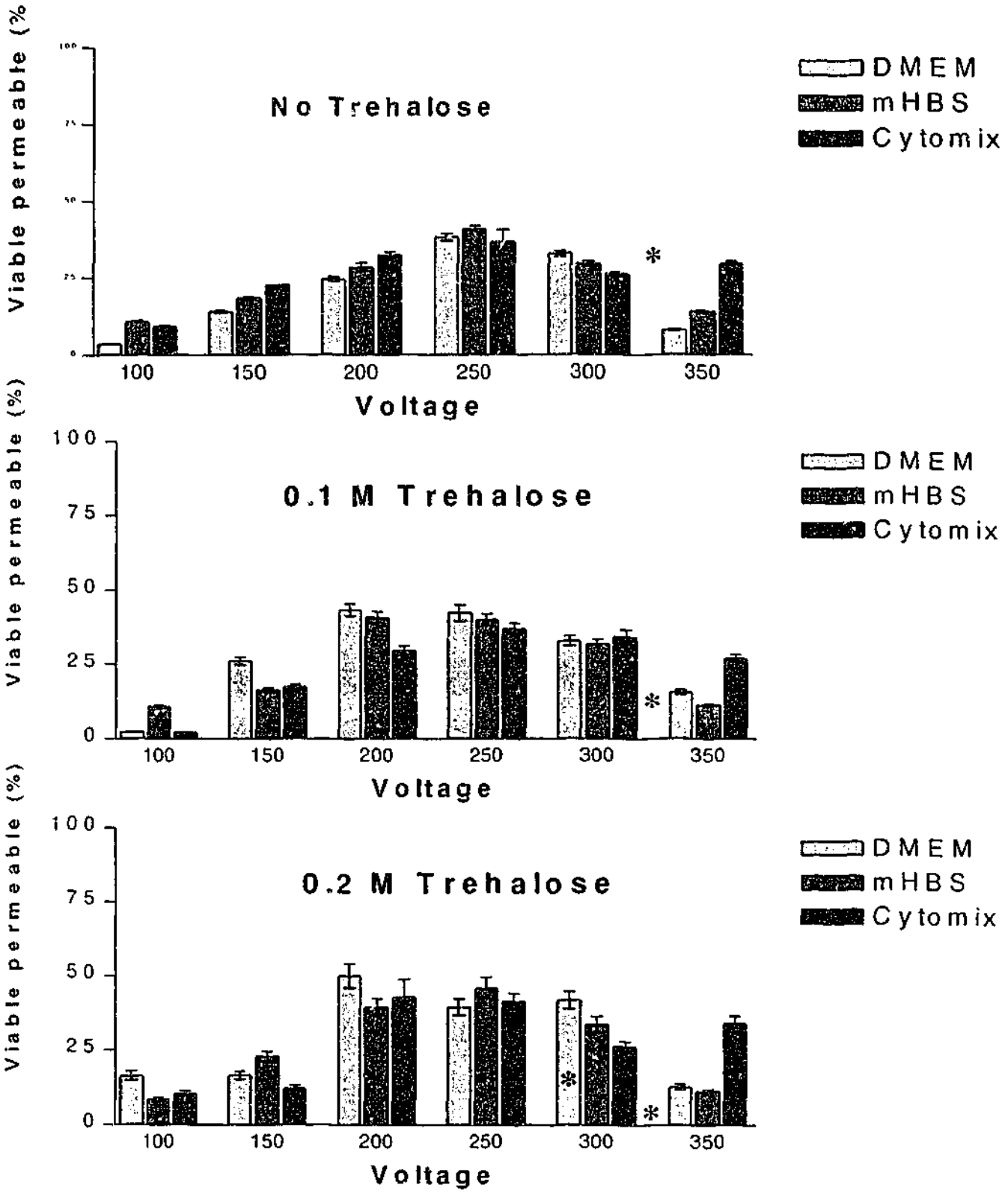
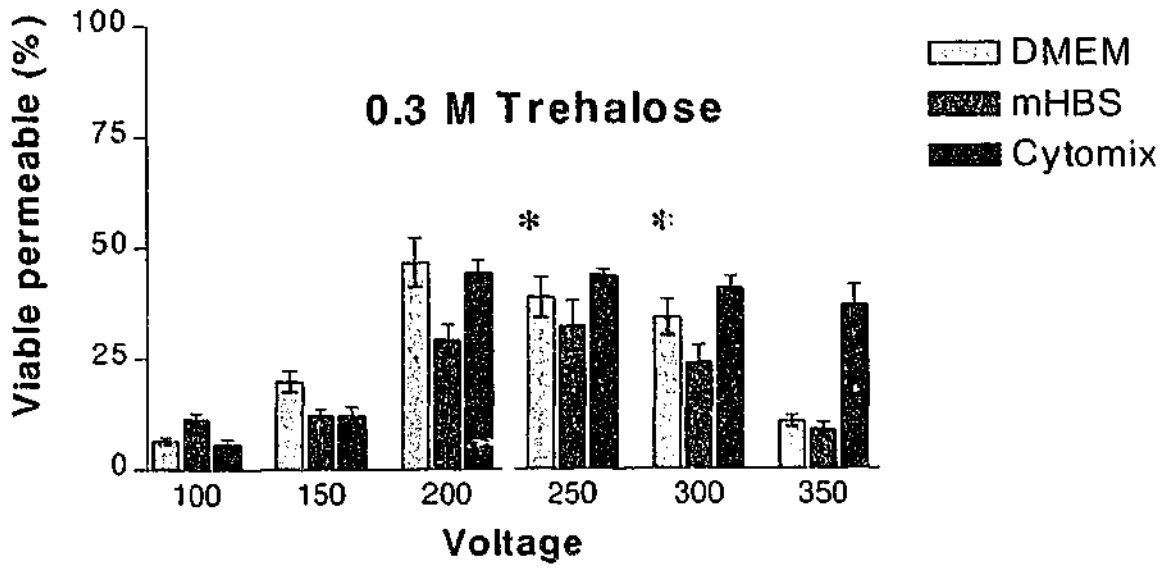


Fig. 5.3. Proportion of viable permeable cells (Data in Appendix 5.D) (continued)



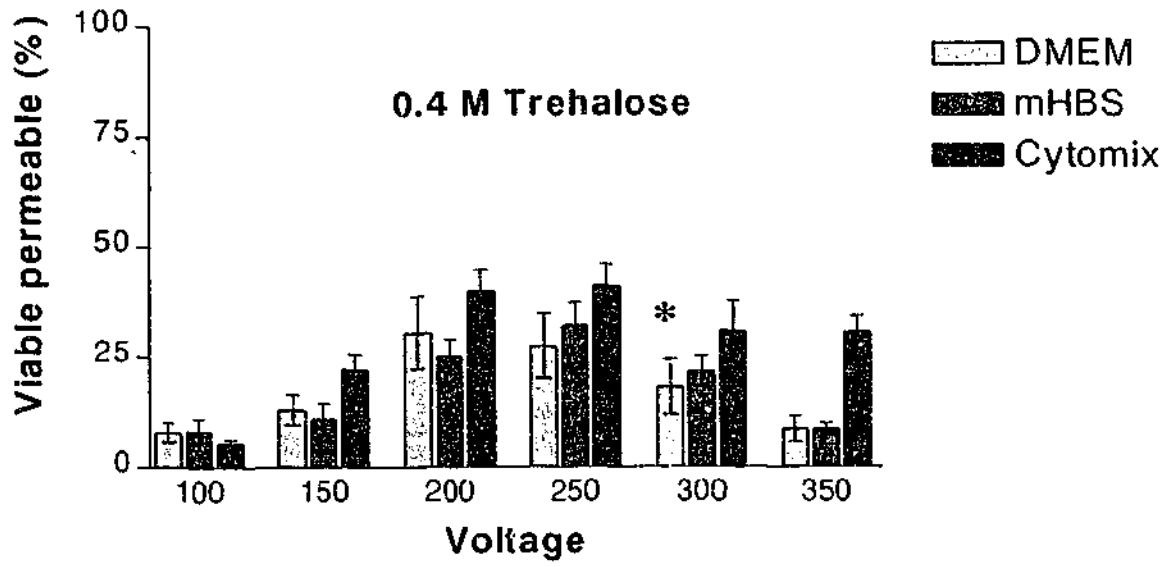


Fig 5.3. (Continued) Proportion of viable permeable cells (Data in Appendix 5D).

Figure 5.3 shows that the optimal permeabilisation of the viable cells (PI positive and TB negative cells) was achieved at 200-250V ($P<0.01$). At 350V the electric pulse made irreversible pores and destroyed the most of the cells. However electroporation in Cytomix produced more ($P>0.05$) viable permeable cells at 300 and 350 V than electroporation in the other media. There was a significant difference ($P<0.01$) between voltages but there were no differences ($P>0.05$) between the three media at voltages of between 100 and 250 V.

5.3.1.2.2. Effect of trehalose concentration on cell permeability

Electroporation in media containing trehalose (Fig. 5.3) gave the maximum proportion of viable but permeabilised cells, as indicated by PI positive and TB negative cells at 250 V ($P<0.01$). The result was not significantly different to electroporation in medium without trehalose. Analysis using two factor ANOVA showed a significant effect between voltages ($P<0.01$) and no significant effect of trehalose concentrations ($P>0.05$). Electroporation at 200-250V gave relatively high numbers of cells that were viable and permeable.

The highest proportion of viable cells was observed in cells electroporated at 100 V and the highest proportion of non viable cells (TB positive) was observed following electroporation at 350V.

5.3.1.2.3. Propidium Iodide retention in the electroporated cells

After 24 hr of in vitro culture, a proportion of the plated cells that had been electroporated at 100-350 V remained PI positive (Fig. 5.4). The PI excitation of plated cells was less intense than 30 min after electroporation (Fig. 5.5). By layering both images (light and epifluorescent microscopy) with Photoshop 5.0 software (Fig. 5.5) and blending the images, it was possible to distinguish between the viable permeabilised, viable-non-permeabilised and non-viable cells. The viable cells that had attached to the dish and formed flattened and/or elongated shapes (plating) and the non-viable cells held their original shape (floating or flying in the medium).

Cells that were electroporated at 100 and 150 V showed a high number of plated cells but few of the cells contained intracellular PI. While cells electroporated at 300 and 350 V, showed a significant ($P>0.01$) reduction in the number of plated cells. Most of th cells were PI positive. Cells electroporated at 200 and 250 V showed a high numbe and proportion of cells retaining intracellular PI.

Higher voltages (300 and 350V) produced fewer cells surviving electroporation as compared to electroporation at 200V or below (Fig. 5.3). There was a negative correlation between voltage and the number of cells surviving electroporation. The data showed a positive correlation between voltage and proportion of PI positive cells.

5.3.1.3. Experiment 2. Multiple pulses studies

Electroporation using more than one decaying pulse at 5 sec, 3 min or 6 min intervals (Fig. 5.7) showed that electroporation at a lower voltage (50 V) did not permeabilise the cells and that there was no difference ($P>0.05$) between single and multiple pulses for any parameter. At higher pulse strengths (250-300V) multiple pulses were detrimental to the cells, since more dead cells were found after electroporation. The results show that giving multiple pulses at high voltages (200-250V) result in significantly ($P<0.01$) more dead cells (TB+) as compared to the lower voltages (50-150V) or a single pulse. The single pulse at 250 V was the most optimal setting for electroporation of STO cells, and gave the highest number and proportion of viable permeabilised cells (Fig. 5.7).

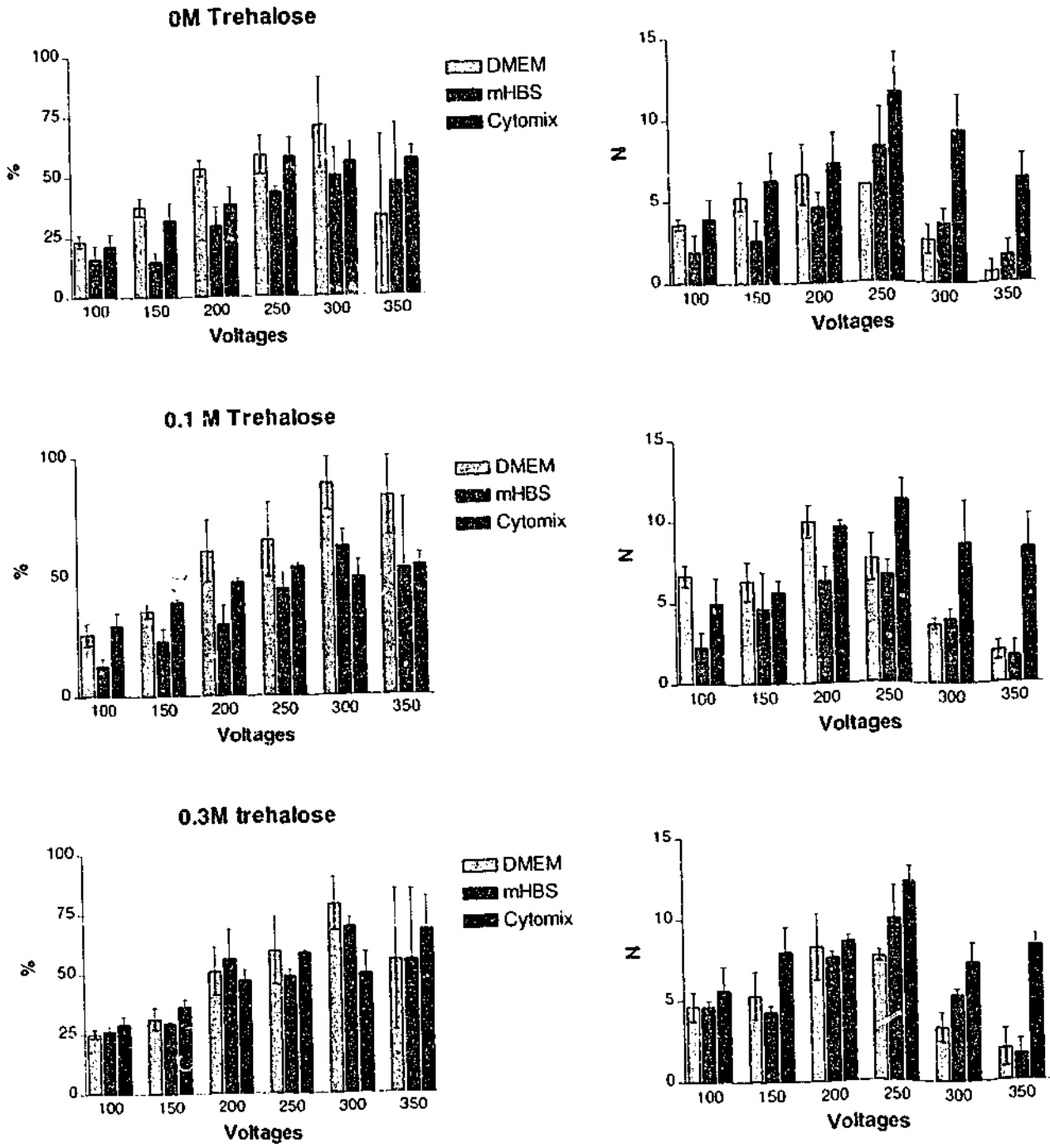


Fig. 5.4. The influence of voltage, EP media and trehalose content of the EP media on the number (graphs on the right) and proportion (graphs on the left) of plated cells that retained PI after in vitro culture for 24 h (data in appendix 5.4) (continued next page).

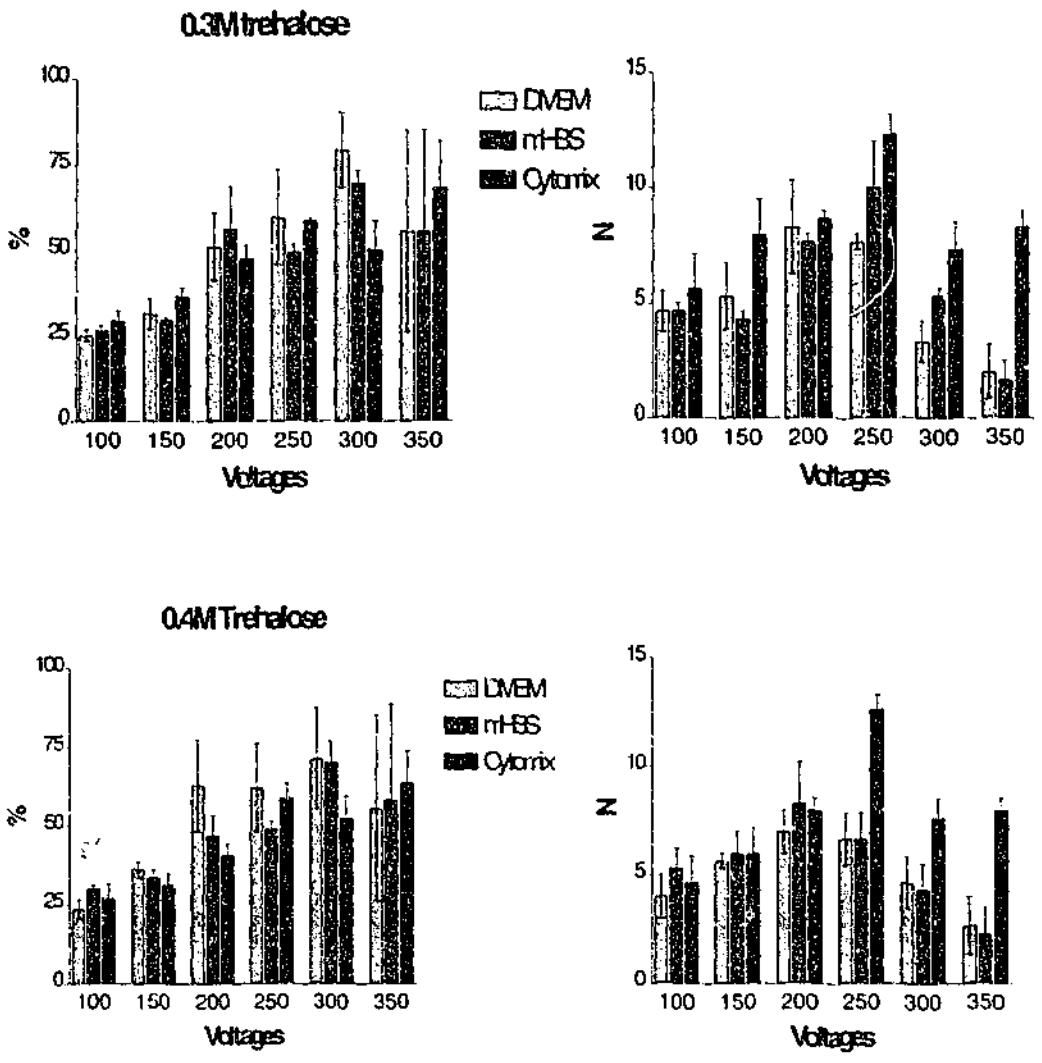
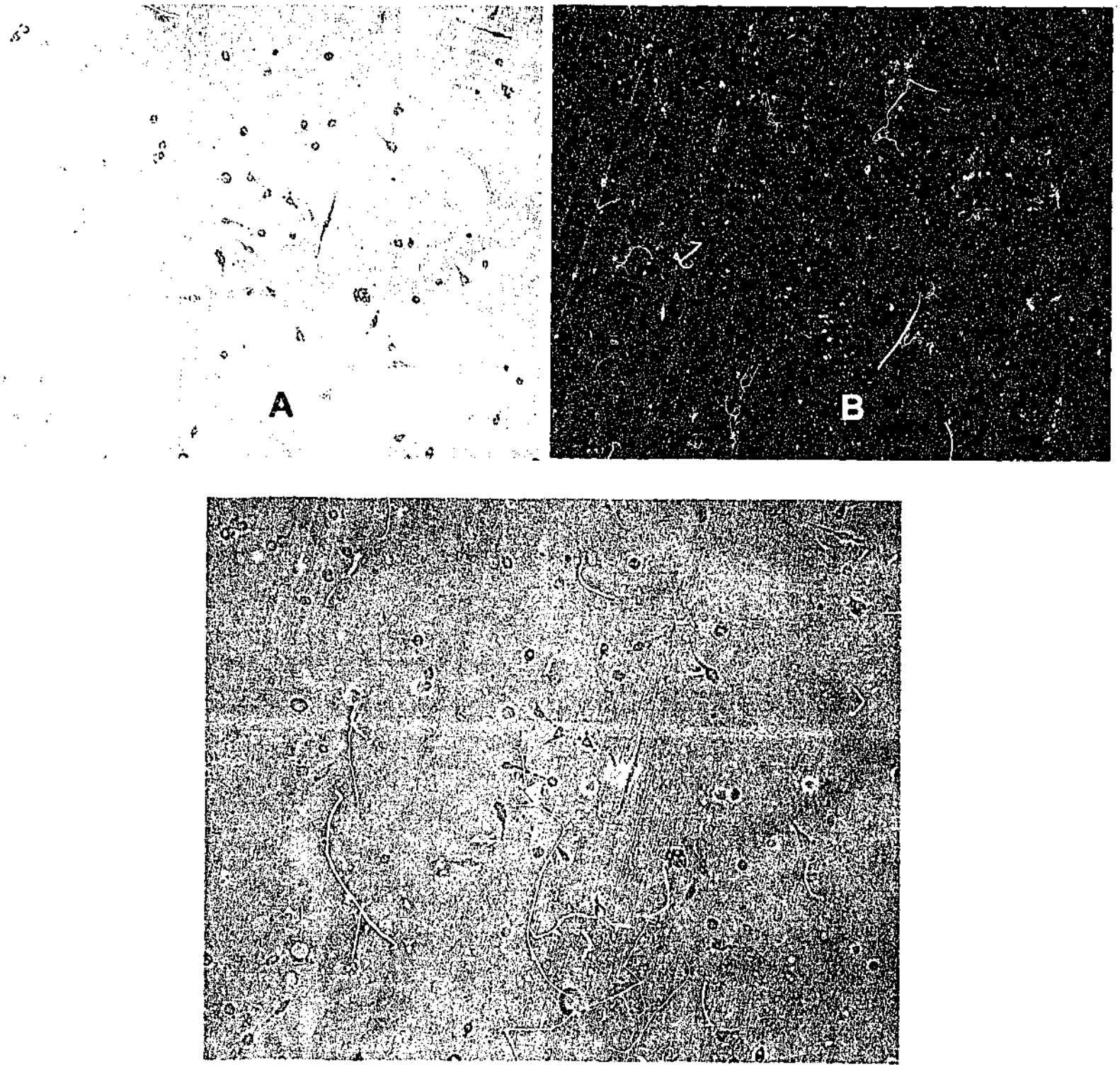
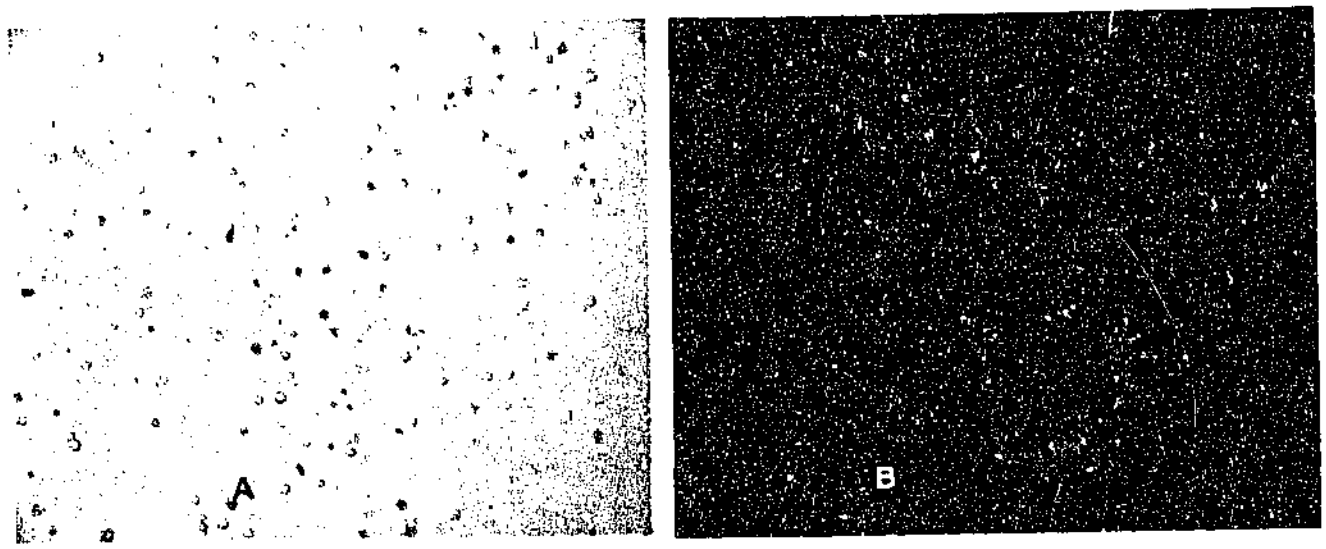


Fig. 5.4 (continued). The influence of voltage, EP media and trehalose content of the EP media on the number (graphs on the right) and proportion (graphs on the left) of plated cells that retained PI after in vitro culture for 24 h. (data in appendix 5.4).



A. Light microscopy of viable plated cell and non-viable cell (spherical); B. Epifluorescent microscopy of viable-plate cell (not bright) and non-viable cell (bright), C. Layered picture with viable-permeable and non viable cell and viable-non-permeable.

Fig 5.6. PI retention by viable plated cell after 24 h in culture



A. Light microscopy of live (TB negative) and dead (TB positive) cells

B. Fluorescence microscopy of both permeable and dead (PI positive) cells

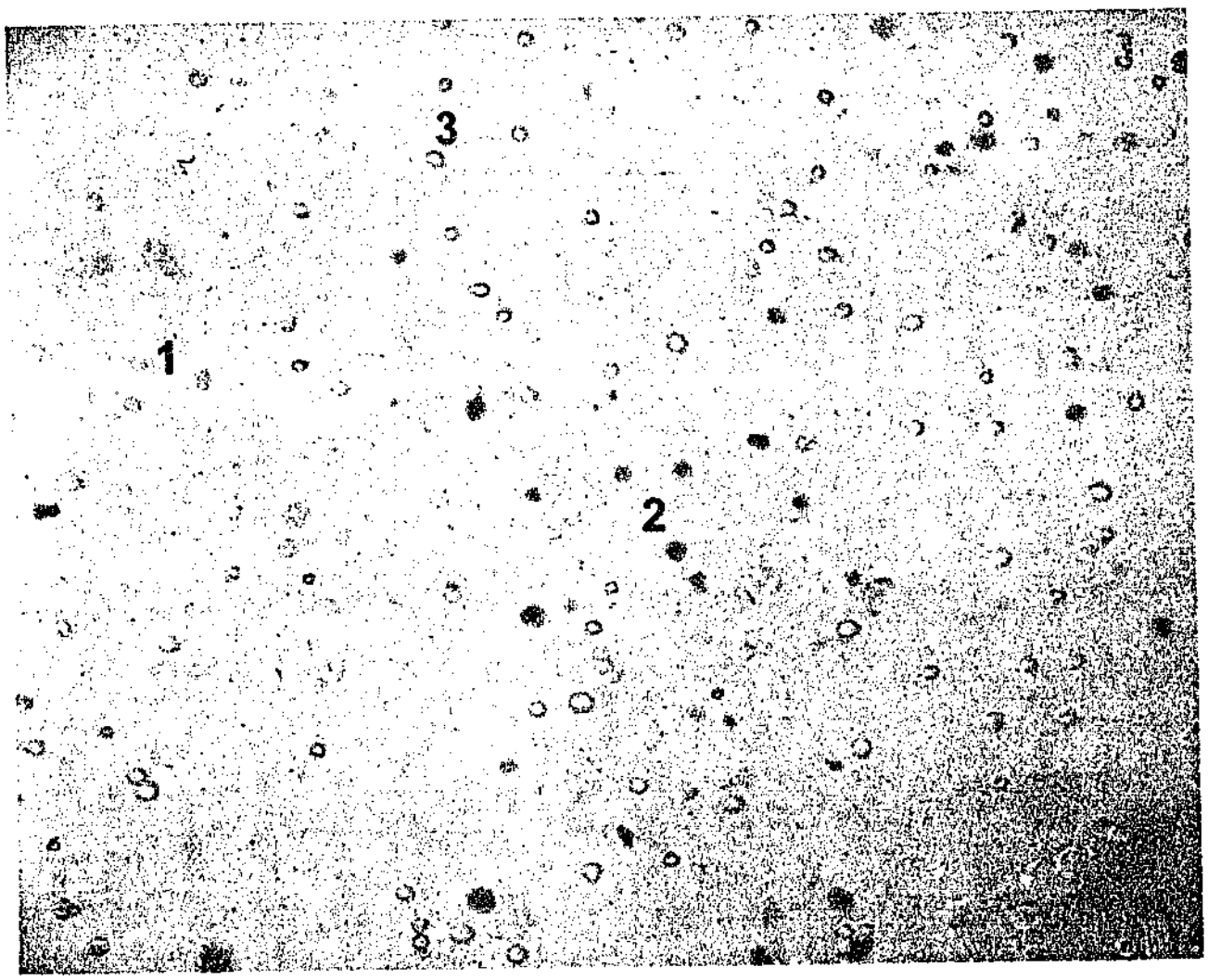


Fig. 5.5. Overlaying both images (Fig. A and Fig. B) showing the viable permeable (PI+ but TB-) and dead (PI+ and TB+) and viable not permeable (PI- and TB-)

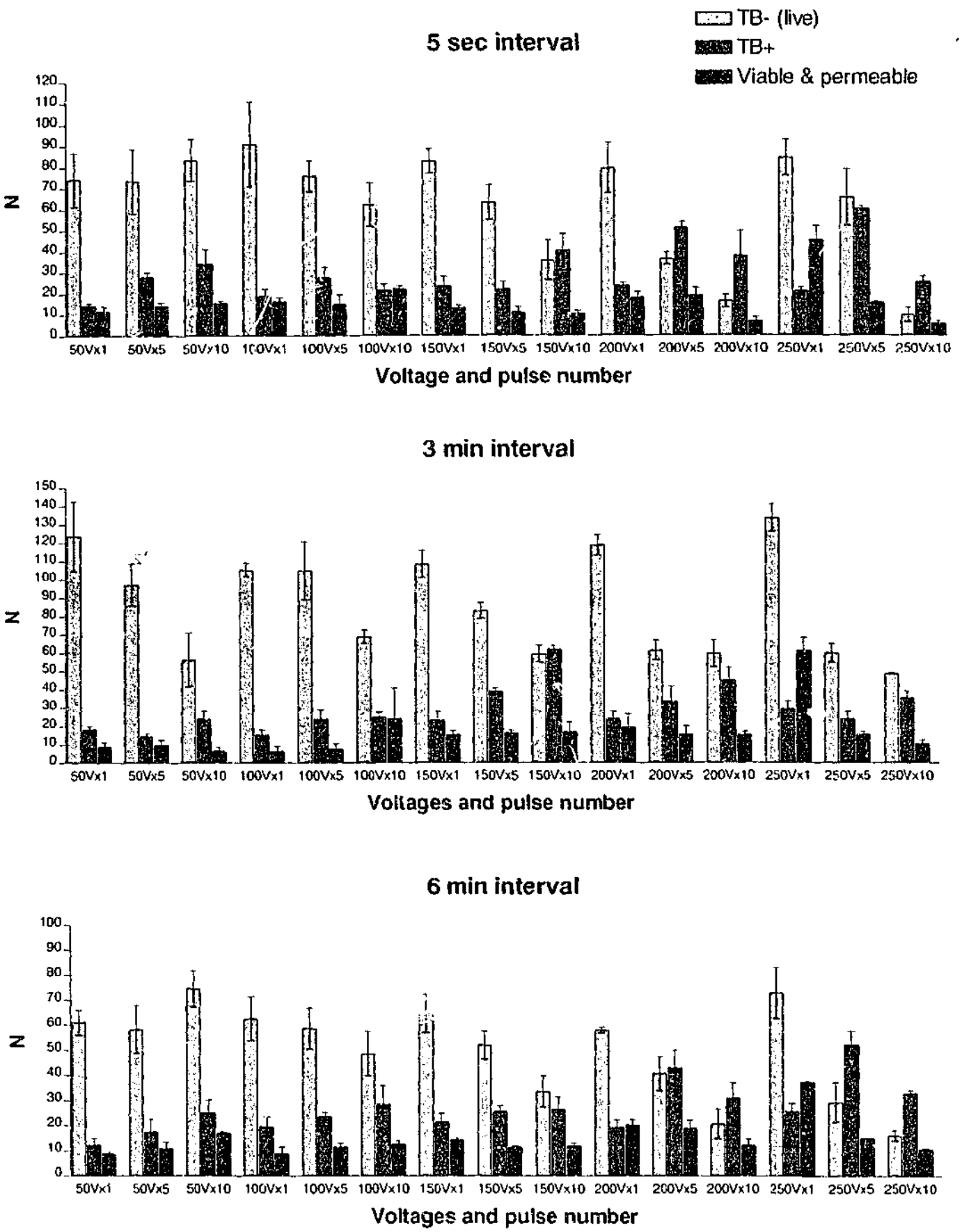


Fig. 5. 7. The number of viable permeabilised cells after electroporation with a single pulse or a single pulse given at 5 sec, 3 min and 6 min intervals for a total of 5 or 10 pulses (Data in appendix 5E. 5F and 5G)

5.3.2. Freezing

5.3.2.1. Electroporated and non frozen cells.

The viability of control (non frozen) cells used or tested immediately after electroporation and the addition of cryoprotectant was significantly higher ($P<0.01$) than for cells used or tested 30 min after electroporation. Cells electroporated in the presence of trehalose (0.3M) had significantly ($P<0.01$) higher cell viability than those electroporated without trehalose. (Fig. 5.8).

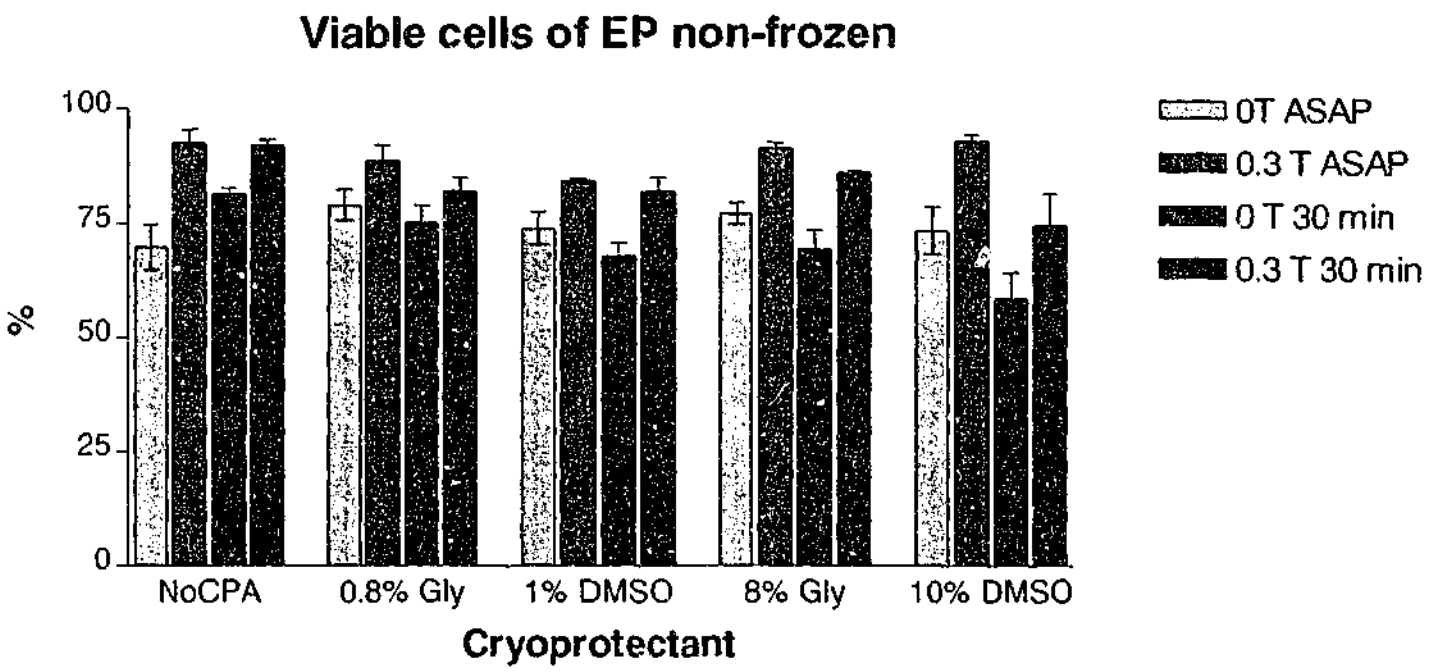


Fig. 5.8. Viability of non frozen cells following electroporation with a single 250V pulse in solutions with or without trehalose (percentage of cells excluding trypan-blue). (Data in appendix 5H)

No significant difference between treatments.

5.3.2.2. Non electroporated cells

The viability of non electroporated cells, was significantly lowered ($P<0.01$) by freezing (slow cooling and snap freezing) in the absence of cryoprotectants (with or without trehalose) and at low concentrations of cryoprotectant (with or without trehalose). This indicates that without electroporation trehalose had no effect on the freeze-thaw outcome (Fig. 5.9). In the presence of a high concentration of cryoprotectants cell viability was high. Results in the next sections showed that electroporation in the presence of trehalose and absence of cryoprotectants protected cells against freezing. However, electroporation itself reduced the cell viability (n.s, $P>0.05$) of the non frozen or cells frozen in the presence of a high concentration of cryoprotectant.

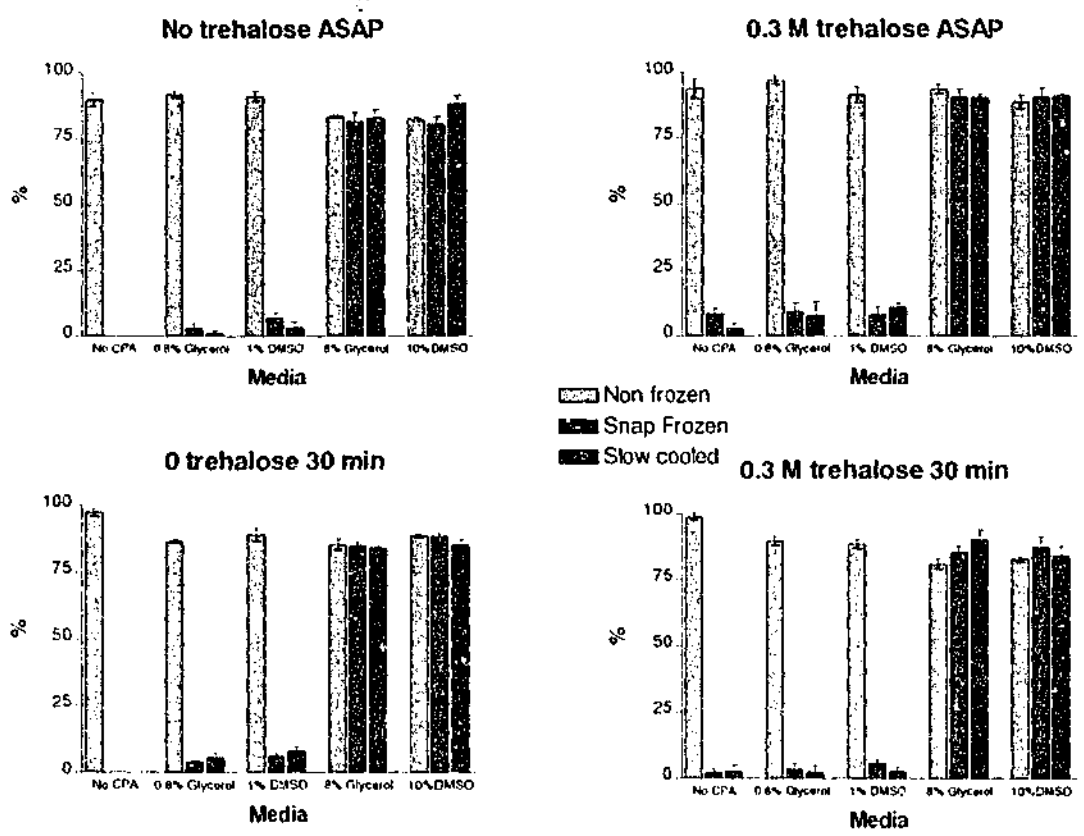


Fig. 5.9. Viability of non electroporated cells after freezing ASAP (top) or 30 min later (bottom), without (left) and with (right) trehalose. Data in appendix 51.

5.3.2.3. Slow cooled cells.

The survival of cells frozen without electroporation was lowest in the absence of a penetrating cryoprotectant, and highest at the highest concentration of penetrating cryoprotectant (Fig. 5.9). Cells which were slow cooled immediately and 30 min following electroporation were protected ($P<0.01$) by trehalose alone (0.00% vs. 23.96% and 0.00% vs. 68.84%). Cell viability was significantly ($P<0.01$) affected by the time interval between electroporation (and cryoprotectant addition) and freezing (immediately vs. 30 min, $P<0.01$). In most groups viability was highest ($P<0.01$) when cells were slow cooled in the presence of trehalose after electroporation.

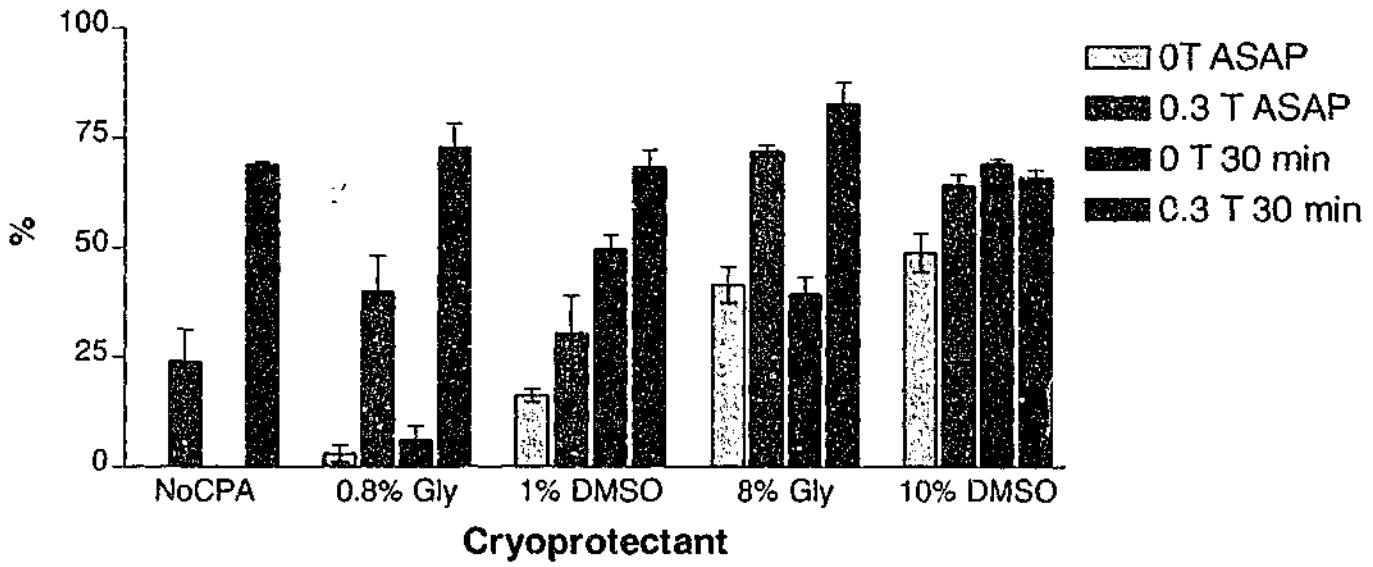


Fig. 5.10. Effect of Slow-cooling (1°/min to -80°C) and storage in LN2 after electroporation with a single 250V pulse on cell viability (percentage of cells exclude trypan-blue) (Data in appendix 5J)

5.3.2.4. Snap frozen cells.

As with slow cooled cells, there was a significant effect ($P<0.01$) of trehalose, on the outcome of snap freezing (Figs. 5.11-5.14).

Trypan blue exclusion was performed 30 minutes after thawing for both freezing methods. Very few control electroporated cells, frozen in the absence of either trehalose or cryoprotectants survived freezing.

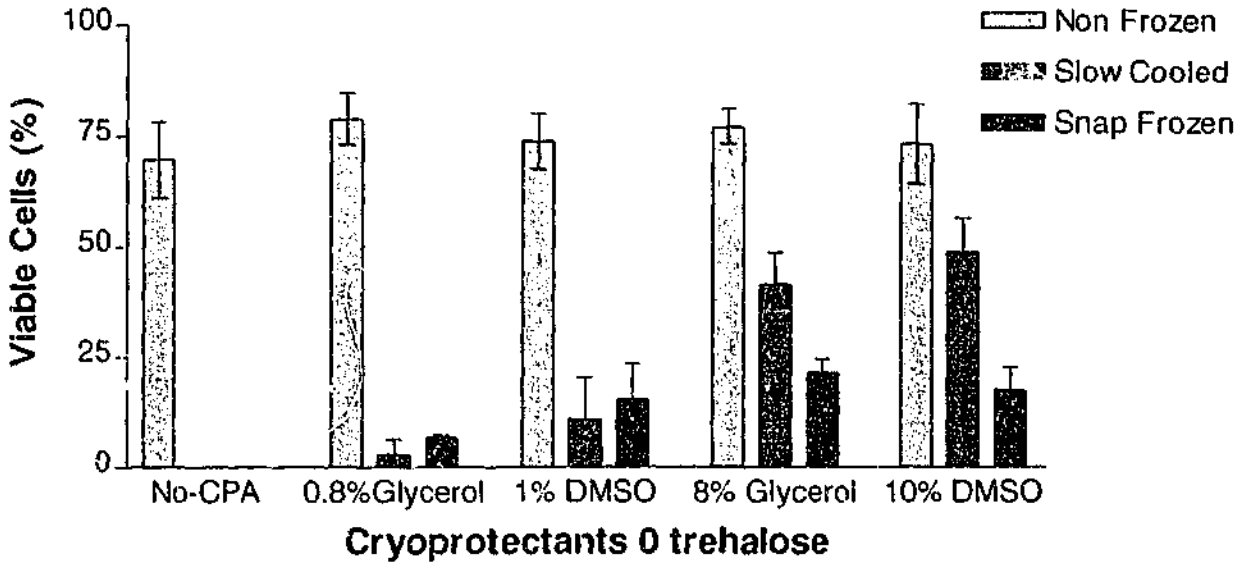


Fig 5. 11. Proportion of viable cells (%) electroporated with a single 250V pulse in medium without Trehalose and used immediately (Data in appendix 5J, 5L and 5M).

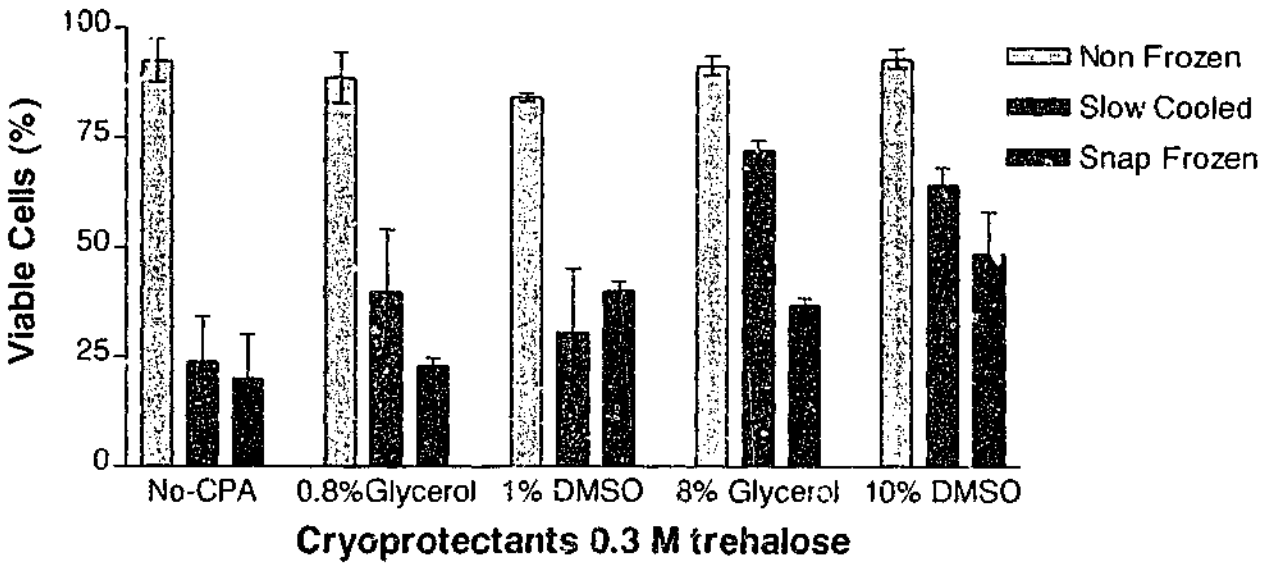


Fig. 5.12. Proportion of viable cells (%) electroporated with a single 250V pulse in medium with 0.3 M Trehalose and used immediately (Data in appendix 5J, 5L and 5M)

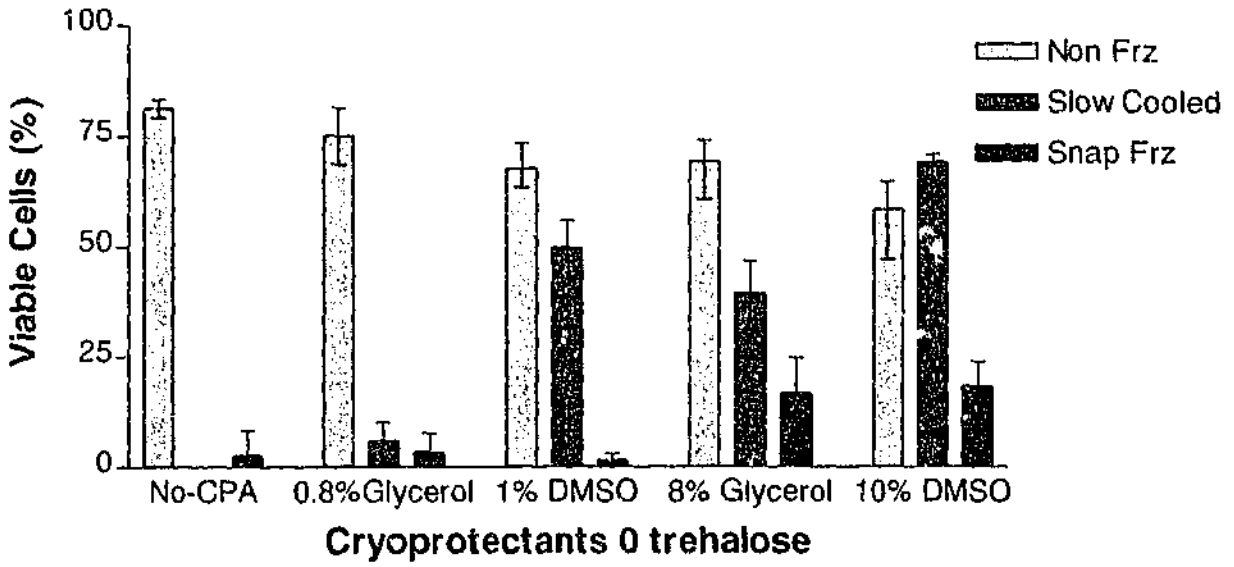


Fig.5.13. Proportion of viable cells (%) electroporated with a single 250V pulse in medium without Trehalose and used 30 minutes later (Data in appendix 5J, 5L and 5M)

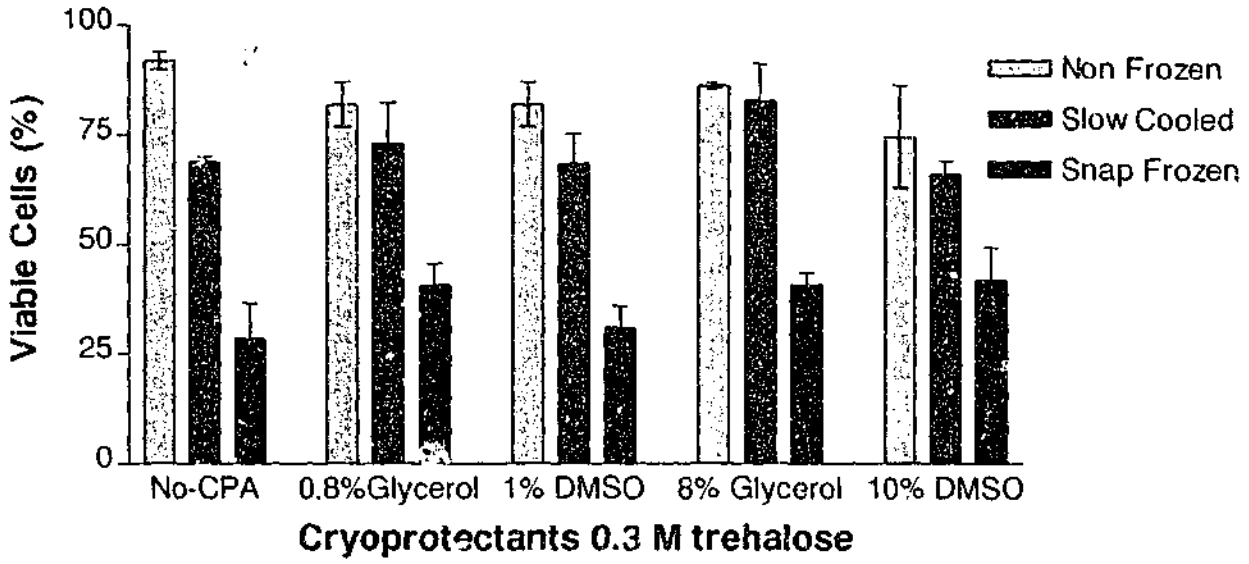


Fig. 5.14. Proportion of viable cells (%) electroporated with a single 250V pulse in medium with 0.3 M Trehalose and used 30 min later (Data in appendix 5J, 5L and 5M)

Statistical analysis using Two-factor ANOVA showed that trehalose concentration, cryoprotectant concentration and the freezing method affected the viability of non-frozen ($P<0.01$), slow cooled ($P<0.01$) and snap frozen ($P<0.01$) cells. Slow cooling gave a higher ($P<0.01$) survival rate compared to snap freezing.

Cryoprotectants at the higher concentration (8% Glycerol and 10% DMSO) and the presence of trehalose (0.3M) gave better ($P<0.01$) protection against slow cooling and snap freezing.

5.3.2.5. Summary of viability outcome for cells frozen in solutions without permeating cryoprotectants.

Data in table 5.2 below summarises the results for cells exposed to, or frozen in, solutions containing no permeating cryoprotectant. It clearly shows that electroporation in the presence of trehalose partially protected mouse STO cells from freeze-thaw injury.

Table 5.2. Summary of the viability outcome for cells in the groups treated in solutions free of permeating cryoprotectant (± electroporation ± trehalose)

Treatment and Time	Cells treated Immediately				Cells treated after 30 min			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
	% Viable	SEM	% Viable	SEM	% Viable	SEM	% Viable	SEM
Non frozen EP	69.7	0.05	92.6	0.03	81.37	0.01	92.0	0.01
Slow No EP	0.0	0.0	3.0	0.02	0.0	0.0	2.8	0.03
Snap No-EP	0.0	0.0	8.6	0.02	0.0	0.0	2.4	0.01
Slow EP	0.0	0.0	24.0	0.07	0.0	0.0	68.8	0.01
Snap EP	0.0	0.0	20.1	0.06	2.78	0.03	28.6	0.05

5.3.2.6. Plating rate of Non electroporated cells

Cells that were frozen in the absence of cryoprotectants (trehalose, Glycerol or DMSO) did not plate. In the presence of trehalose alone, the plating rate was lower ($P<0.01$) as compared to that in the presence of permeable cryoprotectants (Fig. 5.15).

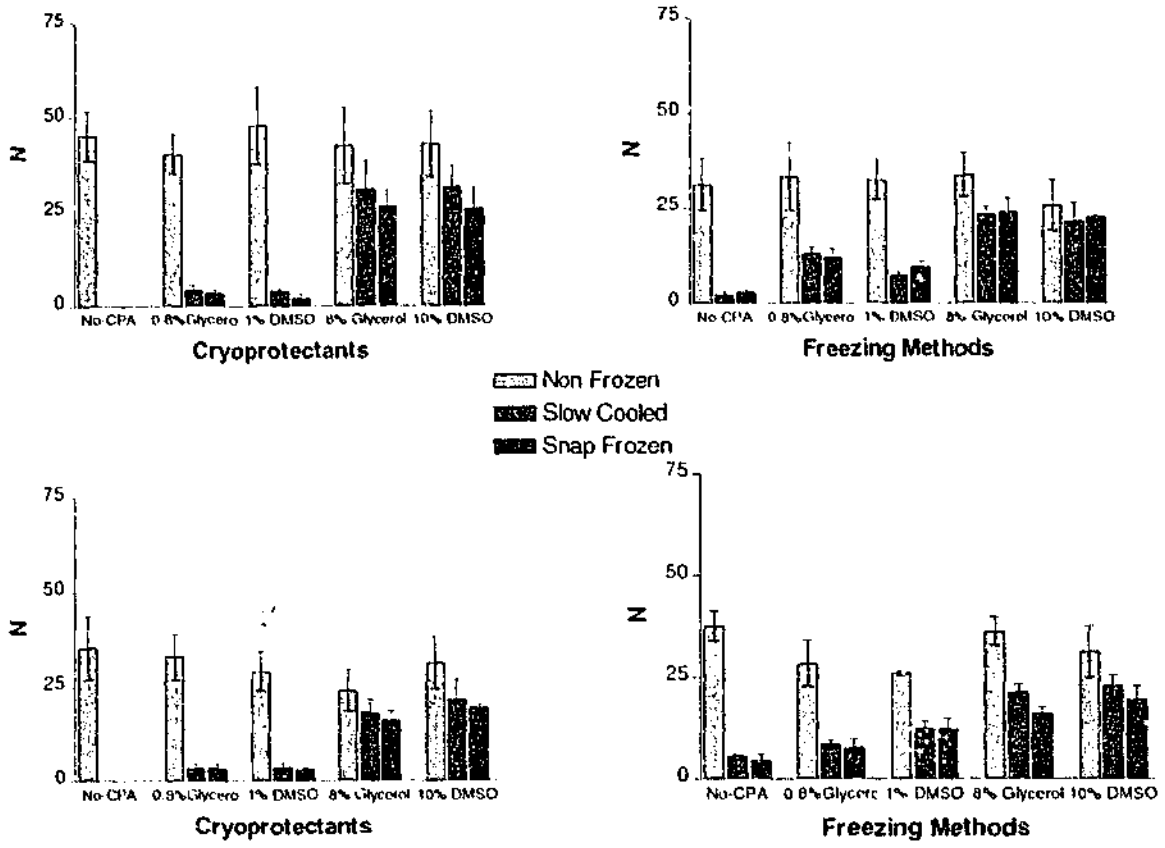


Fig. 5.15. Plating of non electroporated cells after freezing Cells were frozen ASAP (top) or 30 min after adding solutions (bottom), without (left) and with (right) trehalose and cryoprotectant. (Data in appendix 5N).

5.3.2.7. Plating rate of electroporated cells

Following electroporation with a single 250V pulse, freezing (with and without cryoprotectant) and thawing, the cells were cultured in vitro in 96-well TC-dishes. Most of the non-frozen cells, with or without electroporation, plated, proliferated and reached near confluency within 24 hr. Fewer frozen (slow cooled and snap-frozen) cells plated (Fig. 5.16-5.19 and Appendix 8) ($P<0.01$). However some individual treatments showed a high or very variable number of plating cells (observed or plated). This problem may have been caused by cell clumping, as those cells concentrated on a particular spot in the culture well which made it difficult to count individual cells.

Data on the number of plated cells as presented in appendix 8 showed that in the absence of both trehalose and cryoprotectants, none of the cells plated following cooling to -196°C using either slow cooling or snap freezing. In the presence of trehalose the number of viable cells increased significantly ($P<0.01$) (Fig. 5.17 and 5.19). Addition of cryoprotectants at a normal concentration (8% Glycerol and 10% DMSO) increased

(P<0.01) the number of viable plating cells as compared to those with trehalose alone or in the low cryoprotectant concentration (0.8 % Glycerol and 1% DMSO).

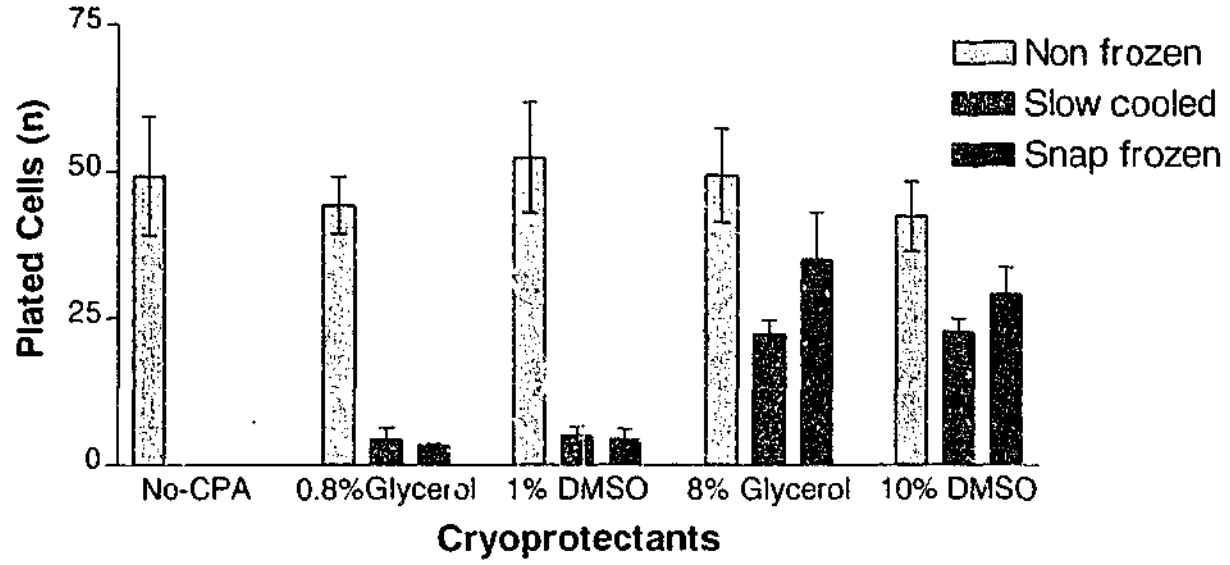


Fig. 5.16. The number of plated cells (%) after 24 h in culture. The cells were used immediately after being electroporated with a single 250V pulse without trehalose (0 M).(Data in appendix 5O)

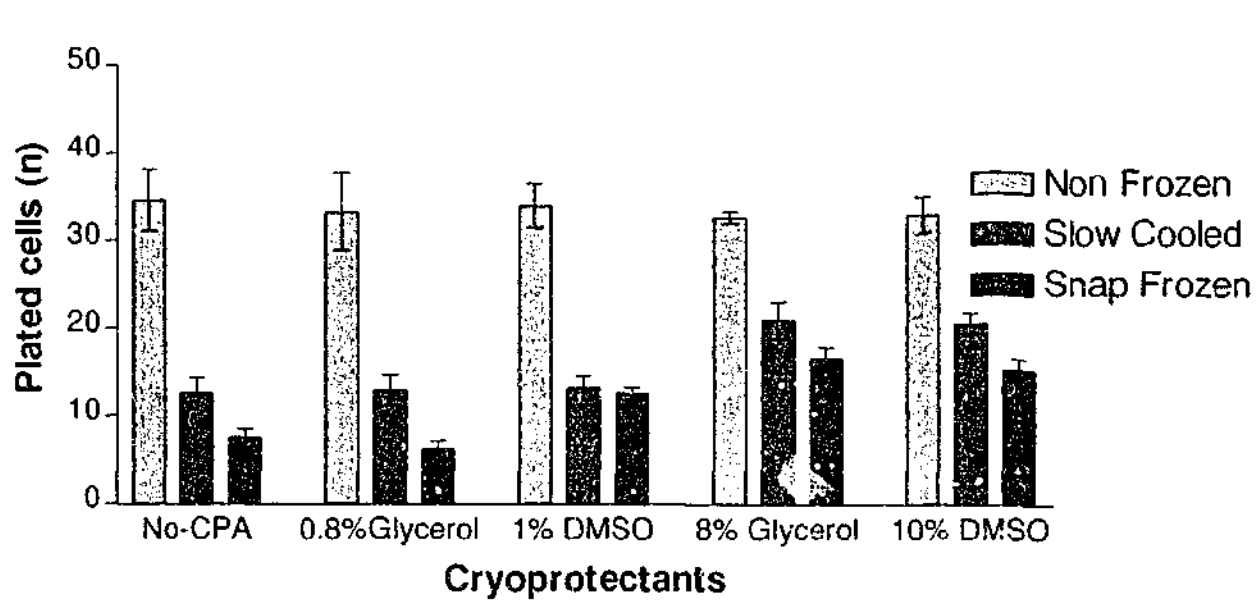


Fig. 5.17. The number of plated cells (%) after 24 h in culture. The cells were used 30 min later after being electroporated with a single 250V pulse without trehalose (0 M). (data in appendix 5O).

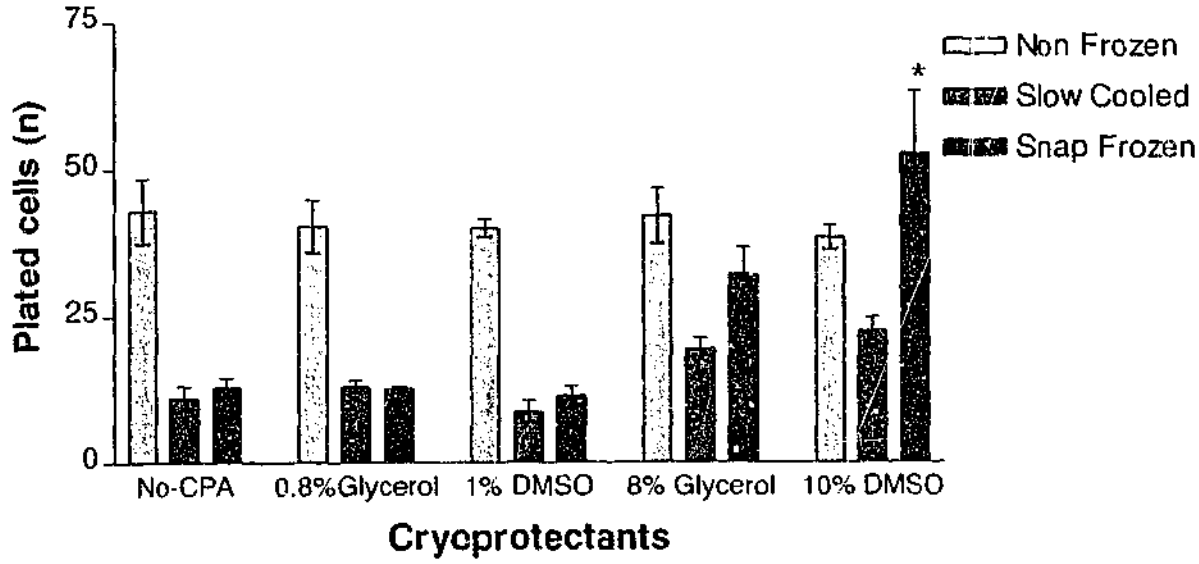


Fig. 5.18 The number of plated cells (%) after 24 h in culture. The cells were used immediately after being electroporated with a single 250V pulse with trehalose (0.3 M). Data in appendix 5O, (*:2 replicates only)

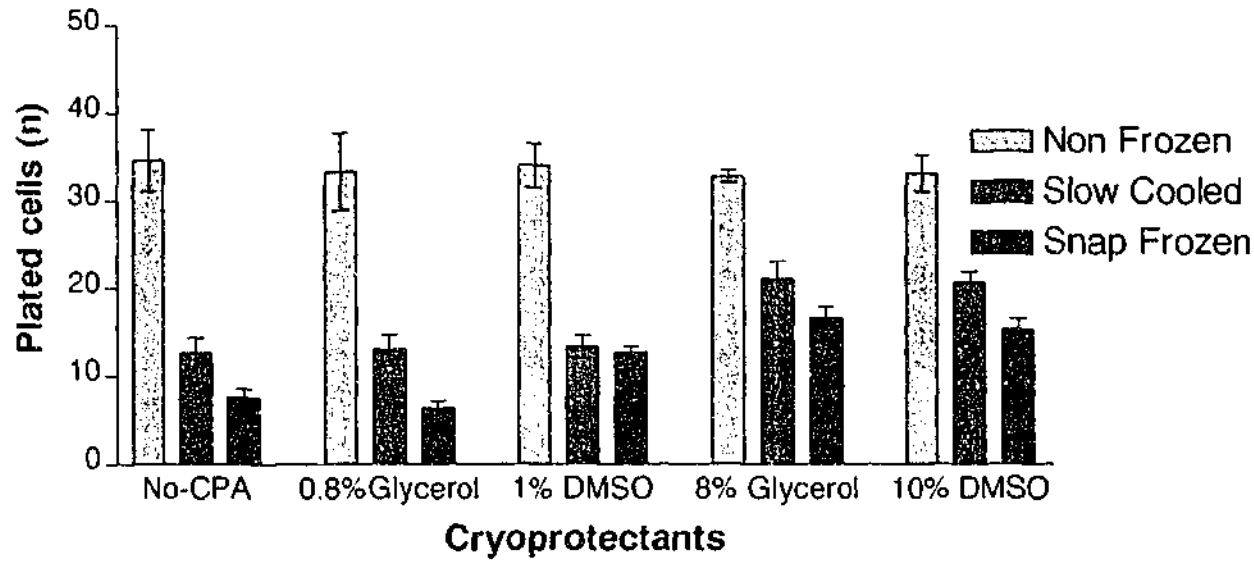


Fig. 5.19. The number of plated cells (%) after 24 h in culture. The cells were used 30 min after being electroporated with a single 250V pulse with trehalose (0.3 M). Data in appendix 5O.

5.3.2.8. Summary of plating outcome for cells frozen in solutions without permeating cryoprotectants.

Data in table 5.3 below summarises the plating outcome for cells treated in solutions containing no permeating cryoprotectant. It clearly shows that electroporation of mouse STO fibroblasts in the presence of trehalose gave partial protection against freeze-thaw injury.

Table 5.3. Summary of the plating outcome of cells in the groups treated in solutions free of permeating cryoprotectant (± electroporation ± trehalose).

Time and Treatment	Cells treated Immediately				Cells treated after 30 min			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
	N	SEM	N	SEM	N	SEM	N	SEM
Non frozen + EP	49.3	10.1	35.7	5.49	43.0	5.5	34.7	3.5
Slow cooled no EP	0.0	3.0	0.01	0.0	0.0	0.0	4.7	0.15
Snap cooled no EP	0.0	2.3	0.01	0.0	0.0	0.0	5.7	0.01
Slow cooled + EP	0.0	0.0	11.0	2.08	0.0	0.0	12.67	1.76
Snap cooled + EP	0.0	0.0	13.0	1.53	0.0	0.0	7.67	0.88

5.3.3. Drying

5.3.3.1. Drying cells in suspension

The trehalose and cryoprotectant free cell suspensions which were air dried or evaporatively dried on a TC dish left a thin crystalline layer of dried cells on the surface of the dish. When trehalose or cryoprotectants were present, a sticky residue formed.

The non-frozen cell suspensions in cryovials formed bubbles when a vacuum was applied. It resulted in rapid dehydration and samples (50μL) dried within 2 to3 hr. Samples at -196°C, or -80°C remained frozen and dehydrated at a slower rate.

All non-frozen and melted samples bubbled under vacuum and formed a spongy residue. This residue was dry as only 10 to 20 % of the original weight remained. This is the equivalent of most of the water as 10-20 of the original solutions were solid (salts and sugars).

Only partial dehydration was achieved for the samples that remained in the frozen state (-196°C and -80°C) during vacuum drying. There was only a small reduction (4 to 6%) in sample weight after vacuum as compared to the initial weight (Table 5.4). The samples did not form a dry residue and melted/thawed after exposure

to room temperature. This indicated that the vacuum was not strong enough to remove the frozen water (ice) from the sample and resulted in incomplete drying.

Samples dried using a Christ freeze drying machine had a different appearance as compared to those dried in the Dynavac freeze dryer. Samples suspended in 0.3 M trehalose or in 0.3 M trehalose with 2 M methanol formed a white powder residue, while samples suspended in medium containing 8% Glycerol or 10% DMSO remained wet, because the glycerol and DMSO did not evaporate.

5.3.3.1.1. Rehydration and cell viability

Residues of dried cell suspensions were easily dissolved in culture medium or freezing medium. Rehydrated cells retained their spherical shape, however staining using trypan blue showed that all cells obtained from dried residues were not viable. However cells obtained frozen residues with reduced water content remained viable. This indicated that the removal of some water from the frozen samples did not harm the cells.

5.3.3.2. Drying and rehydration of adherent cells.

Air drying, evaporative drying with nitrogen and vacuum drying at room temperature was applied to plated cells. Observation under the microscope showed that those cells retained their original shape/morphology (as a plated cell).

Rehydration by adding 700 µL of culture medium (DMEM 10% FBS) did not alter cell morphology, and viability testing using trypan blue showed a small proportion of trypan blue positive cells. Extended culture in vitro did not indicate any changes in cell plate pattern or the color of the culture medium. This indicated either that none of the cells were metabolically active and did not proliferate, or that these cells were non viable.

5.3.3.2.1. In vitro culture

In vitro culture in DMEM 10% FBS at 37°C in humidified air showed that none of the totally dried cells grew in vitro however the partially dried cells from frozen samples did grow.

Table 5.4. Sample weight before and after dehydration and cell viability.

Medium	Drying method*)	Residue (%)	Viable	Grow in culture
No CPA	1	95.08	Yes	Yes
DMSO 10 %	1	94.20	Yes	Yes
Glycerol 8%	1	94.20	Yes	Yes
Methanol 2M	1	95.07	Yes	Yes
No CPA	2	95.75	Yes	Yes
DMSO 10%	2	95.36	Yes	Yes
Glycerol 8%	2	94.11	Yes	Yes
Methanol 2M	2	94.17	Yes	Yes
No CPA	3	14.38	No	No
DMSO 10%	3	13.09	No	No
Glycerol 8%	3	19.20	No	No
Methanol 2 M	3	15.08	No	No
No CPA	4	16.26	No	No
DMSO 10%	4	16.89	No	No
Glycerol 8%	4	20.21	No	No
Methanol 2 M	4	14.79	No	No
No CPA	5	15.78	No	No
DMSO 10%	5	16.12	No	No
Glycerol 8%	5	20.55	No	No
Methanol 2 M	5	15.02	No	No
No CPA	6	15.53	No	No
DMSO 10%	6	16.04	No	No
Glycerol 8%	6	18.01	No	No
Methanol 2 M	6	14.50	No	No

*) Drying methods 1= -196°C for 4 days attached to Dynavac 1mTorr vacuum pressure; 2= -80°C (dry ice for 3hr); 3= -80°C (dry ice for 4hr) then leave for 24hr;
4= Room temperature for 4hr (vacuum drying); 5= Using a Christ F/D machine for 18hr; 6=Using a Christ F/D machine for 3 days.

5.4. Discussion

Electropermeabilisation or electroporation has been described as a multisteps process including the creation, expansion and resealing of transient pores on the membranes (Golzio, *et al.*, 1998; 2001, Knutson and Yee, 1987). The effect of various pulse voltages on cell permeabilisation was indicated by the presence of PI in the cells directly after electroporation and PI retention after 24 hr in culture. Propidium iodide was used because it has higher molecular weight (668.4) than trehalose (378.3). The

detection of PI excitation proved that the larger molecules (PI) had crossed the membrane and that it was retained following plating.

Pulse strength or voltage has a major contribution to the success of electroporation (Hui, 1995). Lower voltages (100 and 150V) do not permeabilise or kill cells. Higher voltages (above 250V) increase the proportion of permeabilised and dead cells following electroporation.

The ionic composition of the electroporation media do affect the permeabilisation rates and survival (Djuzenova, *et al.*, 1996). In this study the solution which mimicked the intracellular ionic composition (Cytomix) was less damaging at the highest voltages as compared to the culture medium. Electroporation under 200V pulse strength with 500μF capacitance did not show any difference between those three media. However, when cells were electroporated at 250V-350V at 500μF, Cytomix produced more viable and permeable cells as compared to either DMEM or mHBS. This agrees with Djuzenova *et al* (1996) that ionic composition affects medium conductivity and electroporation efficiency.

The presence and absence of trehalose or cryoprotectants altered media osmolality. However there was no effect of different trehalose concentrations in the electroporation media. This result is different to Rols and Teissie (1992) and Rols *et al.* (1994) who showed that when cells mixed with plasmids were pulsed and incubated in an iso-osmolar pulsing buffer, the percentage of viable cells expressing β-galactosidase activity reached up to 20-25%, and was 2.5 fold lower when cells were pulsed under hyperosmotic conditions. In this experiment cells that were pulsed under iso-osmotic and hyperosmotic (containing trehalose and or cryoprotectants) conditions had similar permeabilisation efficiency ($P>0.05$). However these results are similar to those of Golzio (1998) who found no effect of osmotic pressure on cell permeabilisation and Mussauer *et al* (2001) who reported that trehalose protected against cell lysis and improved survival of electrotransfected mammalian cells.

The pulse strength in this experiment (100-350 V) was lower than that used by Bright *et al.* (1996) who used 1500V/cm (300V/2mm), but higher than that in Rols *et al.* (1998) who used 300 to 900 V/cm or (60 to 180V/2mm). In my study, I found that pulses less than 200 V (1000 v/cm) were not optimal, while pulse strengths of more than 300 V (1500 V/cm) damaged the cells. Electroporation at 100 V left more cells intact but few of

these were permeabilised. Observations on the in vitro cultured cells 24 hr after electroporation showed that plated viable cells retained PI intracellularly. This indicated that electroporated cells may also retain trehalose intracellularly.

The use of multiple pulses with lower voltage was not as effective as for the single pulse. It may be that the type of the pulse emitted by the electroporation equipment used for this study may have influenced the results. This experiment used a decaying pulse while many other electroporation studies use short (microsecond) square wave pulses (Golzio *et al.*, 1998; Kotnik *et al.*, 2000; Meldrum *et al.*, 1999; Rols *et al.*, 1998). Further research may clarify whether this is important.

Trehalose is a disaccharide that has proved useful in maintaining the integrity of many biological systems submitted to various stresses (De Carlo *et al.*, 1999). Eroglu *et al.* (2000; 2001) showed that the introduction of low concentrations of intracellular trehalose could greatly improve the survival of mammalian cells and human oocytes during cryopreservation. However the strategies used by Eroglu *et al* (2000 and 2001) to introduce trehalose were complex.

In this study electroporation in the presence of trehalose prior to snap freezing by plunging them directly into liquid nitrogen temperature at -196°C or by slow cooling (Fig. 5.11 and 5.13) significantly improved cell viability. The survival and plating rate following a single 250V pulse in the absence of other cryoprotectants, however, was low (40%) as compared to that obtained by Eroglu *et al.* (2000) (80%). The trehalose enhanced viability with all the cryoprotectants. The results showed that in the presence of trehalose, cell viability was higher (25%) compared to when trehalose was absent (0%).

This study showed that the presence of trehalose and electroporation are required to protect cells against snap freezing by direct plunging into liquid nitrogen. While none of the cells were viable after electroporation and freezing without either trehalose or cryoprotectant, nor cell freezing in the presence of trehalose without electroporation. This indicates that intracellular trehalose protected cells against cryopreservation by direct plunging into liquid nitrogen.

The presence of trehalose alone or both trehalose and a cryoprotectant increased post thaw cell viability as compared to those without trehalose. This indicated that

trehalose gave a cumulative action with the cryoprotectant to protect cells against cryopreservation stresses.

Drying of cells electroporated in the presence trehalose failed to produce viable cells. Even though the results for freezing indicated that trehalose had crossed the cell membranes this was not sufficient to provide protection against drying. This result resembled those obtained by Tunnacliffe *et al* (2001) who could not obtain any viable cells after drying L- cells. They transfected L-cells with *ots*A and *ots*B genes to allow cell to produce their own trehalose and dry the cells using air drying, nitrogen drying and freeze drying. My results and those of Tunnacliffe *et al* (2001) differ to those of Guo *et al* (2000), Puhlev *et al* (2001) and Chen *et al* (2001) who showed that the presence of trehalose in both intra and extra-cellular protected cells against drying.

Thus it remains controversial whether mammalian cells can be stored in dry state. However most studies on drying of anhydrobiotic organisms (Crowe *et al.*, 1992), lipid membranes (Tsvetkova *et al.*, 1998) and proteins (Sun *et al.*, 1998) showed that trehalose does play an important protective role.

Sugars may not be the only molecules involved in protecting the molecular structure of the dried cytoplasm of anhydrobiotes, but also proteins. Protein are also abundantly present in the cellular cytoplasm. Kalichevsky *et al.*, (1992); Bell and Hagemen, (1996), Wolkers *et al.*, (2001a) showed that proteins have a considerable effect on the molecular properties of sugar glasses. In seeds and pollen Late embryogenesis abundant (LEA) proteins (Wang *et al.*, 1996 and Wolkers *et al.*, 2001a), accumulated prior to the onset of drying (Close, 1996), and may be involved in protecting such a molecular network. It is likely that proteins and sugars interact through hydrogen bonding in the dry state in the cytoplasm of anhydrobiotes (Levine and Slade, 1988; Slade and Levine, 1991). Both sugars and proteins play an important role in the molecular organization of the dry cytoplasm when they are present in the same cellular compartment (Woelker *et al.*, 2002).

There are several possible explanations for the low survival rate for the cells in this study. Insufficient intracellular trehalose concentration may have lead to this problem. The presence of permeable cryoprotectants that protected cells against freezing may not suitable for drying since they remained in the residue and were not completely removed by vacuum pressure during drying. Alternatively, other permeable

cryoprotectants that are easy to evaporate and do not leave any residue should be used as an alternative to DMSO or Glycerol.

5.5. Conclusion

Electroporation using a single decaying wave, or multiple pulses could be used to create pores and introduce large molecules into living cells. The optimal voltage for electroporation of mouse STO fibroblast was 250 V. Electroporation of cells in the presence of trehalose increased the proportion of cells which survived and plated after freezing (slow cooling and snap freezing), but did not result in cells capable of surviving evaporative or freeze drying.

Chapter 6. Evaluation of equilibration, cryoprotectants, cooling and freezing as strategies for loading mammalian cells with trehalose.

6.1. Introduction

Cell membranes can be permeabilised to allow non-permeable components to cross the membrane. In chapter 5 mouse fibroblasts that were electroporated in the presence of trehalose exhibited improved survival after subsequent slow cooling and snap freezing, but failed to survive drying. Electroporation was however not an ideal strategy for loading trehalose as it caused a significant reduction in the number of viable cells. Further strategies which permeabilise a greater proportion of the initial cell population need to be found.

Freeze/thaw permeabilisation has been used to introduce impermeant molecules into synaptosomes (Nichols *et al.*, 1989) and Sasaki *et al.* (1991) showed that freezing could be used to introduce DNA into the cells. They suggested that the freezing created cracks and or pores in the cell membrane as a result of ice formation. Interestingly some treatments that increased permeation only caused moderate cell death. It is difficult to reconcile this report with others which show that freezing reduces cell viability through cell membrane rupture and altered membrane permeability (Acker and McGann, 2000; 2001; Buck *et al.*, 1981;Fujikawa, 1980; Holt *et al.*, 1992; Ignatov *et al.*, 1982; Mazur, 1984; Mazur *et al.*, 1984; McGann *et al.*, 1988; Muldrew and McGann, 1994; Pegg, 1987; Steponkus *et al.*, 1983; Zhu and Liu, 2000).

Most reports show that membrane damage is caused by intracellular ice formation. Membranes are initially destabilised during the freezing process, both by phase transitions at low temperatures, altered efficiency of trans membrane pumps, and by exposure to high salt concentrations (Holt and North, 1994). Increased osmotic pressure of the extracellular solution in the presence of ice can rupture the plasma membrane, allowing extracellular ice to propagate into the cytoplasm (Muldrew and McGann, 1994). Freezing spermatozoa to temperatures between -10°C and -15°C did not induce significant membrane permeabilisation. However, freezing to below -15°C was followed by membrane permeabilisation immediately after thawing (Holt and North, 1994; Zhu and Liu, 2000). Zhu and Liu, (2000) showed that cryopreservation

causes significant membrane rupture in the head and tail regions of spermatozoa; this damage may occur independently and the presence of an intact tail membrane does not necessarily indicate the intactness of head membrane, and can therefore affect post-thaw motility, without reducing the fertilising potential.

The experiments in this chapter aimed to

1. Determine whether cooling, freezing and cryoprotectants could be used as an alternative to electroporation to introduce non-permeable PI and trehalose into cells.
2. Maximise tolerance to snap freezing without penetrating cryoprotectants.
3. Determine whether the medium surrounding the cells at the time of freezing would influence the post thaw outcome.

The outcome was assessed by monitoring propidium iodide incorporation and plating.

6.2. Materials and methods

The mouse fibroblast (STO cell line), culture and freezing methods are described in chapters 2 and 5.

6.2.1. Cell preparation and suspension

Cells were detached from the culture flasks using 0.25% Trypsin and 1 mM EDTA (Gibco, Invitrogen) and then centrifuged at 1000 rpm for 5 min to obtain a pellet. The pellet was resuspended in different media as specified in each experiment.

6.2.2. Assay for cell viability and permeabilisation

Cell viability was assessed using trypan blue staining and in vitro culture. Viability was assessed in two ways, firstly by staining cells with PI and Trypan Blue to determine cell permeability, and secondly by in vitro development to determine cell development and PI retention.

Cell permeability was assessed using a fluorescent microscope as described in Table 5.1 (Chapter 5). Cells that excluded trypan blue but incorporated PI were classified as viable and transiently permeabilised.

Following a 24 hour recovery period during in vitro culture, the cells were re-evaluated to ascertain how many of the cells still contained intracellular PI (assessed under a fluorescent microscope as described previously). The culture medium/supernatant (containing non-plated/non-viable cells) was then decanted from the culture dish, leaving only viable (plated) cells behind. These plated cells were observed by fluorescence microscopy with a Texas Red filter. Cells that took up PI and plated had a red fluorescent cytoplasm while the non-permeabilised cells had a clear cytoplasm. Assays were performed after each time point in each treatment.

6.2.3. Slow cooling

Slow cooling was performed in a Cryologic Freezing machine (Cryologic, Australia). The cooling chamber of this machine allows controlled cooling from +40°C to -80°C at cooling rates from 0.1°C/min to 3°C/min. During this experiment the cooling rate was controlled by proprietary software for this freezing machine (Cryogenic, Cryologic-Australia). This software allows adjustment of the initial freezing temperature, cooling rate, holding time at any particular temperature, final temperature and final status (hold or free fall to –196°C) also equipped with reminder alarm (bell). The freezing machine was connected to a PC by a RS-232 cable.

The cooling chamber used in this experiment has a maximum cooling rate of 3°C/min and could hold either cryovials or straws. Cryovials (2 ml) were loaded with 100 to 1000 µL of cell suspensions and the lid was closed before loading them into the freezing machine. Slow cooling in straws was performed by loading 20µL of cell suspension into 0.25 ml straws, sealed at both ends with PVA powder.

During cooling, the chamber was immersed in a LN bath.

6.2.4. Snap freezing

Cell suspensions were snap frozen in straws. A total of 20µl of the cell suspensions were aspirated into the lower portion (approximately 1 cm from the end) of 250 µL straws. After loading the straws were sealed using PVA moistened with DMEM and then snap frozen by immersing the straw directly into liquid nitrogen.

6.2.5. Thawing

The frozen cell suspensions were thawed at RT for 5 sec followed by immersion in a 37°C waterbath until melted. The contents were then tested for their viability, permeability or in vitro culture (as in 6.2.2).

6.2.6. Experimental design

Four different approaches to permeabilisation (experiments 1 to 4) were used in this chapter.

Data were collected from 3 replicates and were analysed using two-way ANOVA. All data and graphs were analysed and made using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

6.2.6.1. Experiment 1. Chilling and freezing in the absence of penetrating cryoprotectants

This experiment used the same solutions as in chapter 5 (Cytomix, mHBS, DMEM 10% FBS) as well as FBS as this is routinely used for freezing of this cell line. The media were supplemented with 0 or 0.3 M trehalose but no penetrating cryoprotectants were added to the solution. Cell suspensions were cooled at 1°C/min to temperatures between +25 and -25°C, re-warmed and then snap cooled to ascertain whether sufficient trehalose would enter the cells to protect against subsequent snap freezing immediately or 30 min later.

6.2.6.1.1. Tabular presentation of the protocol for experiment 1 (Figure 6.1 shows the flow chart)

1. Cell preparation and viability test, cells were detached from TC-flasks as described in sections 6.2.1 and 6.2.2 in this chapter.
2. The pellets were suspended in each of four media (100% FBS, DMEM with 10% FBS, Cytomix, or mHBS) with 0 or 0.3 M trehalose at a concentration of 50×10^6 cells/ml at RT.

3. Aliquots (500µL) of the cell suspension were loaded into 2 mL cryovials (Greiner, Interpath) at RT and immediately slow cooled at 1°C/min in a Cryologic freezing machine controlled with Cryogenic software starting from +25°C.

4. For each solution vials were removed at each of 6 set temperatures (+25°; +15°; +5°; -5°; -15° and -25°C) i.e. 0, 10, 20, 30, 40 and 50 minutes after starting the cooling program. These vials were moved to a water bath at 37°C to warm (and thaw) the cell suspensions.

5. After warming/ thawing the cell suspensions were mixed by pipetting and either:

   held at RT for a further 30 min, before adding TB and assessing their TB and PI status,

   aliquoted (20µL) into 200µL DMEM for in vitro culture in 96-well TC dishes.

   aliquoted (20µL) into 250µL straws and sealed with PVA then re- frozen (by snap freezing) immediately or 30 min later after thawing

6. After the second freezing (c), cells were thawed by warming the straws in air for 5 sec at RT followed by immersion in a 37°C waterbath until melted.

7. Cells were tested again for their viability and in vitro cultured in 200µL DMEM 10% FBS in 96-well TC- dish.

6.2.6.2. Experiment 2. Effect of exposing cells to penetrating cryoprotectant and trehalose prior to snap freezing.

This experiment aimed to determine whether permeating cryoprotectants (DMSO and Ethylene Glycol, EG) in the first solution (FBS or Cytomix)would permeabilise cells to trehalose in solution 2. Cells were first placed in FBS or Cytomix with or without (0.6 M) trehalose in the presence of cryoprotectants (20% DMSO; 10% DMSO with 10% EG; 10% DMSO or 10% EG) and 100µg/µL PI at room temperature. The cells were then expelled into a second solution of FBS or Cytomix with (0.6 M) or without (0 M) trehalose which did not contain penetrating cryoprotectants.

6.2.6.2.1. Tabular protocol for experiment 2 (Figure 6.5 shows the flow chart)

1. Cells were detached from the TC-flasks then processed as in section 6.2.1 and 6.2.2 then suspended in solutions containing cryoprotectants (20% DMSO; 10% DMSO with 10% Ethylene Glycol; 10% DMSO or 10% EG) in Cytomix or FBS with 100µg/mL PI at $50x10^6$ cell/mL.

2. The cell suspensions were incubated for 5 or 10 min at RT or placed immediately at 0°C and cooled to -25°C by slow cooling at 1°C/min (100µL in 250 µL straws, without seeding).
3. Frozen straws were thawed by holding them in air and then immersing them into a water bath at 37°C until melted.
4. Aliquots (10µl) of each cell suspension were then removed and the cells tested for their viability (TB), permeability (PI) and growth in in vitro culture.
5. One aliquot (20 µL) of each solution was aspirated into a 250µL straw and snap frozen by plunging into liquid nitrogen.
6. Ten microliters of each cell suspension was then transferred into 90µL of a second solution (FBS or Cytomix containing no or 0.3 M trehalose) in a 96 well TC dish. Cells were held in this second solution for 10 or 60 min, before being loaded into straws (20µL in 250µL) and snap frozen or in vitro cultured.
7. Cells which were snap frozen in the second solution were thawed at RT (5 sec) and then held in a 37°C waterbath until melted then cultured in vitro (20µL in 200µL DMEM) or tested for their viability (TB) and permeability (PI).

6.2.6.3. Experiment 3. Evaluation of the effect of adding Cytomix components to a conventional freezing solution.

Fetal Bovine Serum containing 10% DMSO is very effective and widely used as a freezing solution for mammalian cells. This experiment aimed to establish whether cells became permeabilised during conventional cooling. If permeabilisation did occur then the cells should internalise trehalose and other normally non-permeable components of the cryopreservation medium. Following thawing cells were therefore frozen again (this time by snap freezing) in a solution (Cytomix) without DMSO (the penetrating cryoprotectant). It was hypothesised that cells would be killed by the second snap freeze (as in Figure 5.1 in chapter 5) unless they had internalised trehalose during the first freeze. The first solution (10% DMSO in FBS) was supplemented with individual components of the Cytomix solution to ascertain whether any specific component was associated with the observed difference between Cytomix and mHBS in

experiment 1. The first freeze was performed by suspending the cell pellet in FBS with 10% DMSO with or without 0.3 M trehalose and the individual Cytomix components (Table 6.1). Propidium Iodide (100 µg/mL) was added to the solution when permeability was to be assessed visually. The second freeze for all groups was performed in Cytomix with 0 or 0.3 M trehalose without DMSO.

Table 6.1. Composition of the solutions used for the first freezing.

Cryoprotectant	Trehalose	Element added*)	Symbol
10% DMSO in FBS	0.0 M	Nil	DF
10% DMSO in FBS	0.3 M	Nil	DFT
10% DMSO in FBS	0.3 M	2 mM ATP (1 x)	DFTA1
10% DMSO in FBS	0.3 M	10 mM ATP (5 x)	DFTA5
10% DMSO in FBS	0.3 M	50 mM Glutathione (10 x)	DFTG10
10% DMSO in FBS	0.3 M	5 mM EGTA (1 x)	DFTEG

*) Concentration as compared to the normal concentration in Cytomix medium.

6.2.6.3.1. Tabular presentation of the protocol used in experiment 3 (Figure 6.11 shows the flow chart)

1. Cell preparation and viability testing of the cells which were detached from TC-flasks were as described in sections 6.2.1 and 6.2.2 in this chapter
2. Cells were suspended in 1 ml of 10% DMSO in FBS with 100 µg/ml PI (50×10^6 cells/ml) supplemented with each of 5 different supplements as outlined in table 6.1, equilibrated for 5 min at room temperature and loaded into 2 mL cryovials.
3. The vials were slowly cooled at 1°C/min to -80 at 1°C/min from room temperature in a Mr. Frosty in a -80°C freezer then held at -80°C overnight and then placed in liquid nitrogen.
4. Vials were thawed by each of three warming protocols (0°C for 30 min, RT for 15 min or 37°C for 5 min). Aliquots (20µL) were taken for viability testing and in vitro culture.
5. The thawed specimen/vials were centrifuged at 1000 rpm/min to produce a pellet. The majority (1 ml) of the supernatant was then removed.
6. The pellets were diluted with 1 ml Cytomix containing 0.3M Trehalose without any penetrating cryoprotectant. Aliquots of this cell suspension were taken for viability testing (TB), in vitro culture, and freezing.

7. The cell suspensions were snap frozen by dispensing 200µl into 2 mL cryovials pre-cooled in liquid nitrogen. Cell suspensions (200µl) were slow cooled in 2 mL cryovials placed in a Mr. Frosty at room temperature and then cooled at 1°C/min to -80°C and then placed in liquid nitrogen.
8. Re-frozen (Snap frozen or Slow cooled) cells were thawed and tested as described in 6.2.2 and 6.2.3.

6.2.6.4. Experiment 4. Effect of DMSO, Trehalose, FBS and cooling to temperatures between +25 ℃ and LN_2 on survival after snap freezing.

In this experiment cells were cooled by conventional slow cooling (1°C/min) in 10% DMSO in FBS. At set temperatures (ranging from 0°C to -196°C) vials were re-warmed and expelled into FBS or Cytomix containing trehalose (0, 0.3; 0.5 or 1.0 M) with or without DMSO at room temperature. This cell suspension was then frozen again by snap freezing to evaluate post-thaw survival (resistance to freeze-thaw injury).

The aim was to establish whether the cells would be permeabilised by conventional freezing, and permit trehalose to enter the cell from the diluent solution after thawing.

6.2.6.4.1. Tabular presentation of the protocol used in experiment 4. (Figure 6.18 shows the flow chart)

1. Cells were detached from TC-flasks as described in sections 6.2.1 and 6.2.2 in this chapter
2. Cells were suspended in 10% DMSO in FBS with 100 µg/ml PI (50×10^6 cells/ml) and 1mL of cell suspension was loaded into 2 mL cryovials.
3. The vials were slowly cooled at 1°C/min from RT to -80 °C in a Cryologic freezing machine controlled with Cryogenic software then placed in liquid nitrogen.
4. Every 10°C from 0°C to -80°C a vial was removed from the freezing machine and thawed by holding the vial for 5 sec in air at RT and then in water at 37°C until melted. Aliquots (20µL) were tested for their viability and development in in vitro culture as described in 6.2.2. and 6.2.3.

5. Aliquots (100 µL) of the thawed specimens were placed into 1 ml of the second solution and then centrifuged at 3500 rpm/min to obtain a pellet.
6. The supernatant (1 mL) was removed and the remaining 100 µL was aspirated (as 20 µL aliquots) into 250µL straw, and then snap frozen.
7. These snap frozen straws were thawed and tested as described in 6.2.2. and 6.2.3.

6.3. Results

6.3.1. Initial observations

Observations on the cell quality prior to freezing showed that 97.2% of the cells were viable. None of these cells (0%) were both viable and permeabilised (TB- and PI+).

6.3.2. Experiment 1. Chilling and freezing in the absence of penetrating cryoprotectants

This experiment aimed to evaluate whether the type of solution in which cells were held would influence trehalose loading of cells while the cells underwent slow cooling (1°C/min) from 25°C to -25°C (Fig. 6.2.) in the absence of a penetrating cryoprotectant. The outcome was assessed by re-freezing (by snap freezing directly into LN) immediately after thawing, or 30 minutes later.

During cooling at 1°C/min from 25°C to -25°C, the cell suspension remained in the liquid state until -10°C. At -15°C a small amount of ice had formed and the suspension was completely frozen at -25°C. This indicated that cells experienced super cooling before ice crystallisation

Observations at each temperature point showed that the cells were not initially permeable to PI in any solution (Fig. 6.3). There was no consistent effect of trehalose, on the number of viable cells which were PI positive, or the proportion of cells which survived freezing, though there was a marginal protective effect of trehalose for groups frozen in FBS, Cytomix and DMEM.

Light microscopy observations after the first incubation and cooling showed that the viable permeabilised cells had a less well defined membrane than the viable non permeable cells. Observations on cells cultured for 24 h in vitro revealed no plated cells retaining PI, while the dead (non viable) cells retained PI. This may indicate that the viable permeable cells that were found after thawing failed to recover during the subsequent in vitro culture step.

Thawing of cells that had been snap frozen immediately after thawing, gave a higher ($P<0.01$) proportion of viable cells as compared to those snap frozen 30 min after thawing. Cells suspended in medium without serum or trehalose were not viable (Fig. 6.3.) after snap freezing. This indicated that either serum or trehalose provided some protection against snap freezing.

In mHBS with or without trehalose more than 10% of the cells died even before cooling commenced. At each temperature, the proportion of dead cells remained consistently higher for mHBS than for the other solutions. Cell deaths were consistently lowest for FBS with or without trehalose, Cytomix with trehalose and DMEM with trehalose.

Subsequent in vitro culture of snap frozen cells (Fig. 6.4) showed that the cells that were suspended in medium containing trehalose and /or serum could plate and develop in vitro but none of these plated cells retained PI. However the dead (non viable) cells retained PI.

Cells suspended in

FBS	Cytomix	MTBS	DMEM	FBS	Cytomix	MTBS	DMEM
No trehalose				0.3 M trehalose			

Containing 100µg/ml Propidium Iodide

500 µL of cell suspension was loaded into each 2mL cryotube.

1. Slow cooling at 1°C/min from +25°C to -25°C using Cryologic freezing machine.
2. Samples were taken at temperature observation points (+25°C, +15°C, +5°C, -5°C, -15°C and -25°C).

Warming and thawing samples taken at each temperature observation point.

Re-Frozen by snap freezing

(20µL thawed cell suspension aspirated into 250µL straw) either at ASAP or 30 min after thawing

Fig. 6.2

Thawing 5 sec in air and then 37°C water bath until melted

Fig. 6.3

Fig. 6.4.

Viability test (by mixing 10µL cell suspension+10µL 4% Trypan Blue in PBS)

In vitro culture by placing 20µL cell suspension in 200µL DMEM in each well of 96-well TC dish).

Fig. 6.1. Flow chart for experiment 1.

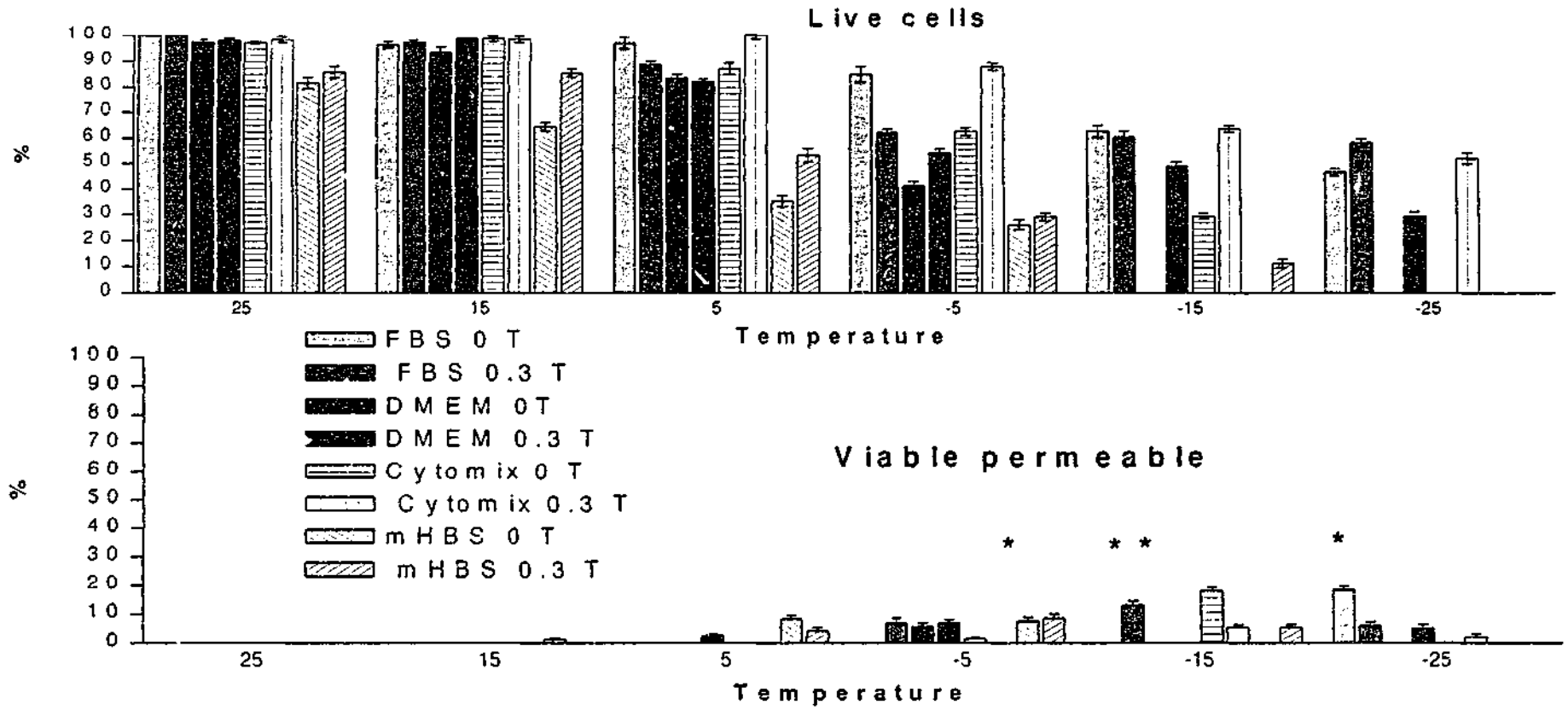
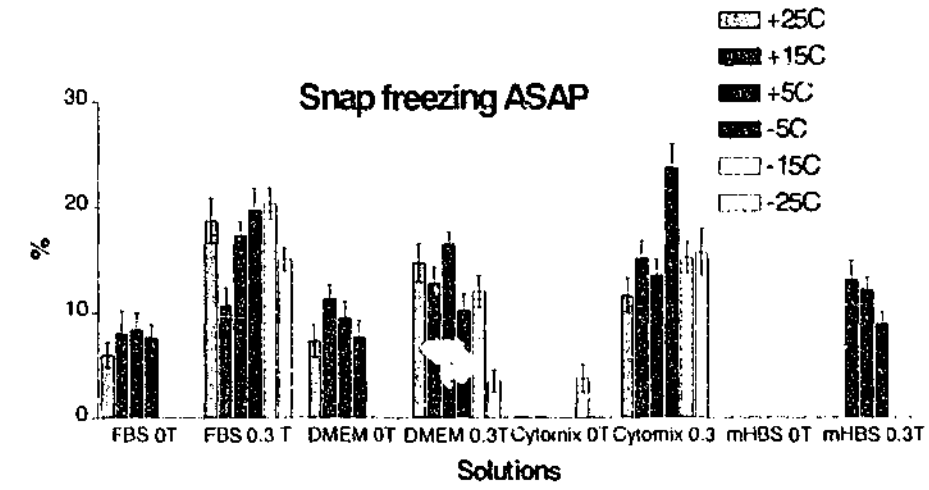


Fig. 6.2. Proportion of viable (TB-), and viable permeable (TB-; PI+) cells after cooling at 1°C/min from +25 C to -25°C (data in appendix table 6.1A.); *) significantly different (P<0.01).



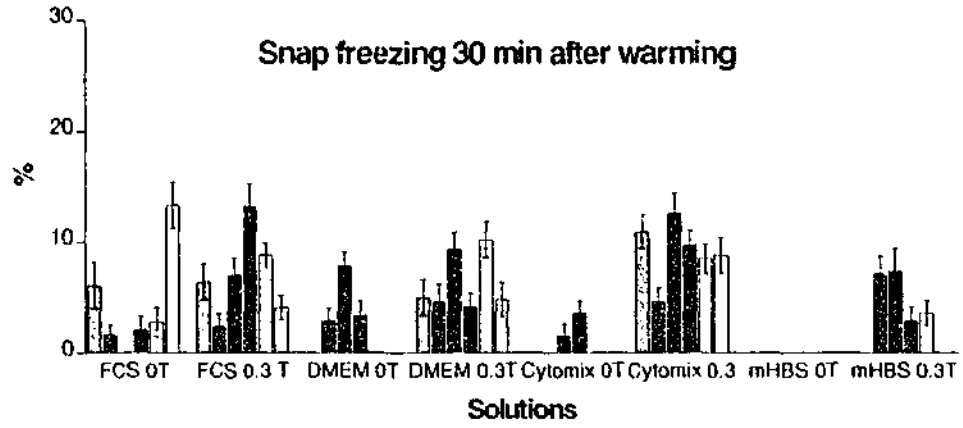
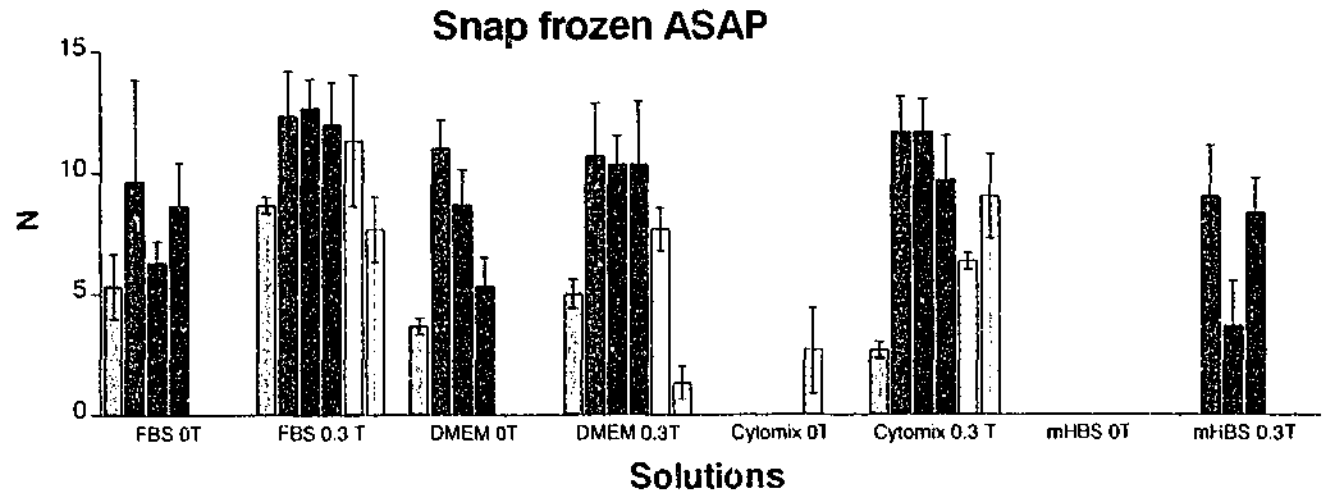


Fig. 6.3. Viability (TB-) of cells that were snap frozen in the absence of penetrating cryoprotectants. Showing the effects of previous slow cooling step (1°C/min between +25°C to -25°C) in each of 4 different media with or without trehalose.(data in appendix table 6.1B)



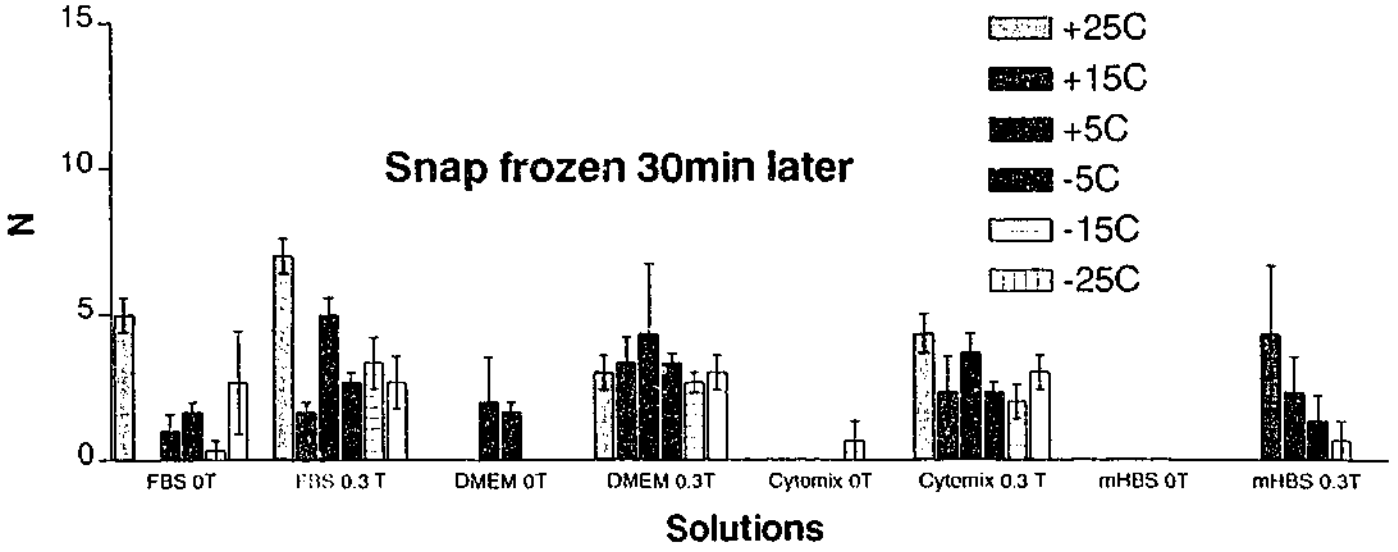


Fig. 6.4. The number of plated cells after thawing of re-frozen (snap frozen) ASAP (top) and 30 min (bottom) after equilibration in the first solution. Data in appendix 6.1C.

Plating in in vitro culture by cells that were snap frozen in the absence of penetrating cryoprotectant. Showing the effect of the previous slow cooling step for cells in 4 different media with or without trehalose.

6.3.3. Experiment 2: Effect of exposing cells to a penetrating cryoprotectant and trehalose prior to snap freezing.

6.3.3.1. Incubation in solution 1 (first solution).

Incubation of cells in solutions (Cytomix or FBS) containing penetrating cryoprotectant, with or without trehalose (0.6 M) for 5 min and 10 min at room temperature or slow cooling at 1°C/min from +25°C to –25°C did not have a toxic effect on the cells. Results in Fig. 6.6 and 6.7 showed no difference in cell survival between incubated (non frozen) and slow cooled cells in the absence (Fig. 6.6) and presence (Fig. 6.7) of trehalose.

The presence of permeable cryoprotectants (DMSO and ethylene glycol) did not significantly alter cell membrane permeability of cells incubated at room temperature. Cooling cells to -25°C in the presence of permeable cryoprotectants and the absence of trehalose could permeabilise the cell membranes (Figs. 6.6 and 6.7). Slow cooling to -25°C significantly ($P<0.01$) increased the number and proportion of viable permeabilised cells as compared to those incubated at room temperature (Figs. 6.6 and 6.7). The number of non viable cells increased (n.s) after slow cooling to -25°C than the viable permeable cells (Fig. 6.6 and 6.7). There was no significant difference between FBS and Cytomix in the proportion viable and viable permeable cells.

Cooling cells to -25°C in the presence of trehalose gave a lower proportion of dead (non viable) cells and viable permeable cells (Fig. 6.7) as compared to cooling without trehalose (Fig. 6.6), but the difference was not significant ($P>0.05$).

Most cells plated in in vitro culture after incubation in the first solutions. The plated cells accumulated in the center of the dish making a very dense cell population which made it difficult to perform cell counts. In these groups there was no effect of equilibration time, temperature and the presence of trehalose on plating. Extending the period of in vitro culture to 24 h resulted in confluent cell cultures. This indicated that cells did proliferate.

6.3.3.2. Incubation in the second solution (Solution 2)

Incubation in the second solution showed a significant effect ($P<0.01$) of time and the presence of trehalose. Cell suspended in FBS had a higher ($P<0.01$) proportion of viable cells as compared to Cytomix.

The presence and absence of trehalose in solution 1 did not have any effect if the solution was based on FBS (Fig. 6.8 and 6.9). There was a significant difference ($P<0.01$) when solution 1 was based on Cytomix.

Table 6.2. Group codes used for the treatments in Fig. 6.8.

First solution (Solution 1)*)	Second solution (Solution 2) **) (min)	Trehalose concentration (M) in the second solution	Group code
5 min incubation at RT	10	0	5O10LNoT
		0.3	5O10L03T
	60	0	5O60LNoT
		0.3	5O60L03T
10 min incubation at RT	10	0	10O10LNoT
		0.3	10O10L03T
	60	0	10O60LNoT
		0.3	10O60L03T
Cooled 1°C/min from +25° to -25°C	10	0	-25O10LnoT
		0.3	-25O10L03T
	60	0	-25O60LnoT
		0.3	-25O60L03T

*) Solution 1 could be with or without trehalose; **) Solution 2 could be FBS or Cyto mix.

6.3.3.3. Snap freezing after incubation in the second solution (Solution 2)

This step, ascertained whether cells could survive snap freezing in solutions without penetrating cryoprotectant if they had first been incubated in solutions containing penetrating cryoprotectant with or without cooling at 1°C/min from 25°C to -25°C. It was designed to test the hypothesis that trehalose uptake into cells would be enhanced by the addition and removal of a penetrating cryoprotectant.

Results in Figs. 6.10 and 6.11 show that both FBS and trehalose in the second (penetrating cryoprotectant free) solution had an effect on cell survival following snap freezing. In the absence of FBS and trehalose in the second solution, none of the cells survived snap freezing.

The type and concentration of the penetrating cryoprotectant in solution 1 in which the cells were pre-incubated, had no consistent effect on the outcome when the subsequent snap freezing steps were performed without a penetrating cryoprotectant.

Those results showed that FBS alone protected cells against snap freezing regardless of the presence of trehalose (Fig. 6.10 and 6.11). However trehalose needed to be present in the Cytomix medium to protect the cells against snap freezing.

Observations following 12 h of in vitro culture showed that only the dead cells were PI positive. None of the plated cells were PI positive.

The presence of trehalose in the first solution did not affect the cell viability and plating rate following incubation in solution 2 and snap freezing (Figs.6.8; 6.9, 6.10 and 6.11). Cytomix based second solutions protected cells against snap freezing only when trehalose was present in the second solution (Fig. 6.10 and 6.11). Cells suspended in Cytomix based solution 2 in the absence of trehalose did not survive snap freezing (Figs. 6.9 and 6.10). None of the cells that were suspended in Cytomix based solution 2 plated after in vitro culture. The presence of FBS in solution 2 protected cells against snap freezing regardless the presence of trehalose (Fig. 6.10 and 6.11).

Composition of solution 1 (containing 100μg/mL PI)

FBS								Cytomix							
No trehalose				0.6 M trehalose				No trehalose				0.6 M trehalose			
20% D	10% D 10% EG	10% D	10% EG	20% D	10% D 10% EG	10% D	10% EG	20% D	10% D 10% EG	10% D	10% EG	20% D	10% D 10% EG	10% D	10% EG

Equilibration 100μL cell suspension at

RT for 5 min	RT for 10 min	Immediately cooled at 1°C/min from 0° to -25°C then thawed for 5 sec at RT and 37°C waterbath

Composition of solution 2 (containing 100μg/mL PI)			
FBS		Cytomix	
No trehalose	0.3 M Trehalose	No trehalose	0.3 M Trehalose
Incubate for 10 min or 60 min at RT			

Protocols:

- Dilute 10μL of cell suspension in solution 1 into 90μL of solution 2 then incubate for 10 or 60 min at RT.
- aspirate 20μL cell suspension into 250μL straw.

Snap freeze into Liquid Nitrogen

Thawing 5 sec in air and then 37°C water bath until melted

Fig. 6.8
Fig. 6.9

Fig. 6.6
Fig. 6.7

See text

Fig. 6.10
Fig. 6.11

Fig. 6.12

Viability test (by mixing 10μL cell suspension+10μL 4% Trypan Blue in PBS)

In vitro culture by placing 20μL cell suspension in 200μL DMEM in each well of 96-well TC dish).

Fig. 6.5. Flow chart for experiment 2

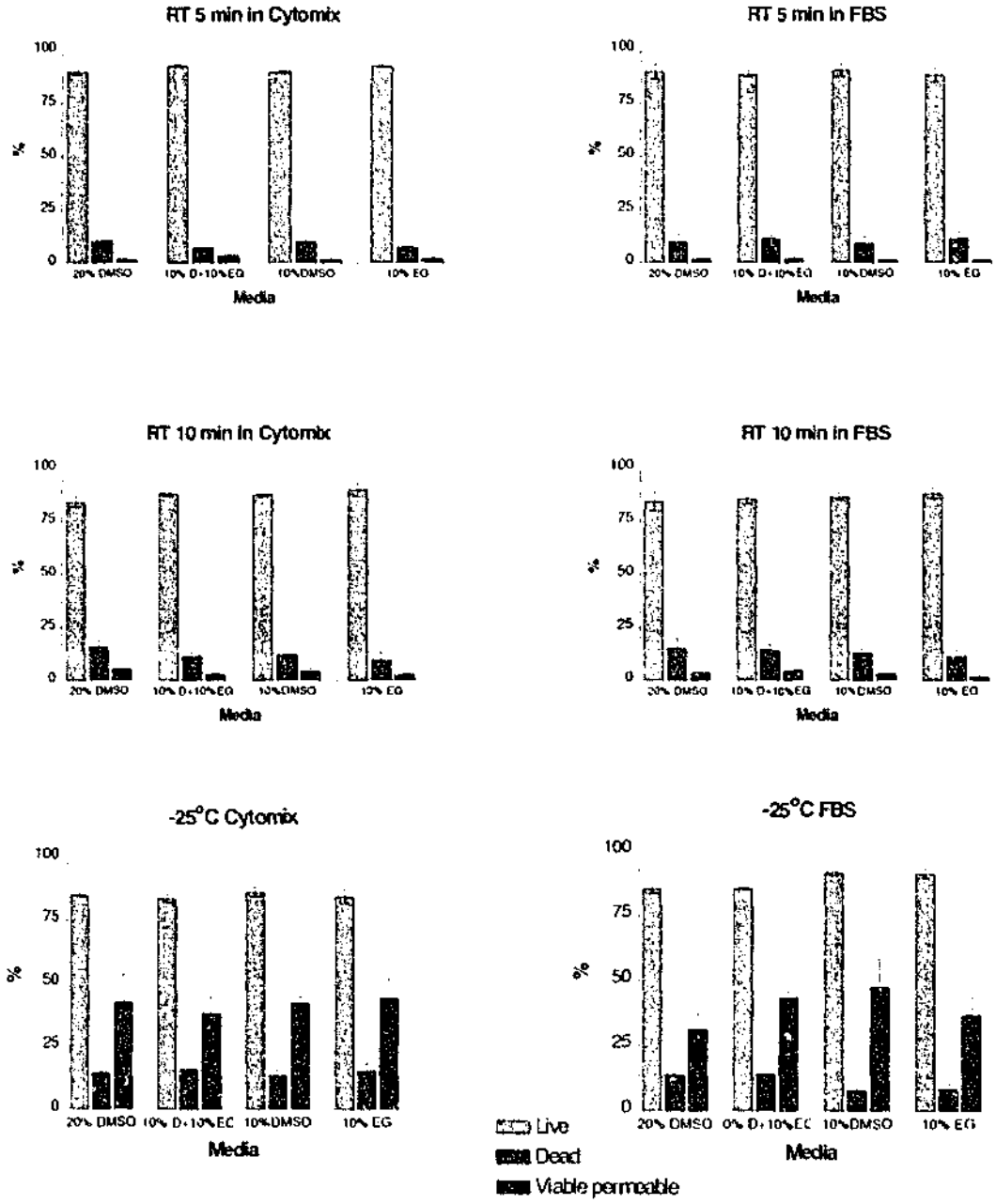


Fig. 6.6. Cell viability after equilibration in the first solutions without trehalose (data in appendix 6.2A)

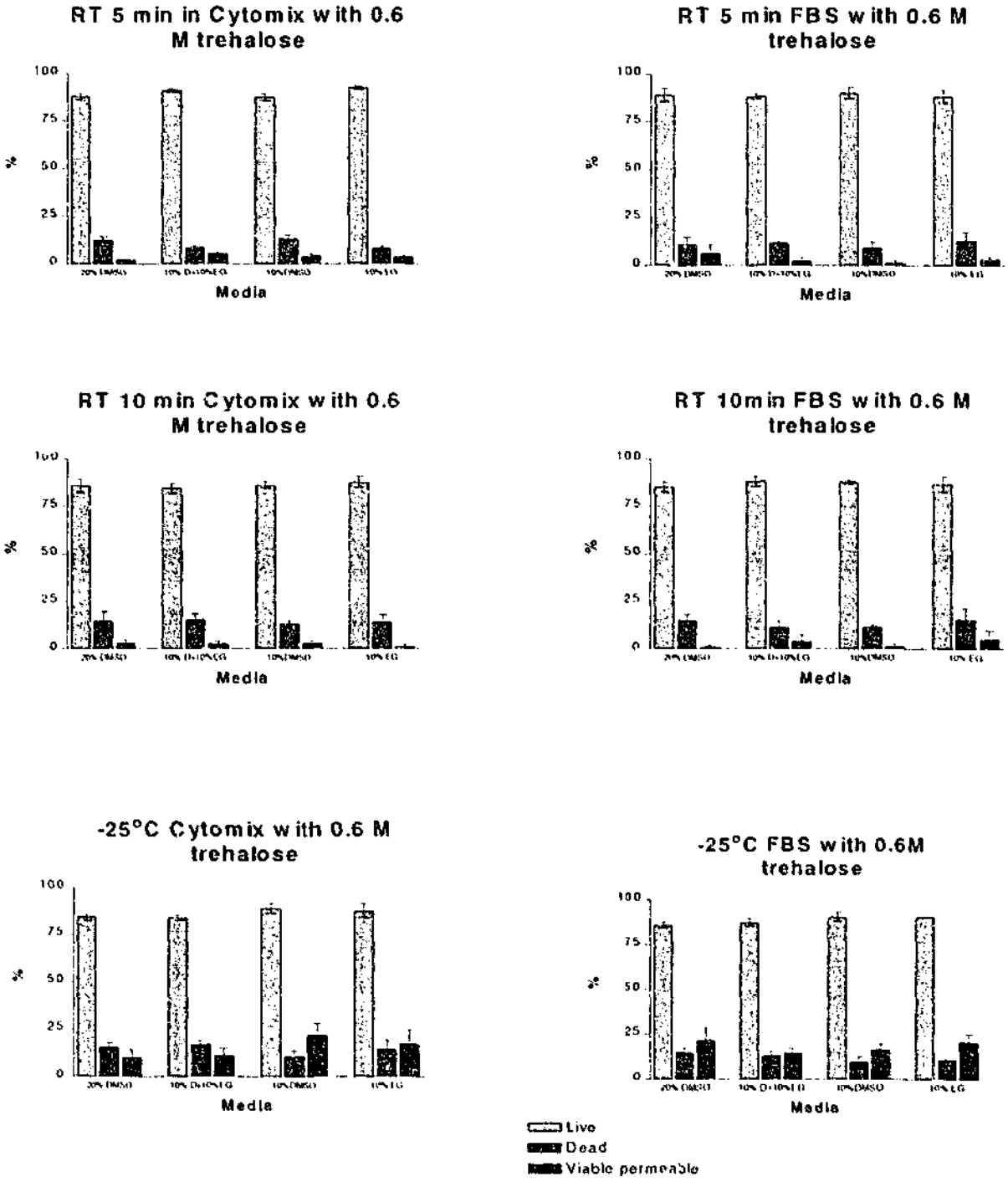
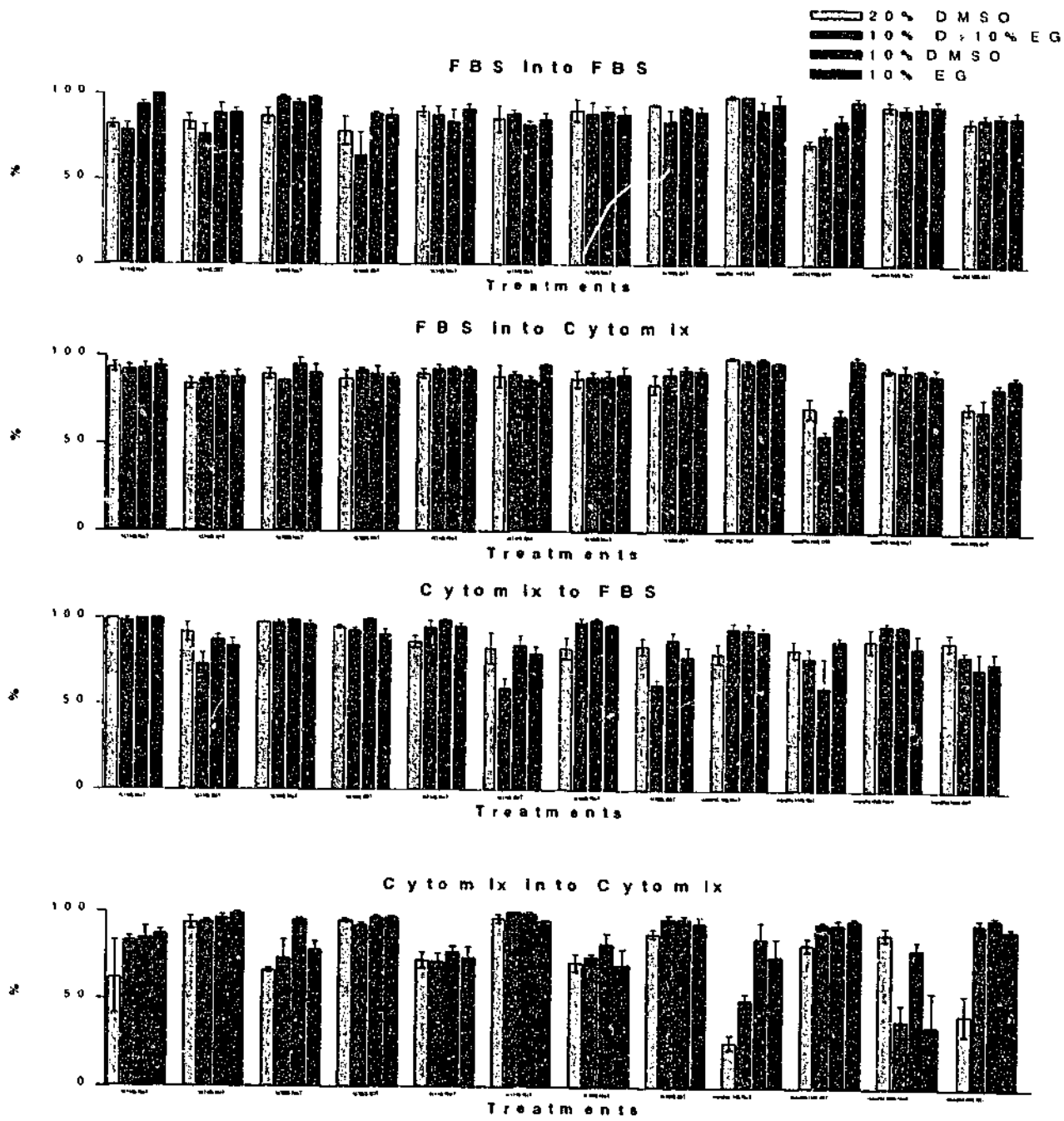
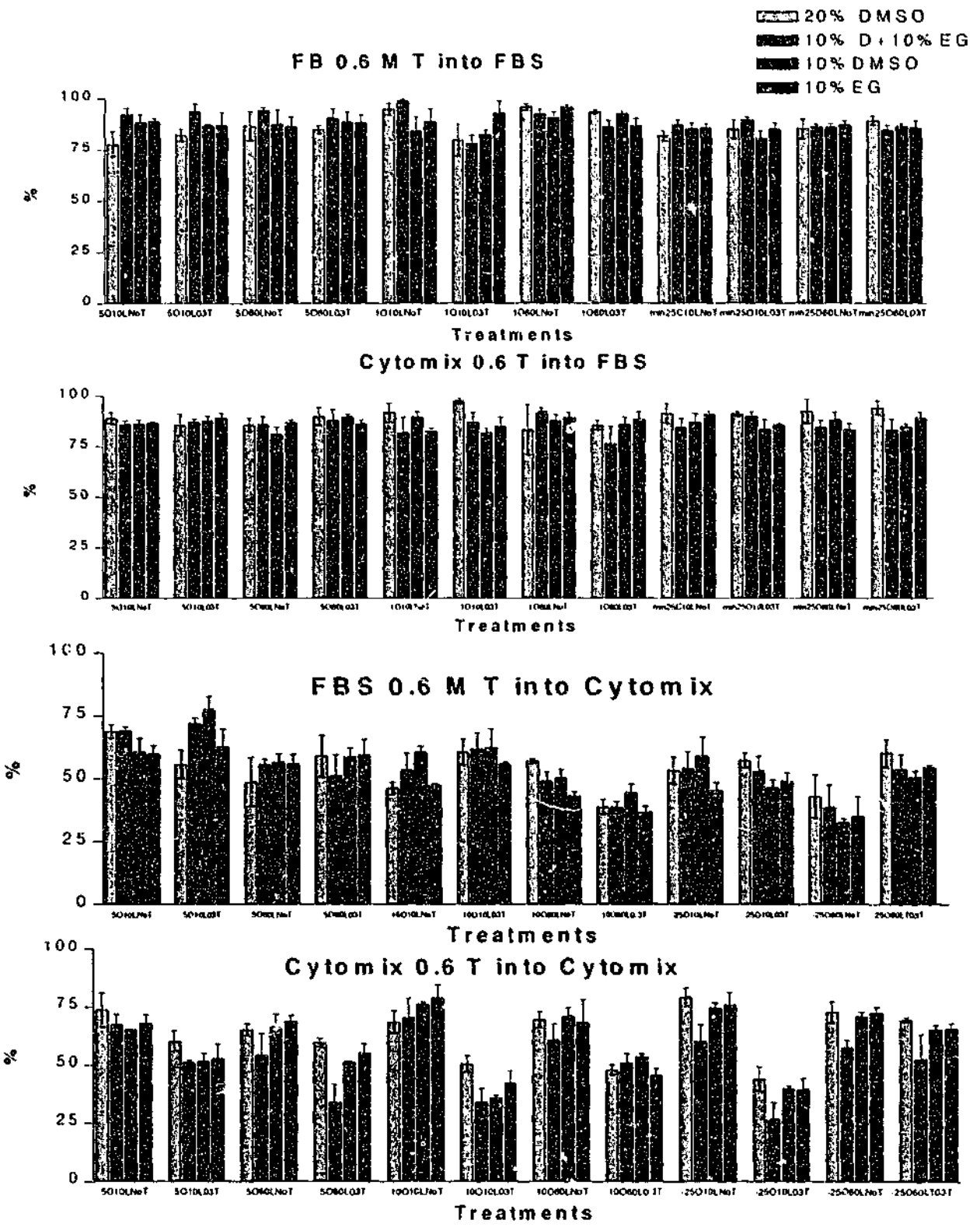


Fig. 6.7. Cell viability after equilibration in the first solutions containing 0.6M trehalose (data in appendix 6.2B).



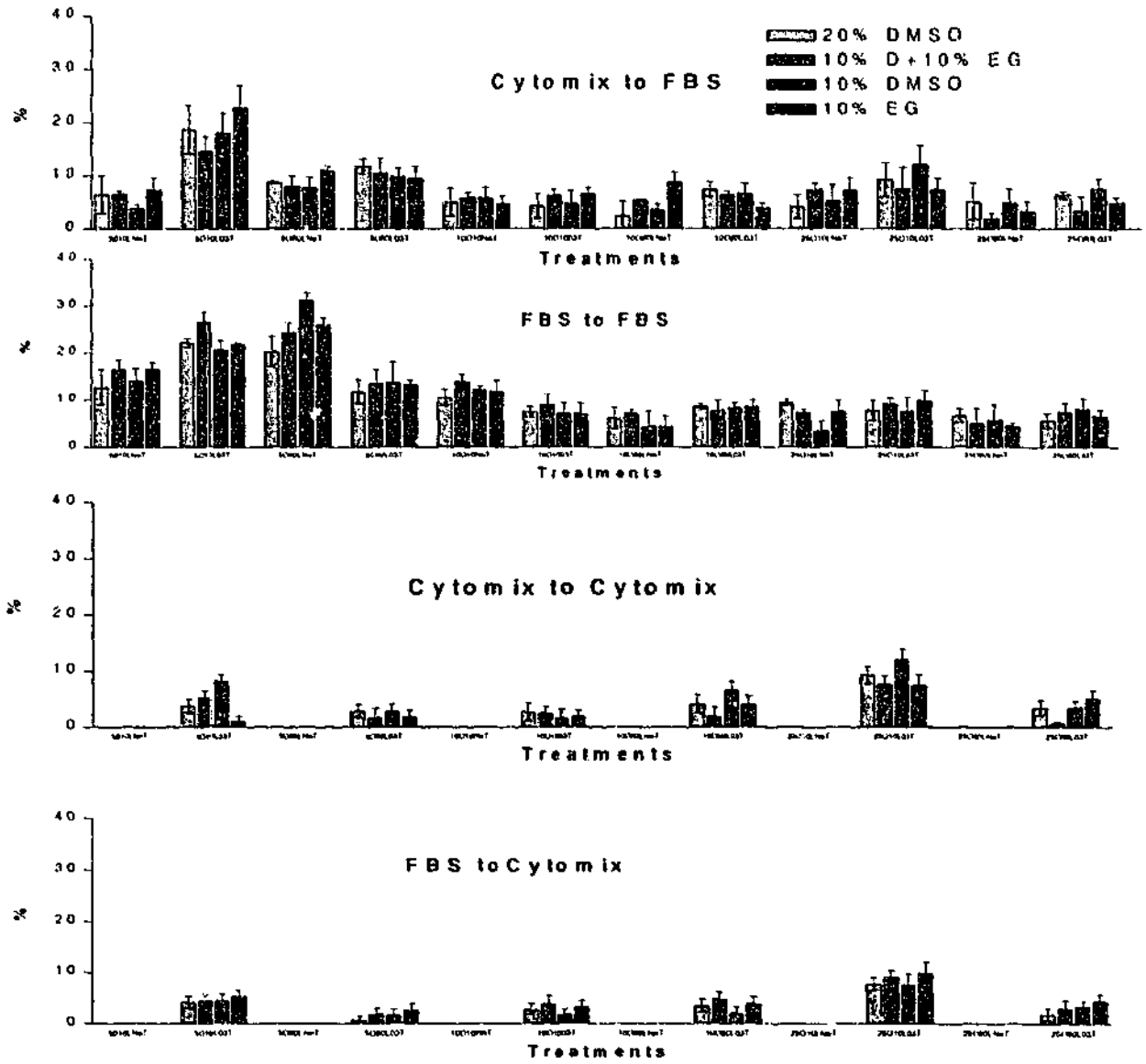
Trehalose in solution 2 (M)	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3
Time in Solution 2	10 min		60 min		10 min		60 min		10 min		60 min	
Equilibration in Solution 1	RT for 5 min				RT for 10 min				Slow cool 1°C/min to -25°C			

Fig. 6.8. Proportion of viable cells after equilibration in both solution 1 (without Trehalose) and solution 2 at different time and temperatures. (Data in appendix 6.2D) (Solution control).



Trehalose in solution 2 (M)	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3
Time in Solution 2	10 min		60 min		10 min		60 min		10 min		60 min	
Equilibration in Solution 1	RT for 5 min				RT for 10 min				Slow cool 1°C/min to -25°C			

Fig. 6.9. Proportion of viable cells after equilibration in both solution 1 containing 0.6 M Trehalose and solution 2 at different time and temperatures. (Data in appendix 6.2C) (Solution control).



Trehalose in solution 2 (M)	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3
Time in Solution 2	10 min		60 min		10 min		60 min		10 min		60 min	
Equilibration in Solution 1	RT for 5 min				RT for 10 min				Slow cool 1°C/min to -25°C			

Fig. 610. Cells surviving (TB-) snap freezing in solution 2 in relation to the equilibration protocols, and the cryoprotectant type and concentration in solution 1 without trehalose (Cytomix or FBS). Data in appendix 6.2E.

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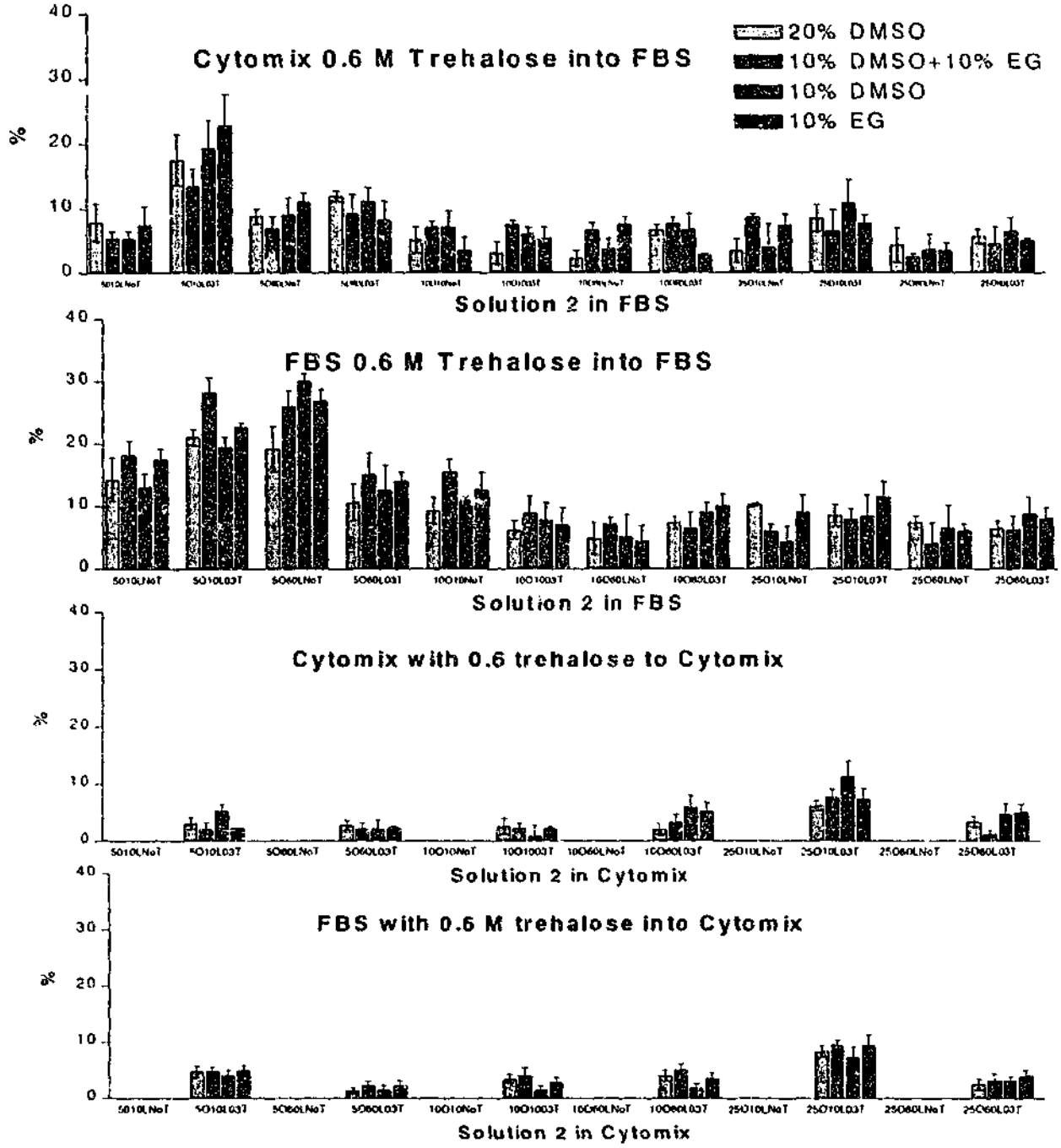
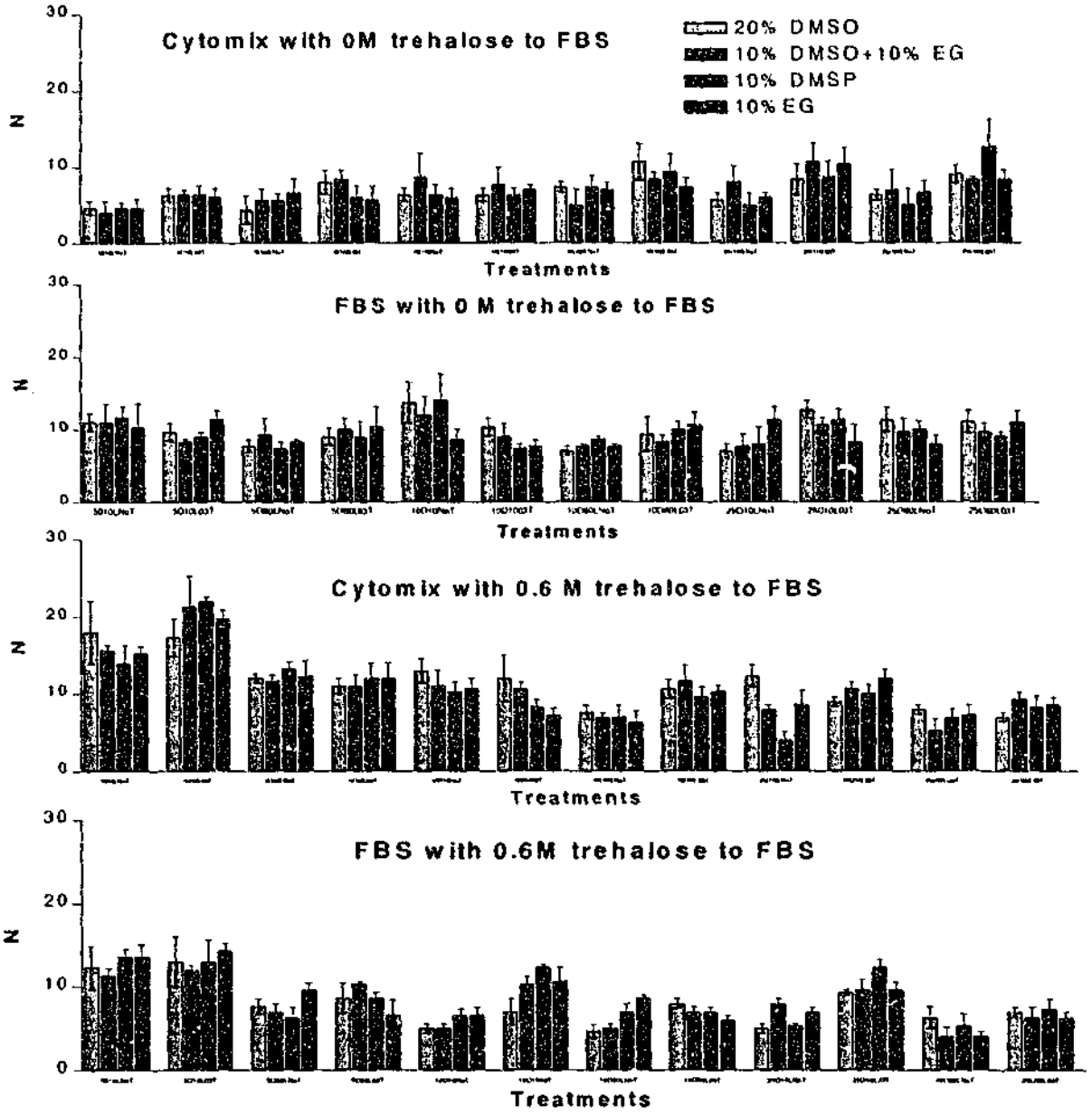


Trehalose in solution 2 (M)	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3
Time in Solution 2	10 min		60 min		10 min		60 min		10 min		60 min	
Equilibration in Solution 1	RT for 5 min				RT for 10 min				Slow cool 1°C/min to -25°C			

Fig. 6.11. Proportion of cells surviving (TB-) snap freezing in solution 2 in relation to the equilibration protocols, and the cryoprotectant type and concentration in solution 1 with trehalose (Cytomix or FBS). Data in appendix 6.2F.



Trehalose in solution 2 (M)	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3
Time in Solution 2	10 min		60 min		10 min		60 min		10 min		60 min	
Equilibration in Solution 1	RT for 5 min				RT for 10 min				Slow cool 1°C/min to -25°C			

Fig. 6.12. The number of plated cells observed in 96-well TC-dish after incubation in solution 1 and 2 then snap frozen

6.3.4. Experiment 3 Evaluation of the effect of adding Cytomix components to a conventional freezing solution.

6.3.4.1. Cell viability

Suspending cells in 10% DMSO in FBS with or without 0.3 M trehalose and other Cytomix components had no effect ($P>0.05$) on the viability of non-frozen cells (Fig. 6.14). The high proportion of viable cells showed that those solutions were not toxic to the cells.

Only a low proportion ($P>0.05$) of the viable cells were permeable to PI. However these viable permeable cells had an altered membrane appearance as in experiment 1 (section 6.3.2).

6.3.4.2. Cell viability and permeability after slow cooling and thawing at different temperatures

The solution in which the cells were conventionally slow cooled at 1°C/min to -80°C and the thawing protocols did influence cell viability ($P<0.01$) and permeability (Fig. 6.15).

Freezing resulted in the permeabilisation of viable cells. The presence of 10 mM ATP in the medium reduced ($P<0.01$) cell viability and the proportion of viable and permeabilised cells irrespective of the thawing method.

Thawing at 37°C for 5 min gave the highest proportion of viable ($P<0.01$) and viable permeabilised ($P<0.01$) cells. .

6.3.4.3. Plated cells

In vitro culture in DMEM 10% FBS at 37°C with 5% CO_2 in air within 96 well-TC plates showed a significant effect of the thawing methods on cell plating ($P<0.01$). Cells thawed at 37°C for 5 min following slow cooling at 1°C/min to -80°C with storage in liquid nitrogen gave a similar plating result ($P>0.05$) to the control. Thawing at RT for 15 min and 0°C for 30 min gave lower ($P<0.01$) numbers of plated cells (Fig. 6.16).

6.3.4.4. Cells were re-frozen by snap freezing or slow cooling in 0.3 M Trehalose in Cytomix

When the conventionally frozen cells (from section 6.3.4.2 and 6.3.4.3) were diluted in Cytomix containing 0.3 M Trehalose without serum or DMSO the initial thawing method (section 6.3.4.3) affected ($P<0.01$) cell viability and permeability when they were subsequently re-frozen (Figs. 6.17 and 6.18). The presence of 10mM ATP reduced cell viability. Adding 10 times the normal concentration of Glutathione did not protect cells against re-freezing.

Figures 6.8 and 6.9 show that the results for cells re-frozen by snap freezing in Cytomix containing 0.3 M trehalose was affected by the initial freeze-thaw procedure. Since the viable cells from the initial thawing were not separated from the non-viable cells, then the observation on the re-frozen groups include non-viable cells from previous treatments.

Slow cooled cells, like snap frozen cells, showed a significant reduction ($P<0.01$) in the number of viable and viable-permeabilized cells as compared to the initial thaw (Fig. 6.16). After both freezing protocols, all viable (TB-) cells excited PI, meaning that all the cells had all become permeable to the dye (PI). (Figs. 6.15 and 6.16). However more cells thawed at 37°C survived ($P>0.05$) snap freezing when trehalose was present in the cell suspension (DFT). The presence of components of cytomix such as ATP, EGTA and Glutathione (in high concentrations) were all toxic to the cells.

6.3.4.5. Plated cells following re-freezing in Cytomix contains 0.3M Trehalose

In vitro culture was used to confirm whether the viable cells could develop in vitro. Observations on the re-frozen cells after in vitro culture for 24 hr showed a difference ($P<0.01$) in the number of plated cells (Fig. 6.19). Cells that had previously been thawed at 37°C showed a higher ($P<0.01$) number of plated cells as compared to those thawed at RT or 0°C. Cells frozen in the presence of a higher concentration of ATP and Glutathione formed fewer ($P<0.01$) plating cells as compared to those frozen in the other solutions. However cells that were frozen in the presence of trehalose on the both sides of the membranes showed the highest plating rate as compared to the other treatments.

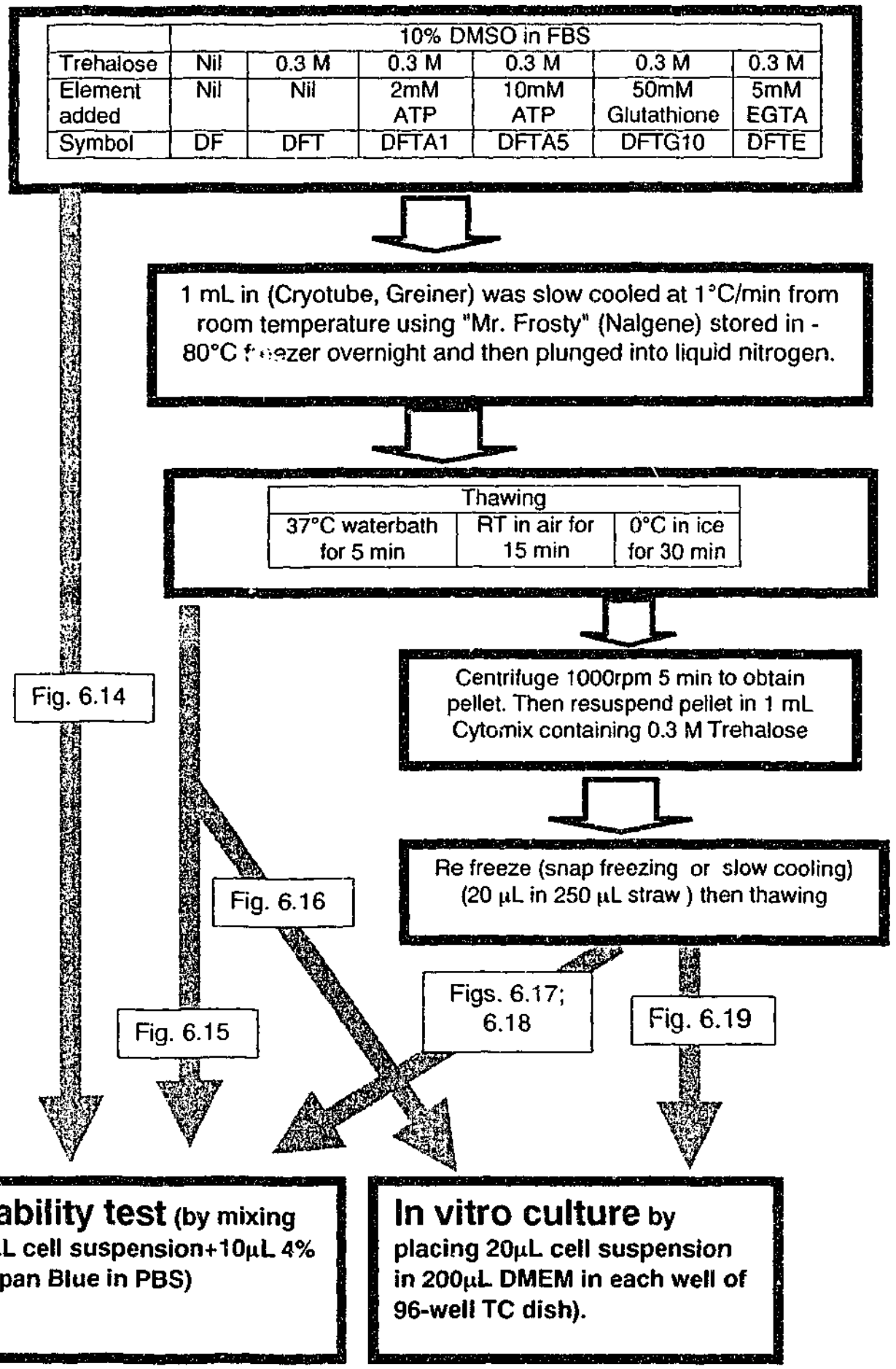


Fig. 6.13. Flow chart for experiment 3

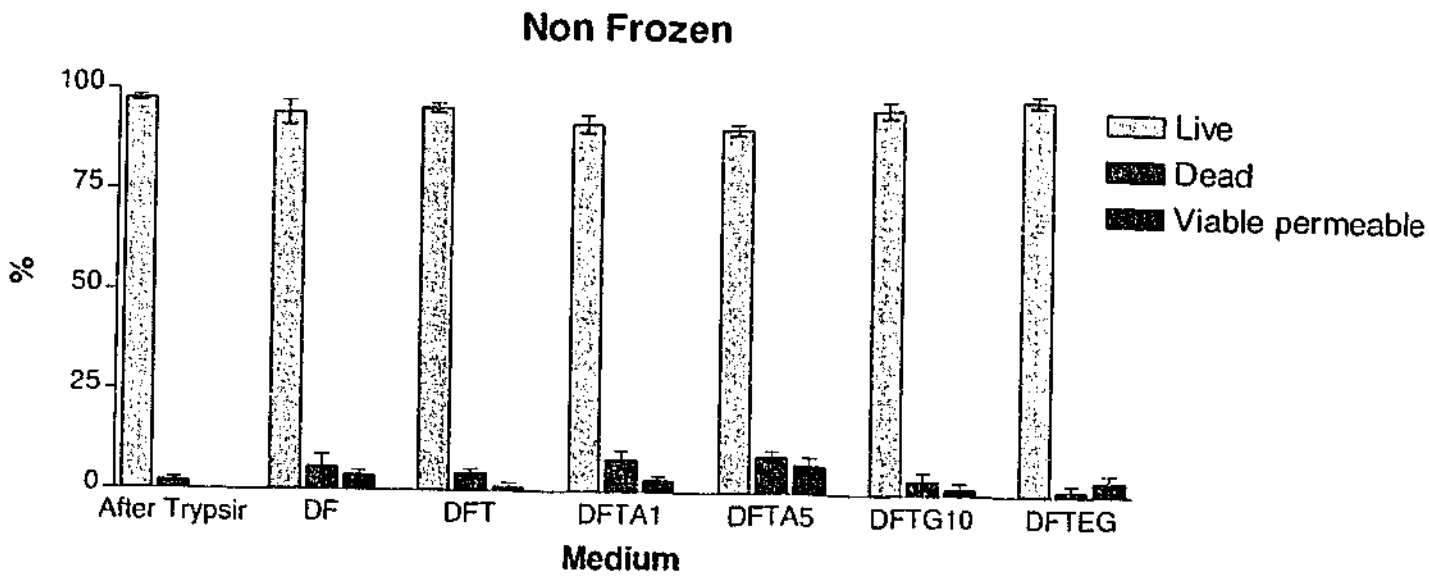


Fig 6.14. Proportion of viable and permeable of non-frozen, diluted, cells prior to freezing. (Data in appendix 6.3A).

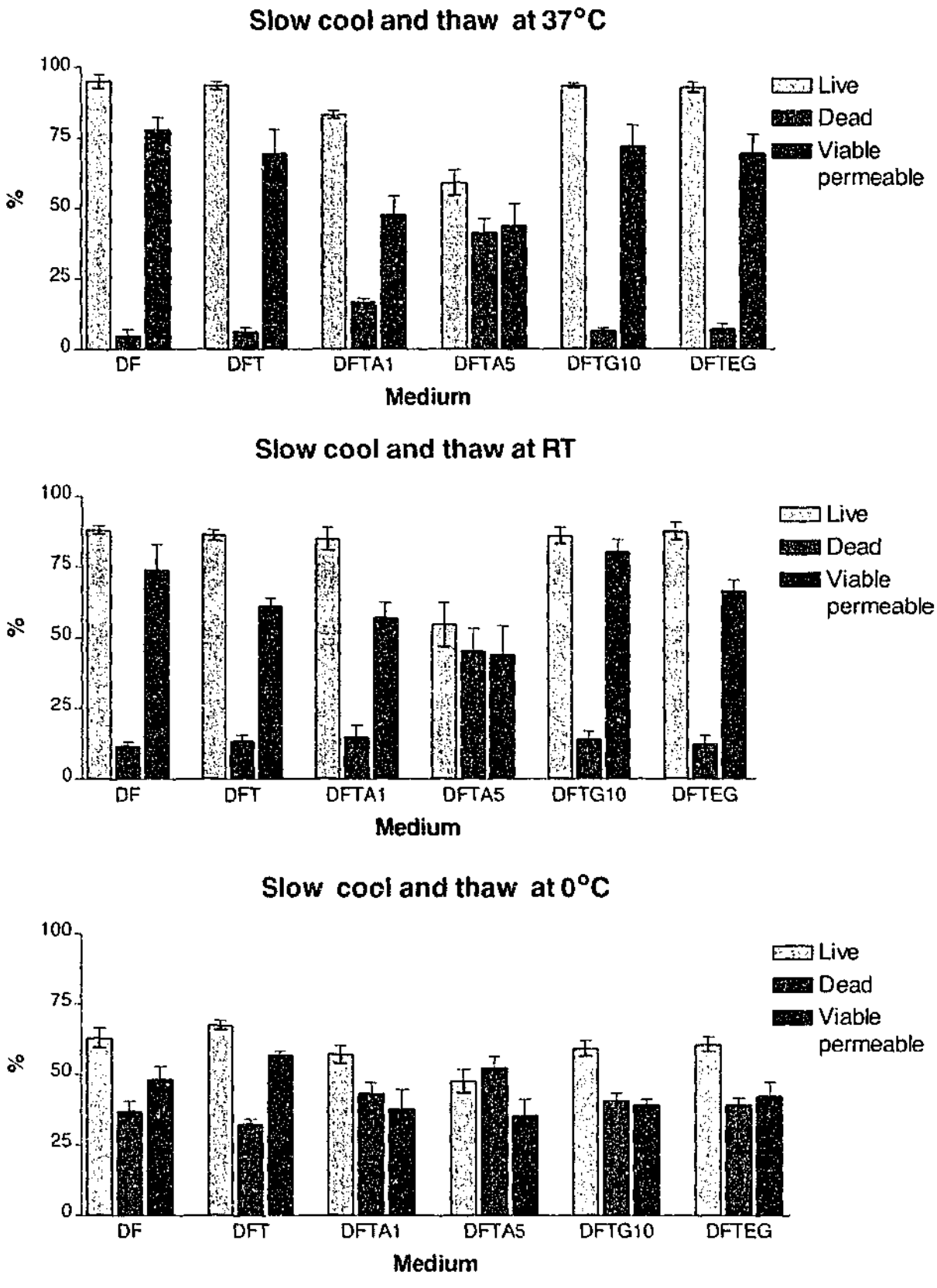


Fig. 6.15. Cell characteristics after conventional slow cooling and thawing (Sata in appendix 6.3B).

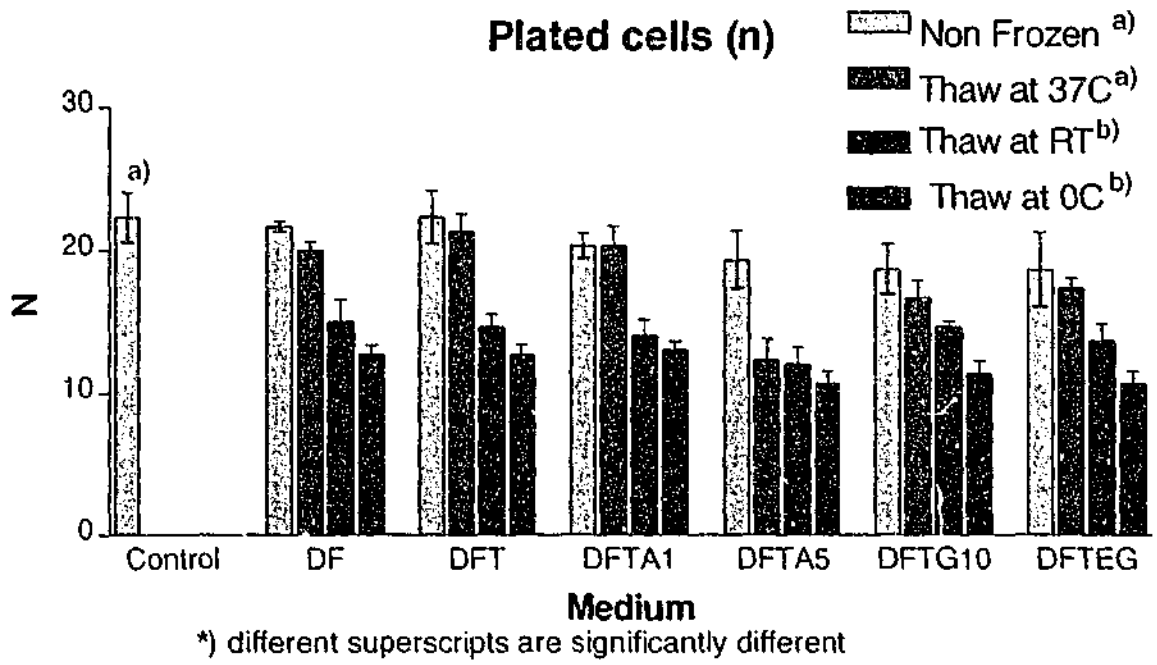


Fig. 6.16. Cell plating by control non-frozen cells as compared to conventionally slow cooled cells thawed by each of three different thawing protocols (Data in appendix 6.3C).

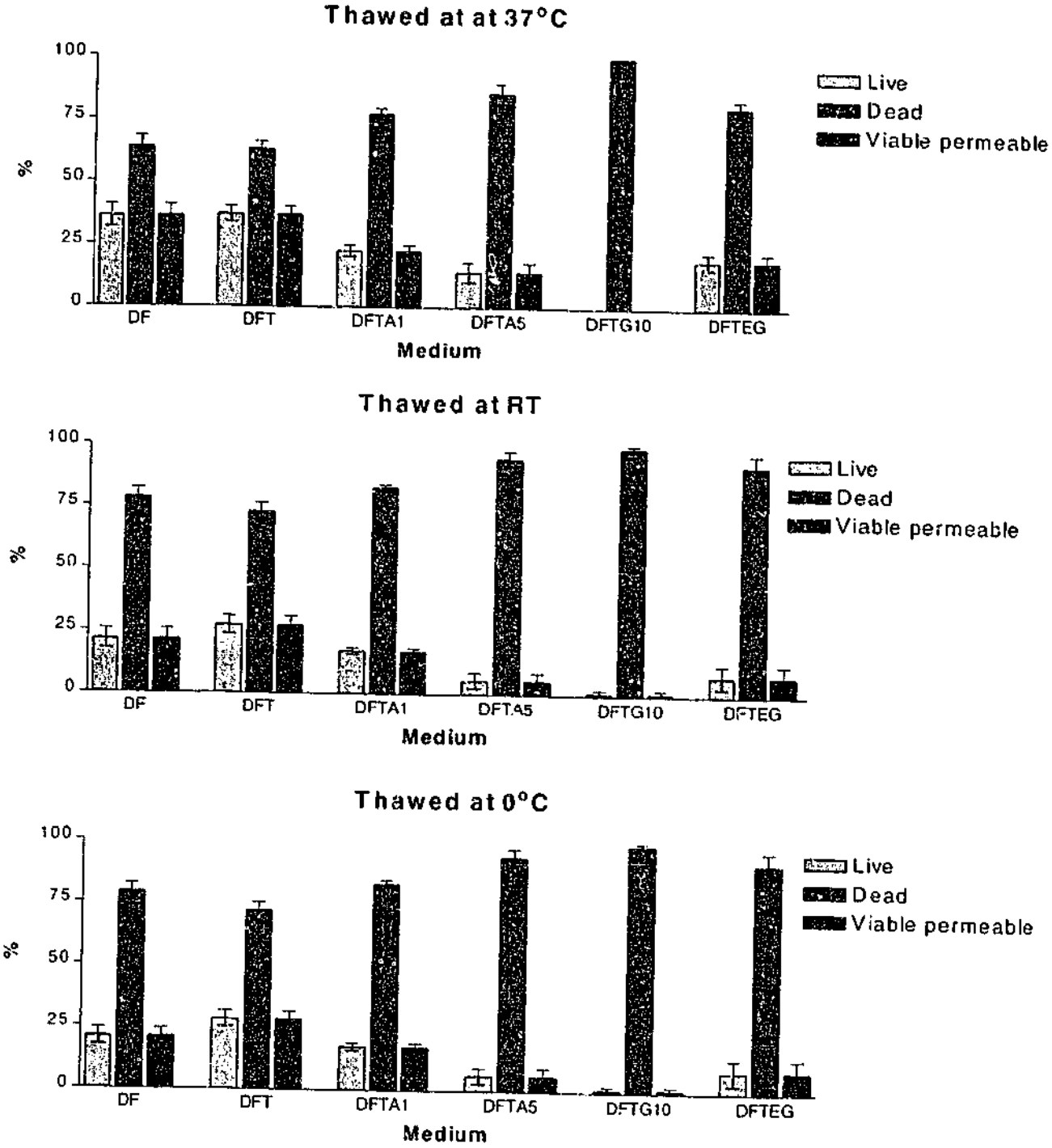


Fig. 6.17. Viability and permeability (%) of cells that were re-frozen (snap frozen) in Cytomix containing 0.3M Trehalose after an initial slow freezing showing an effect of the first thawing method. (Data in appendix 6.3D).

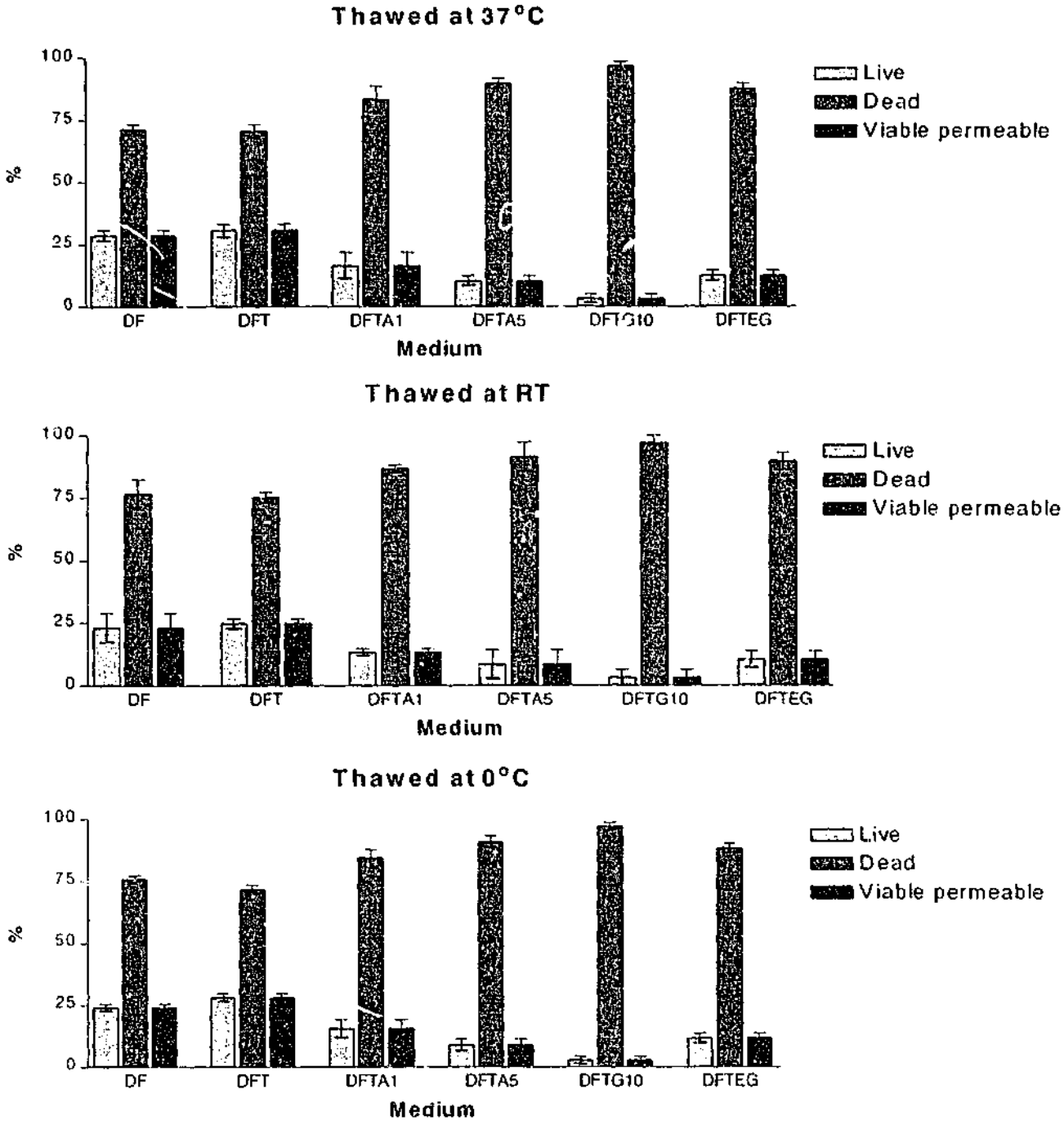


Fig. 6.18. Viability and permeability (%) of cells re-frozen using slow cooling in Cytomix containing 0.3M Trehalose in relation to the initial thawing methods (Data in appendix 6.3E).

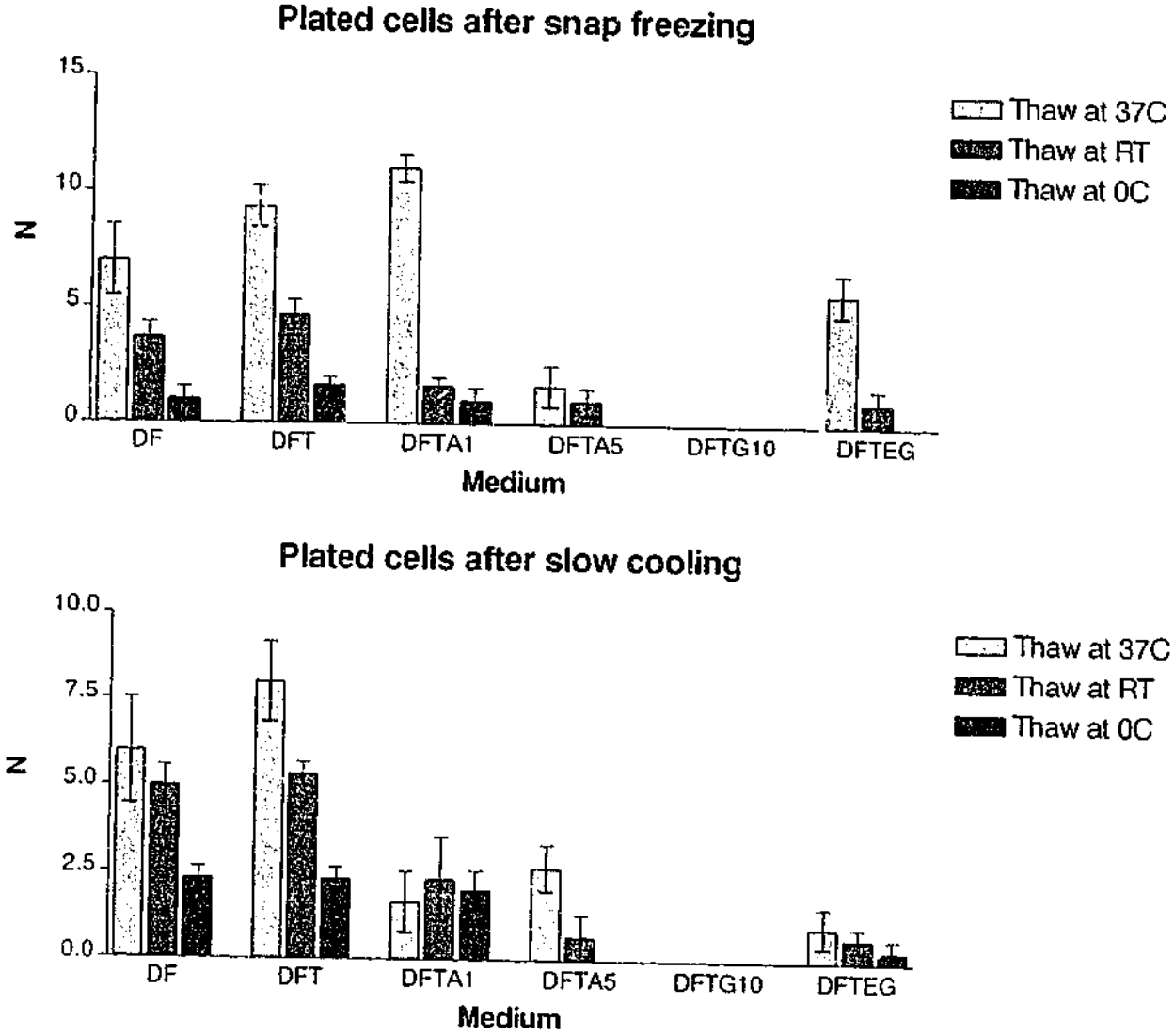


Fig. 6.19. Number of cells plating after re-freezing (snap freezing and slow cooling) in Cytomix containing 0.3M Trehalose (showing an effect of the thawing protocol used after the first freezing) (Data in appendix 6.3F).

6.3.5. Experiment 4. Effect of cooling to temperatures between +25°C and LN_2 in FBS, DMSO and trehalose on membrane permeabilisation and post thaw survival

Slow cooling in 10% DMSO in FBS gave significant protection against freezing damage and is highly suitable for cell preservation. Slow cooling of 500 µL of cell suspension in 10% DMSO in FBS in a cryovial at 1°C/min using a Cryologic freezing machine without seeding, showed that the suspension always formed ice crystals by -20°C. Cooling from RT to -10°C reduced the proportion of live cells ($P>0.05$), but did not significantly ($P>0.05$) alter cell membrane permeability to PI. Once the temperature reached -20°C, the proportion of dead (TB+) cell and viable permeable cells significantly increased. Cooling below -30°C increased the proportion of dead and viable permeable cells, but the ratio then remained steady ($P>0.05$) for all temperatures down to -196°C (liquid nitrogen) (Fig. 6.21). The presence of viable permeabilised cells showed that cooling, particularly below -20°C, made the cell membrane leaky and permeable to PI. The cells that became permeable remained viable as they retained intracellular PI but excluded TB upon thawing. This also indicated that the membrane of viable permeable cells had reclosed within 15-30 min after thawing.

In vitro culture of cells sampled (10µL in 200µL DMEM 10% FBS) from each temperature gave similar rates of plating and proliferation. No quantitative observation could be performed since all groups became sub-confluent within 24 h and became confluent within 72 hr.

DMSO was removed from the cells by layering 100 µL of the suspension over 1 mL of FBS or Cytomix with 0, 0.3 M; 0.5 M; or 1.0 M trehalose. Cells were washed by centrifugation at 3000 rpm to obtain a pellet. Cells were centrifuged at 3000 rpm because centrifugation at less than 3000 rpm did not allow cells resuspended in 1.0 M trehalose to form pellet. After removal of the supernatant, the pellet was tested again for cell viability (Fig. 6.22 and 6.23).

Viability and permeability tests on the cells after centrifugation showed no significant reduction ($P>0.05$) in the number and proportion of live cells (Figs. 6.22 and 6.23). However there was a significant reduction in cell number in the groups washed in 1 M trehalose, because the high density of the media (both Cytomix or FBS) containing 1 M Trehalose prevented the cells from forming a pellet.

In vitro culture of cells (20μL) from the pellets in 96-well TC-dishes with 200 μL DMEM 10% FBS showed that the cells could develop and proliferate. Cells plated within 6 hr and proliferated and became sub-confluent within 48 hr. Again, due to the cell density it was not possible to determine the number of plated cells. Cells centrifuged in medium containing 1 M trehalose were shrunken by comparison to those in the other solutions. The ability to form sub confluent cultures indicated that incubation in either Cytomix or FBS with or without trehalose was not toxic to the cells.

Cells snap frozen after centrifugation and washing of the cryoprotectant killed all cells resuspended in Cytomix without trehalose and most cells resuspended in Cytomix containing trehalose (Fig. 6.22 and 6.23). All cells became PI positive. Although the cells were classified as viable, their membranes were not clear and bright as would be expected.

Cells resuspended in FBS survived snap freezing with or without trehalose (Fig. 6. 23) and there was no difference in the proportion of viable cells between temperatures. As with cells resuspended in Cytomix, cells resuspended in FBS all became PI positive indicating that all cells had leaky cell membranes.

In vitro culture of the cell snap frozen in FBS showed that cells in all treatments plated and proliferated. However the number of plated cells was low as compared to the non frozen groups. There was no effect ($P>0.05$) of the temperatures at which cooling had stopped but there was an effect ($P<0.01$) of the trehalose concentration in which the cells were diluted. This differences was due to the lower cell number obtained after centrifugation in FBS with 1 M Trehalose. These cells plated and proliferated even though they had shrunken due to the extreme hyperosmotic conditions.

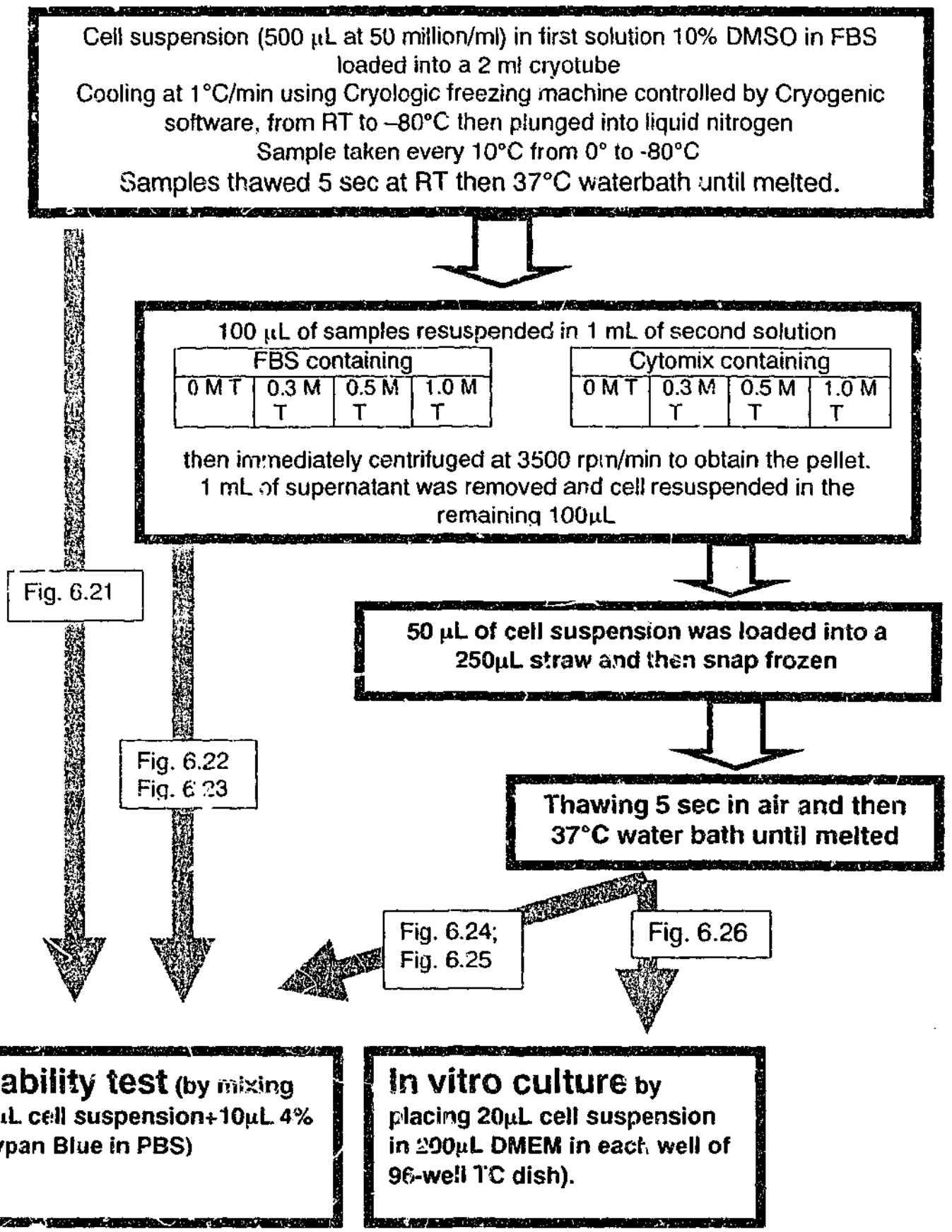
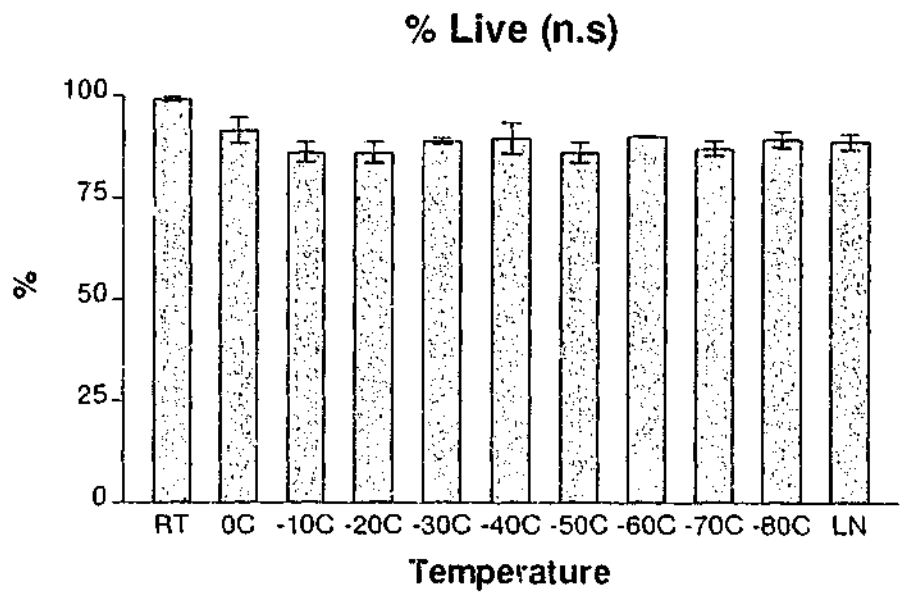


Fig. 6.20. Flow chart for experiment 4.



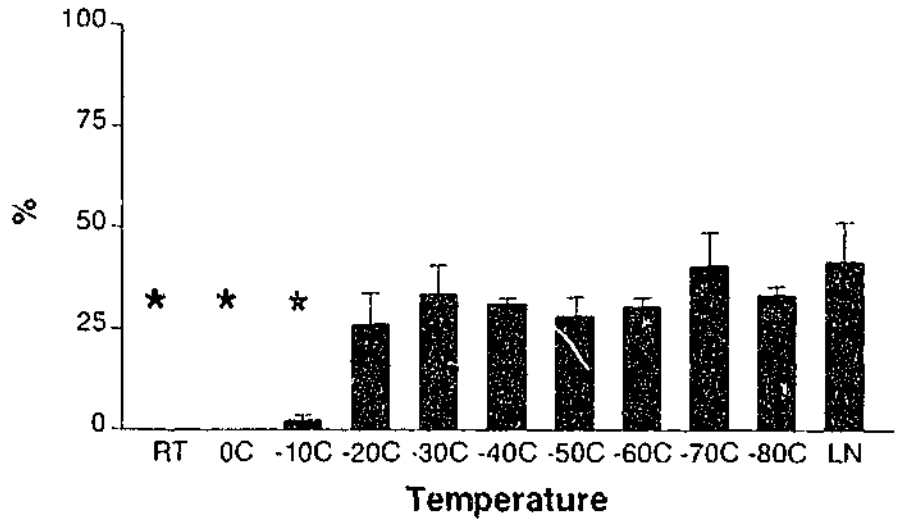


Fig. 6.21. Proportion (right) of live (TB-) and viable permeable (TB-; PI+) cells in relation to the temperature at which slow cooling at 1°C/min was stopped (Data in appendix 6.4A).

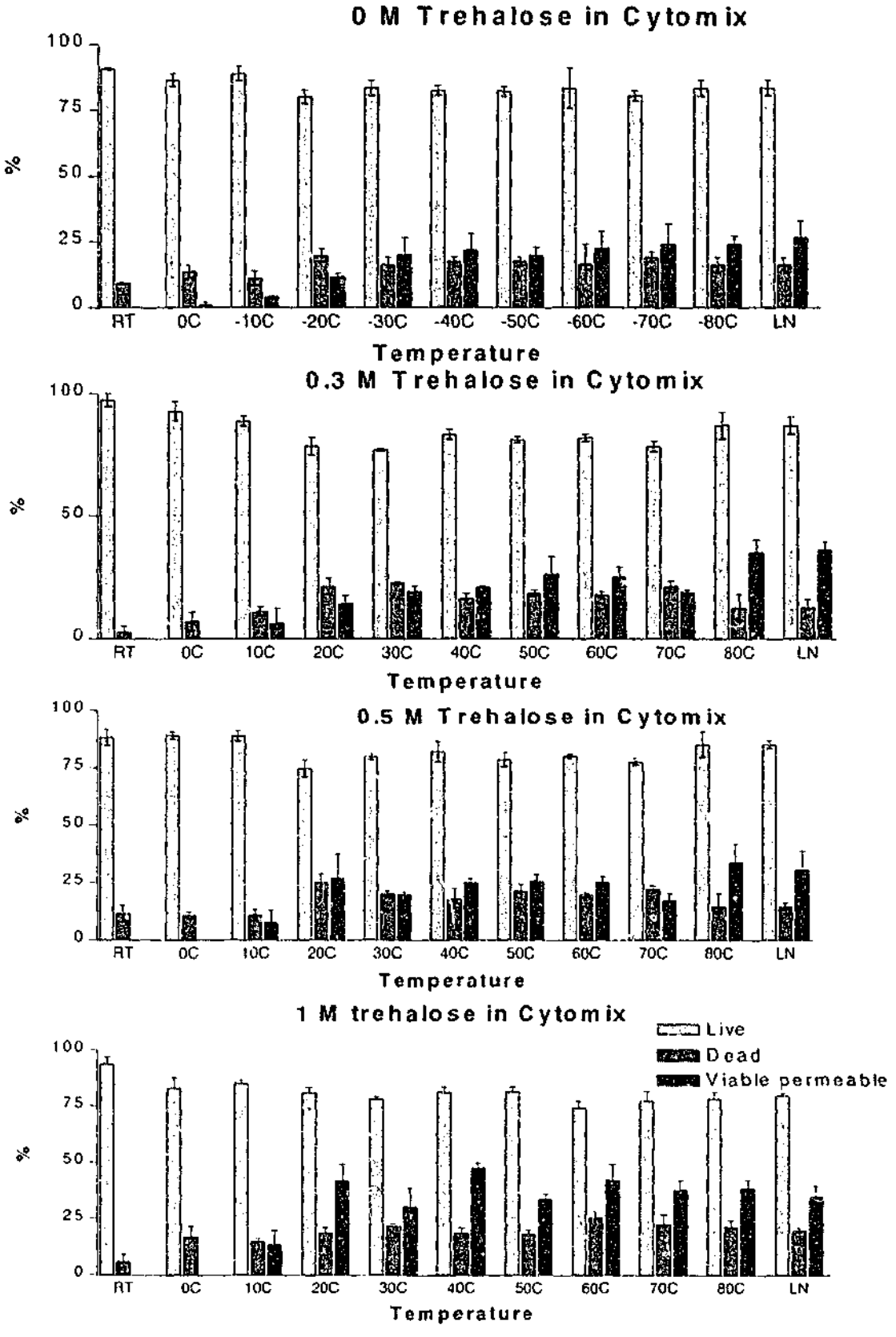


Fig. 6.22. Effect of the temperature to which cells were cooled and the trehalose concentration in the dilution solution (Cytomix) on the proportion of viable, dead and viable permeable cells (Data in appendix table 6.4B).

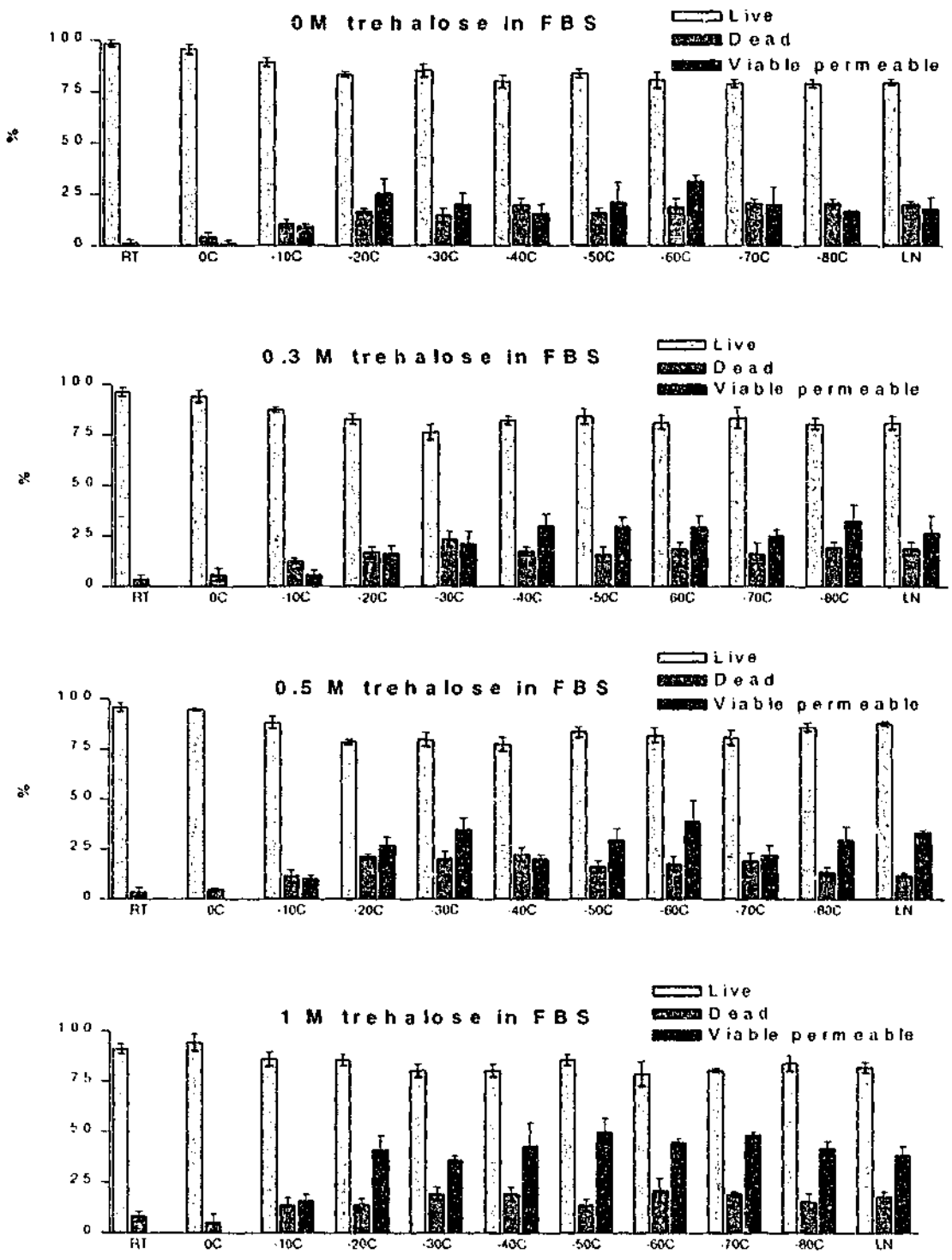


Fig. 6.23. . Effect of the temperature to which cells were slow cooled and the trehalose concentration in the dilution solution (FBS) on the proportion of viable, dead and viable permeable cells (Data in appendix table 6.4C).

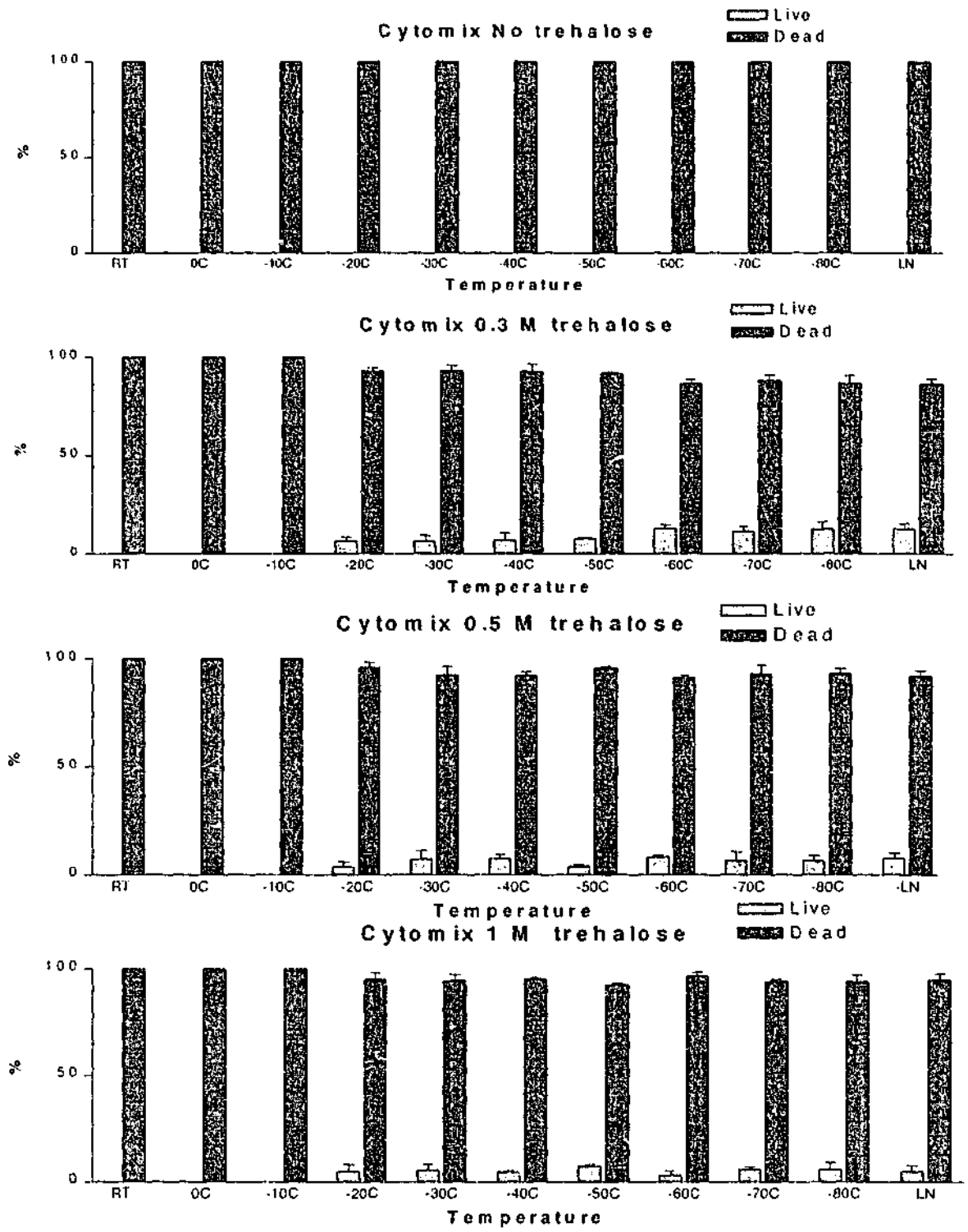


Fig. 6.24. Number of viable and dead cells after re-freezing (snap freezing) cells in Cytomix with or without trehalose in relation to the temperature to which they were originally slow cooled (Data in appendix 6.4D).

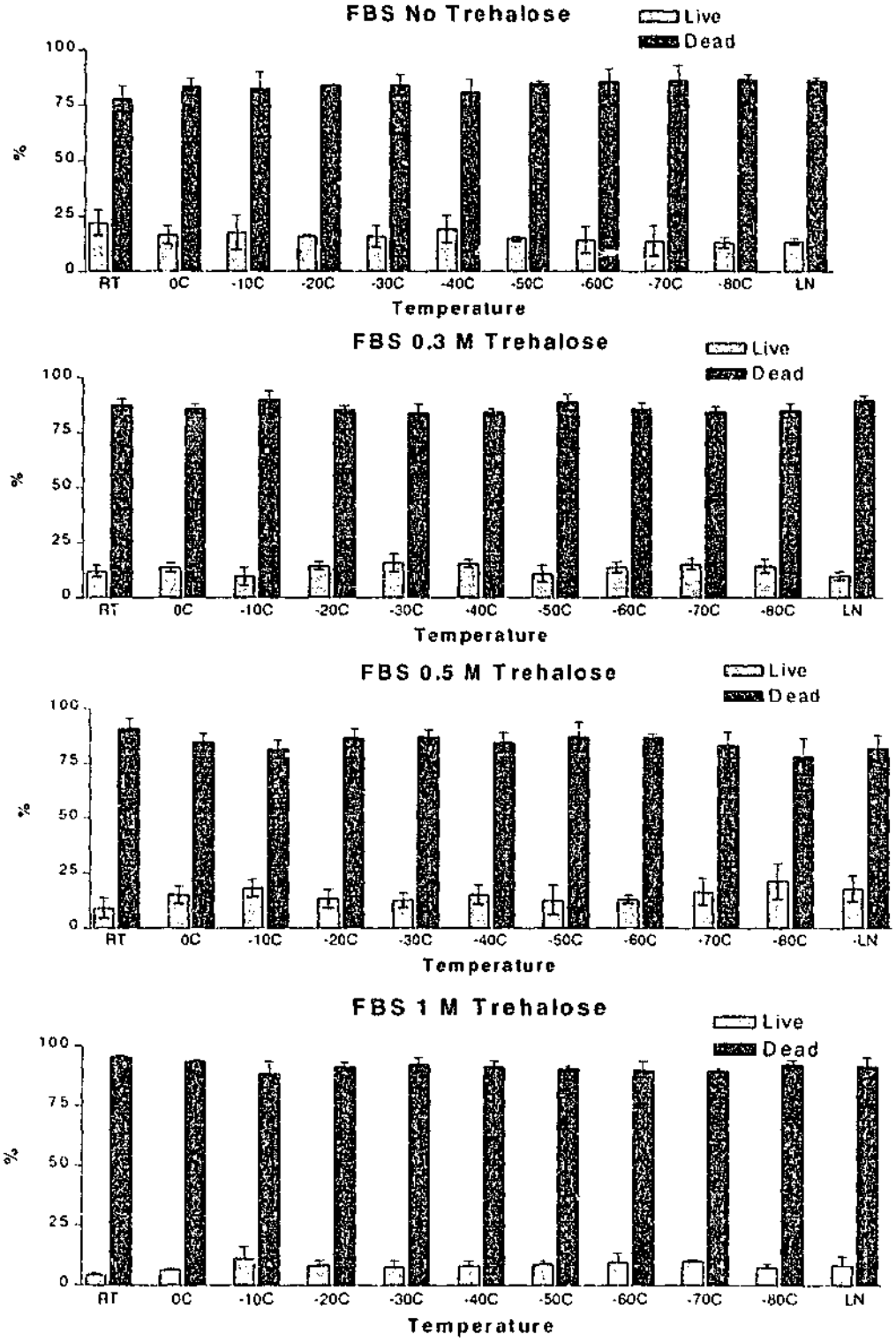


Fig. 6.25. Number of viable and dead cells after re-freezing (snap freezing) cells in FBS with or without trehalose in relation to the temperature to which they were originally slow cooled (Data in appendix 6.4E).

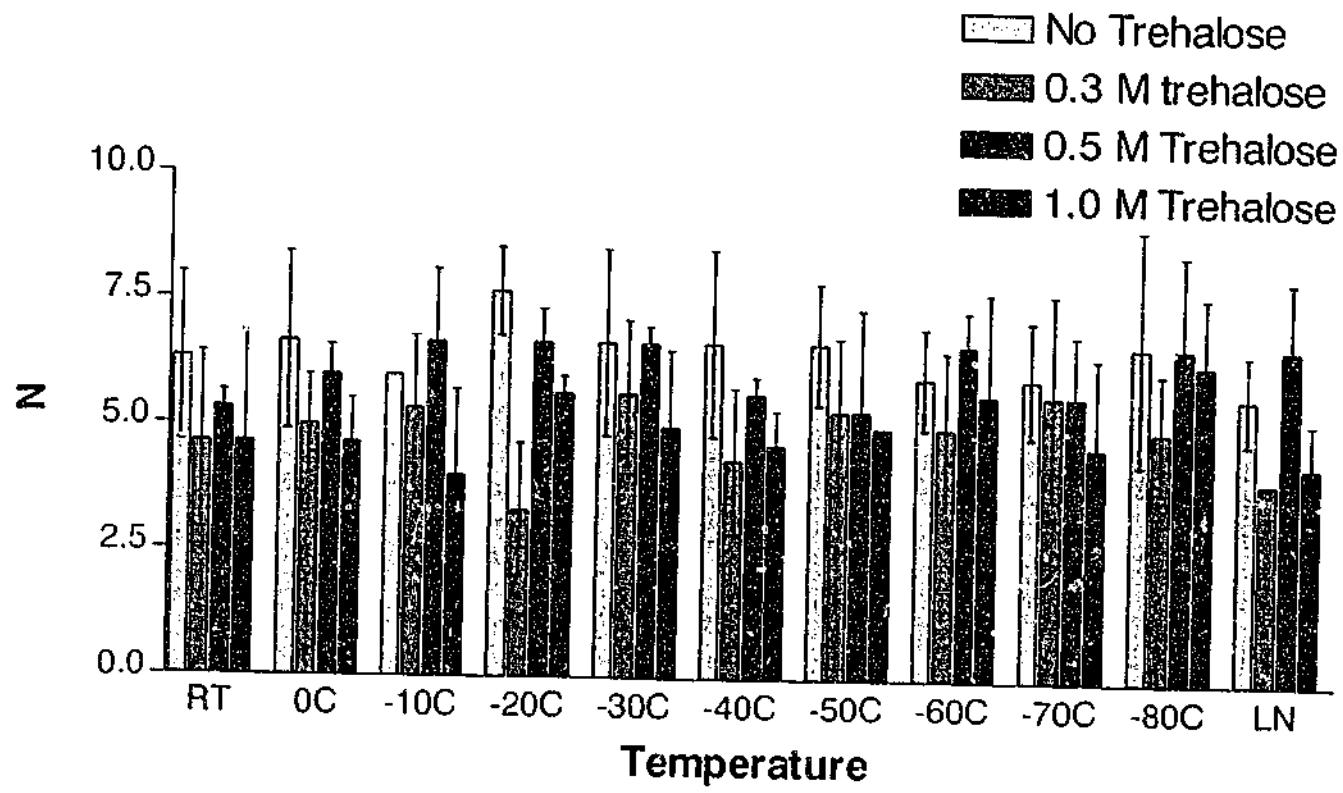


Fig. 6.26. Number of cells plating from cells slow cooled in 10% DMSO in FBS then diluted in FBS containing 0, 0.3, 0.5 or 1 M trehalose and then re- frozen by snap freezing (Data appendix 6.4F).

6.4. Discussion

The results of all 4 experiments confirm previous reports (Holt and North, 1994, Muldrew and McGann, 1994) which show that cells can be permeabilised by cooling. The results showed that cell permeabilisation started at +15°C, this indicated that cooling alone could initiate permeabilisation of the cells possibly by inducing membrane phase transition(s). Cryoprotectants and the Cytomix additive (ie. ATP) have previously been shown enhance permeability (Arav and Friedberg, 1996), but in this study cooling induced membrane permeability in both the presence and absence of these compounds.

In this chapter, freezing by snap freezing was used to indicate whether cells had accumulated intracellular trehalose, as it was assumed that in the absence of a permeating cryoprotectant, only cells with intracellular trehalose would survive.

Experiment 1 compared the media (Cytomix, DMEM and mHBS) which were used in chapter 5 for electroporation but also evaluated FBS as it is routinely added to cryopreservation solutions for cells. It is likely that in this experiment the proportion of viable cells fell as the temperature decreased because the solutions did not contain a permeating cryoprotectant. The absence of PI from most of the cells indicated that few cells became permeabilised under these conditions with the result that little if any protective trehalose would have entered the cells.

Survival did differ in the four tested media. Survival was lowest in mHBS even though this solution contains an ionic balance which is comparable to the inside of the cell. Cytomix also contains a high potassium concentration but resulted in higher survival rates than mHBS, possibly because of a protective effect of other Cytomix components such as Glutathione, ATP or EGTA or the absence of sodium. In this and all the other experiments survival was high in FBS. The higher survival of cells in DMEM than in mHBS may be because of a protective effect from the 10% FBS that it contained. Further studies would be needed to clarify why the FBS was protective. It is possible that the protective effect is at least in part due to its protein content (e.g. albumin) but many other components such as

lipids, fatty acids and ionic composition could play a role. Trehalose was marginally protective but its effect was only evident in the absence of FBS.

In experiment 2 cells were suspended in FBS or Cytomix containing DMSO or Ethylene Glycol. Cooling cells to -25°C in the presence of these penetrating cryoprotectants significantly increased the proportion of viable permeable cells as compared to incubation at RT for 5 or 10 min without cooling. This indicated that the cells were not permeabilised by cryoprotectants alone at RT. This also confirmed that cooling alone or together with ice formation permeabilised cell membranes. The proportion of viable permeable cells and dead to live cells were similar in medium with or without trehalose at RT. But cooling to -25°C with trehalose appeared to stabilise the cell membranes as significantly fewer permeabilised cells were present when cells were slow cooled in trehalose containing solutions. Subsequent snap freezing showed the survival of cells that had been resuspended in FBS was comparable either with or without trehalose. By contrast snap freezing in Cytomix killed cells unless trehalose was present during freezing. This indicated that both FBS and trehalose protected cells against snap freezing. It is not clear why the group pre cooled to -25°C which had more cells with intracellular PI did not have better survival rates than the other groups after re freezing unless it indicates that PI but not trehalose entered the cells or that the trehalose did not provide protection. The results therefore differ from those achieved by electroporation in chapter 5.

Experiment 3 showed that the addition of individual Cytomix components to FBS at a normal or higher than normal level did not affect the cell viability or permeability at room temperature. However slow cooling to -80°C, additives and the thawing method did affect the proportion of viable and viable-permeable cells. Thawing at 37°C produced the highest proportion of viable cells, possibly because this thawing strategy minimized damage caused by ice crystal growth during warming.

ATP can induce membrane permeability (Arav and Friedberg, 1996; Cutaia et al., 1996; de Macedo et al., 1993; Kimmich et al., 1989; Piantadosi et al., 2002; Saribas et al., 1993). The addition of 2 mM (the standard concentration) or 10 mM ATP (5 times the normal concentration of Cytomix) to FBS did not have a toxic effect on non-frozen cells. However when cells were slow cooled the proportion of viable cells was lowered showing

that ATP was toxic to the frozen cells. Freezing causes oxidative damage (Lasso et al., 1994) and ATP converts necrosis to apoptosis in oxidant-injured cells (Lelli et al., 1998), but these factors are not likely to be the reason for the damage seen in this study, as the cells died within minutes of thawing.

Glutathione is a free radical scavenger (Sierra-Rivera et al., 1994). The addition of 50 μM Glutathione or 10 times the Glutathione concentration of Cytomix was non toxic for the non frozen cells. Glutathione protected cells against slow cooling, but they failed to survive re-freezing (slow and snap freezing), and none of them plated indicating that the high concentration (10 times) of Glutathione was more detrimental than beneficial.

EGTA at 50 mM has been used to maintain integrity of freeze dried mouse spermatozoa (Kusakabe et al., 2001), while 10 mM EGTA can protect DTT treated human spermatozoa (Ohsumi et al., 1988) and demembranated *Xenopus laevis* spermatozoa (Lohka and Maller, 1985). In this experiment the addition of 5 mM EGTA, as used in Cytomix, into FBS containing 10% DMSO, with 0.3 M Trehalose did not give higher cell survival as compared to the absence of EGTA. Protection may be evident if 50 mM was used for the cells as in Kusakabe et al. (2001), as the higher concentration of EGTA may be more effective in stabilising chromatin integrity.

The highest proportion of viable permeable cells were obtained when cells were cooled in conventional freezing medium (10% DMSO in FBS) with or without trehalose and thawed at 37°C. After re-freezing all the viable cells had became permeable, but the total number of viable cells was reduced as compared to the first freezing. The finding that all viable cells were PI positive, may indicate that only the cells with intracellular trehalose (PI+) could survive the subsequent freeze in Cytomix containing 0.3 M trehalose.

Experiment 3 showed that most cells remained viable and that a large proportion of the viable cells were PI positive after the first freeze. After the first freeze alonethere was little (only a 10% reduction) difference between the non- frozen and the best frozen groups in terms of plating after 24 h in vitro. However damage was sustained during re-freezing as there was a more than 50% reduction in the number of plated cells as compared to those after the first freeze (Fig. 6.15). The addition of other components such as EGTA

(Kusakabe et al., 2001) or antioxidants did not have an effect in the presence of FBS, but might protect cells in other, less protective, media.

Experiment 4 confirmed and showed that slow cooling to below -20°C in 10% DMSO in FBS did not significantly reduce cell viability, but resulted in a stable proportion of viable permeabilised cells.

Interestingly, survival following snap freezing was less dependent of the first cooling step than on the solution in which the cells were subsequently cryopreserved. FBS provided uniform protection to all groups, while Cytomix protected cells only in the presence of trehalose. This shows that trehalose does protect cells against snap freezing.

Results in all 4 experiments showed that FBS protected cells against freezing. In the presence of FBS incubation at RT or -10°C did not permeabilise the cells, but when these cells were subsequently snap frozen in FBS the survival was high either with or without trehalose. The presence of 100% or 10% FBS (in DMEM) did protect cells against freezing, except when cells were frozen in FBS supplemented with 50 µM Glutathione. While re freezing in Cytomix without trehalose was highly detrimental (experiment 2), interestingly some cells survived re freezing in Cytomix in the presence of trehalose, but none of those cells plated. FBS and other additives such as skim milk powder are widely used in freezing solutions (Nakagata and Takeshima, 1993; Sztein et al., 2000 and 2001). However, further research is still required to identify the particular components in skim milk or FBS that protect cell or spermatozoa against freezing.

The presence of the cryoprotectant DMSO improved the survival rate of cells after initial slow cooling and subsequent re-freezing. This may make it difficult to develop freeze drying strategies if the freezing is to be combined with drying the presence of those types cryoprotectant should be avoided because DMSO is a solvent which is difficult to evaporate.

Quinn (1985) showed that cryoprotectant agents modify the lipid phase transition properties of cells. Experiments 2,3 and 4 showed that in the presence of penetrating cryoprotectants cell permeabilisation started at a lower temperature (-10°C) and higher cell survival was obtained as compared to the -5 to +15°C seen in Experiment 1 (without

cryoprotectants). The lower proportion of viable permeable cells in experiment 1 might due to inadequate protection against freezing, leading to the death of most cells. This is likely since many structures other than membranes, such as microtubules (Aman and Parks, 1994) and the cytoskeleton (Albertini, 1995; Overstrom et al., 1990) are sensitive to low temperatures.

Attempts to obtain viable permeabilised cells by incubation at RT in experiments 2 and 4 were not successful even in the presence of permeable cryoprotectants

Cooling cells to -20°C increased the proportion of viable permeable cells for cells suspended in FBS or trehalose which may reflect the combined effects of both ice crystal formation and membrane transitions. These effects were influenced by the presence of permeable cryoprotectants (Experiment 2). This agrees with Nichols et al (1989) and Sasaki et al (1991) who showed that freezing could be used to introduce DNA into the cells.

McGann et al (1988) indicated that the plasma membrane was only one site for injury sustained during slow cooling as cooling could disrupt other cellular components. In this study the cells were refrozen immediately or 30 min after thawing. It may be that the cells required a longer time to recover after the first freezing, particularly in the absence of serum. Further studies should investigate whether extended in vitro culture to allow cell recovery before subsequent freezing would lead to improved results.

In chapter 5, cells which became PI positive by transient permeabilisation after electroporation retained PI intracellularly for at least 24 h. By contrast, further in vitro culture of freeze thawed PI positive cells showed that the plated cells did not retain intracellular PI. This indicated that although cooling had permeabilised the cell membrane and allowed PI to cross the membrane, and that these loaded cells survived in vitro culture (Experiment 3) the PI was lost during culture. There are some possibilities that could explain this phenomenon, it could be that the membranes remain leaky after being placed in in vitro culture. Low level leakiness is not normal for fibroblasts, but if such a phenomenon exists then it may explain how Wolkers et al. (2001) succeeded in loading trehalose into platelets, which are very small, solely by incubating the platelets in trehalose at 37°C. Another possibility, though it is unlikely, is that at 37°C the fibroblasts could

expel the intracellular PI. This seems particularly unlikely since the electroporated cells (chapter 5) retained PI in culture.

Freshney (1994) showed that 90% of cells that excluded TB after freezing and thawing, but only 60% developed further when placed in in vitro culture for 24 h. Similar losses were evident for the refrozen cells that failed to develop in vitro in this study.

Wolker et al (2001b and 2002) showed that platelets have a very efficient uptake of trehalose (more that 50%) from the extracellular environment after 4 hr incubation at 37°C, and claimed that this allowed them to survive freeze drying. It is however possible that the also cells took up trehalose when they were being cooled to -30°C (the temperature at which the platelets were freeze dried).

Chapter 5 showed that electroporation could be used to stimulate PI (or trehalose) uptake, and protect cells from snap freezing. This chapter showed that cooling also lead to cells accumulating intracellular PI. It was however, difficult to show that permeabilisation gave protection against freezing since re freezing (by snap or slow cooling) killed most of the cells. It is possible that the cells were very slow to recover from the first freeze or that PI, but not the trehalose, had crossed the membranes of the viable permeable (PI+/TB-) cells. Further studies are needed to determine whether (1) cells had accumulated intracellular trehalose, (2) trehalose had crossed the cell membrane after freezing.

In this study cells were not dried, but it was assumed that viable permeable cells (PI+/TB-) cells which were loaded with trehalose during cooling or freezing would have gained some protection against desiccation injury. All the experiments showed that thawing of slow cooled cells was incompatible with subsequent re-freezing, however normal freeze drying may be less detrimental since in freeze-drying the cells are slow cooled and then dried (without warming). However the current freeze drying strategies are unlikely to be suitable for solutions containing DMSO, as this penetrating cryoprotectant is less volatile than water with the result that it is likely to reach toxic or lethal concentrations as the water is removed. As a result future studies should replace the DMSO with other cryoprotectant(s) such as one that is easy to remove or which causes less harm to the cells in the absence of water. Alcohol (methanol and ethanol) might be a possible substitute for DMSO, as alcohol is easy to evaporate.

Arav et al. (1996) showed that the plasma membrane is the primary target of irreversible damage expressed shortly after exposure to low, but not freezing temperatures. The changes he observed are known as direct chilling injury (DCI), or cold shock. There is an association between DCI and the thermotropic phase transition of membrane lipids. Arav et al. (1996) found that the phase transition of the membrane lipids of immature and in vitro-matured bovine oocytes during cooling could be determined using Fourier Transform Infrared Spectroscopy (FTIR) and showed that holding oocytes at the phase transition temperature was more damaging to their membranes than exposure to temperatures above or below phase transition temperature.

Using FTIR it would be possible to determine the temperature at which membrane phase transition(s) occurred and since membranes are particularly leaky when incubated at that temperature it could be used to increase trehalose uptake

Alternatively altering the lipid membrane composition using polyunsaturated fatty acids (PUFA) may change the cells susceptibility to low temperatures (Shinitzky, 1984; Stubbs and Smith, 1984).

The results show that cooling can be used to load trehalose into cells, but they also demonstrate that the cell viability and permeability varied. This study was not able to optimise a chill or freezing permeabilisation strategy for use with freeze drying, but indicates that further research is warranted. It is suggested that further research should aim to:

1. Identify how FBS protects against snap freezing.
2. Establish if cells can be dried in FBS alone or FBS with trehalose.
3. Establish whether highly volatile cryoprotectants such as methanol and ethanol, can be used instead of DMSO as this may be compatible with drying.
4. Ascertain whether the use of e.g. PUFA to modify the cell membrane affects permeabilisation.

6.5. Conclusion

This study showed that freezing could be used to permeabilise cell membranes to normally non-permeable compounds. Trehalose protected against subsequent freezing, but FBS also protected cells against refreezing regardless of the presence of trehalose. The presence of permeable cryoprotectants improved cell viability and the proportion of viable permeable cells. It remains to be established whether freezing with FBS and trehalose can be used in combination with freeze drying.

Chapter 7. General discussion

Drying biological material is an important tool in clinical medicine, the pharmaceutical industry and agriculture because it is low in cost and without space restrictions. The first part of this thesis attempted to develop simplified protocols to preserve functional mouse spermatozoa in a dry state. The second part attempted to develop drying protocols for somatic cells (fibroblasts).

7.1. Mouse spermatozoa drying

Several studies have now aimed to preserve cells including spermatozoa, in a desiccated state. Early attempts to freeze-dry spermatozoa gave some success (reviewed by Smith, 1961), with some researchers reporting the presence of motile spermatozoa after the freeze-drying process (Meryman and Kafig, 1959; and Juschenko, 1959; both were reviewed in Smith, 1961). These results were however not replicated until Katayose *et al.* (1992), Wakayama and Yanagimachi (1998) and Kusakabe *et al* (2001) showed that freeze dried spermatozoa could fertilise oocytes, and developed into normal offspring.

This study, which aimed to evaluate low cost drying strategies, was not able to produce the highly repeatable results reported by Wakayama and Yanagimachi (1998). As in Wakayama and Yanagimachi's (1998) study, none of the dried spermatozoa were motile and many spermatozoa had bent or broken tails or had heads separated from their tails (chapter 3). The outcome of the evaporative drying was dependent on the gas used for drying. None of the spermatozoa that were evaporatively dried using oxygen containing gas, including atmospheric air, medical air mixed gas containing only 5% oxygen or CO_2, supported full embryo development after ICSI (chapter 3). Some but not all experiments using spermatozoa dried with nitrogen gas gave results indistinguishable from results for fresh spermatozoa The reason for the variable outcome of these experiments could not however be established. ICSI using vacuum dried spermatozoa allowed the oocytes to develop into blastocysts at a rate comparable to fresh spermatozoa, but there was a delay in sperm head decondensation after ICSI using dried spermatozoa (chapter 4).

Normal atmospheric air contains ~21% oxygen and the damage caused by the air-drying processes is likely to have resulted from both oxidative damage (Lavelli, *et al*, 1999), and desiccation damage to proteins, nucleic acids, and membranes (Potts 1994). The oxidative damage is most likely to have been mediated through reactive oxygen species (Brawn and Fridovich, 1981). Reactive oxygen metabolites are known to disrupt spermatozoa-oocyte fusion, spermatozoa motility, and DNA integrity; however, the relative sensitivities of these elements to oxidative stress are unknown (Aitken, *et al*, 1998 and Lopes, *et al.*, 1998). Ward *et al.* (2000) showed that the structural integrity of the mouse sperm nuclear matrix may be necessary for the proper unpacking of sperm DNA for participation in embryogenesis. It is likely that the sperm nuclear matrix contributes to the organisation of the sperm DNA and its disturbance can seriously damage the paternal genome or its expression. They further suggest that even very subtle changes in the sperm nuclear structure may have a significant impact on emlryo development (Ward, *et al.*, 1999 and 2000). Such changes may have lead to the poor results with air dried mouse spermatozoa seen in this study.

In this study 14 offspring were born after ICSI of spermatozoa evaporatively dried using nitrogen. The successes occurred over a short period of time, and subsequent attempts to repeat those results using other batches of spermatozoa gave rise to embryos at a high rate but these did not give rise to live young after transfer (chapter 3).

A series of experiments then investigated the addition of other types and batches of albumin and modifying the embryo culture conditions and ascertained that this could influence the outcome of ICSI with dried sperm and generate embryos, but none resulted in live pups (chapter 3). Until the reasons for these variable results can be ascertained, this method even though it is cheap and easy, is not yet an effective alternative to the method developed by Wakayama and Yanagimachi (1998).

Kusakabe *et al* (2001) showed that mouse spermatozoa freeze dried in a medium containing EGTA had improved chromosomal integrity and improved ICSI outcome as compared to spermatozoa freeze-dried without EGTA, or at non-optimal concentrations of EGTA. In this study mouse spermatozoa which were dried in a medium (Cytomix) containing EGTA levels similar to those used by Kusakabe *et al* (2001) did produce

embryos at a comparable rate to sperm dried in other media, but these embryos did not develop into offspring after transfer into recipient foster mothers (chapter 3). It is possible that the EGTA provided protection against freezing rather than desiccation damage.

Vacuum drying is an integral part of freeze drying protocols. Removal of water using vacuum pressure at 1 mTorr for 8 h at room temperature left a dry powder of spermatozoa and remaining salts of the medium. In this study, ICSI of vacuum-dried mouse spermatozoon into mouse oocytes allowed 9.7% of the oocytes to develop into blastocysts, even when the powder was stored in vials containing atmospheric air (chapter 4). This finding indicates that vacuum drying might not disrupt the integrity of the sperm nuclear matrix, and that the oxygen caused the damage during the drying rather than after the samples were dry. Ward *et al.* (1999) indicated that the only component of the spermatozoa that is crucial for participation in embryo development is the sperm nucleus with a stable nuclear matrix. This study did however not ascertain whether these embryos would develop into fetuses or live young following transfer.

7.2. Introducing intracellular trehalose and cell freezing

In the second part of the thesis, attempts were made to store somatic cells in the dry state. Previous reports had shown that trehalose can help to protect mammalian cells against osmotic injury (de Castro and Tunnacliffe, 2000), and in some studies conferred protection against freezing (Eroglu *et al.*, 2000; 2001) and / or drying injury (Table 8.1), but de Castro and Tunnacliffe (2000; Tunnacliffe *et al.* (2001) reported that trehalose alone did not protect mammalian cells against drying.

In this study electroporation was used to introduce trehalose into the cells. This technique is usually used to introduce DNA or plasmids into cells. Mouse STO fibroblasts were electroporated with or without trehalose (in the presence or absence of PI) and showed evidence of temporary membrane breakdown, and long term (24h) retention of the foreign molecules (PI and possibly trehalose) (chapter 5).

Mouse STO fibroblasts were electroporated using one or multiple decaying pulses in different media contained different levels of trehalose. Optimum permeabilisation was

found in cells electroporated in Cytomix containing 0.3 M trehalose, electroporated at 250 V with 500 μF capacitance at room temperature (chapter 5). These settings were therefore used in the subsequent experiments.

Table 7.1. Summary of reports on the use of trehalose in mammalian cell drying

Methods & Reference	Cell type	Treatments
Genetic engineering Guo *et al.*, 2000	human embryonic kidney cell line 293 and human fibroblast cell line 12F	Cells were transfected with *otsA* and *otsB* from *E. coli* using adenoviral vector. Air dry 72 h later by removing the culture medium and storing cells at room temperature. Result: Viable and proliferate cells
Incubation Puhlev *et al* 2001	Human fibroblast	Thermal shock 50mM T in DMEM followed by vacuum dry and storage Result: 60% viable day 1, 10% viable day 8.
Incubation Matsuo, 2001	Human corneal epithelial cells	0.2 M Trehalose in PBS followed by air drying for 30 min Result: Viable cells after staining
Incubation Wolkers *et al.* 2001	Human platelets	52mM Trehalose in buffer at 37°C followed by freeze drying. Result recovery >80% intact platelets.
Incubation Gordon *et al.*, 2001.	Human mesenchymal stem cells	50mM T; 3% Glycerol in hMSC medium 24h, vacuum storage. Result: Up to 45% recovery of viable cells, but decreased later.

To establish whether the electroporation lead to the internalisation of trehalose, which would protect the cells against freezing stresses, cells were electroporated and frozen with or without trehalose and/or added permeating cryoprotectants. The results (chapter 5) showed that cells electroporated in the absence of trehalose were killed by freezing (slow cooling or snap freezing), whereas up to 68.8% of those electroporated in the presence of trehalose survived freezing (slow cooling or snap freezing, table 5.4). Addition of cryoprotectants enhanced the survival rate of cells frozen in medium containing 0.3 M trehalose. At low concentrations of penetrating cryoprotectant (0.8% Glycerol and 1% DMSO) the addition of trehalose significantly improved the survival rate (chapter 5).

In the presence of trehalose in the electroporation medium cells survived snap freezing and slow cooling (1°C/min). Eroglu *et al* (2000) showed that intracellular trehalose protected human 3T3 fibroblasts and human keratinocytes against snap freezing.

The results of the present study agree generally with those of Eroglu *et al* (2000) however the survival rate of snap frozen cells in this study (chapter 5) was low (up to 28.6%) compared to their results. The use of multiple pulses (5 and 10 times) did not improve the results in this study (chapter 5).

This study showed that the presence of trehalose and electroporation were both required to protect cells against snap freezing by direct plunging into liquid nitrogen (chapter 5). None of the cells were viable after either electroporation and freezing without either trehalose or cryoprotectant, or cell freezing in the presence of trehalose without electroporation. This indicates that intracellular trehalose protected cells against cryopreservation by direct plunging into liquid nitrogen.

The presence of trehalose alone or both trehalose and a cryoprotectant increased post thaw cell viability as compared to those without trehalose. This indicated that trehalose gave a cumulative action with the cryoprotectant to protect cells against cryopreservation stresses (chapters 5 and 6).

Freezing has been used to introduce impermeant molecules (Nichols *et al.*, 1989) or DNA into cells (Sasaki *et al.*, 1991). Most reports show that cooling induces membrane phase transitions and that freezing reduces cell viability through cell membrane rupture and altered membrane permeability (Acker and McGann, 2000; 2001; Buck *et al*, 1981;Fujikawa, 1980; Holt *et al.*, 1992; Ignatov *et al.*, 1982; Mazur, 1984; Mazur *et al.*, 1984; McGann *et al.*, 1988; Muldrew and McGann, 1994; Pegg, 1987; Steponkus *et al.*, 1983; Zhu and Liu, 2000).

This study therefore evaluated whether cooling or freezing could be used as an alternative method to introduce trehalose into mammalian cells. The results showed a complex interaction between serum and trehalose. When trehalose was present in the solution, the proportion of viable permeabilised cells could be lower than when trehalose was absent (e.g. Figs. 6.6 and 6.7). This may indicate that the extracellular trehalose protected the cell membrane during cooling/freezing and thereby prevented other molecules such as PI from crossing the membrane.

When cells were cooled in the absence of trehalose and then re-frozen the outcome was influenced both by the medium and on whether trehalose was present or absent

(chapter 6). Re-freezing cells in FBS was particularly protective and allowed the cells to develop after in vitro culture (chapter 6). It is however not known how the serum provided this protection.

7.3. Fibroblast cell drying

Changes that take place during drying are analogous to those that take place during slow cooling. During slow cooling cells must dehydrate to lose their freezable water before reaching their internal ice-nucleation temperature (Mazur *et al.*, 1984). Hyperosmotic extracellular solutions need to dehydrate the cells during cooling until the amount of intracellular water is reduced to a level that is compatible with the cell survival after storage in liquid nitrogen (Shaw, *et al.*, 2000).

During freeze-drying biological materials, the water is firstly converted into ice crystals by freezing and then removed by a vacuum while gradually raising the temperature of the materials to remove the remaining moisture. Under vacuum, ice can sublimate straight into vapor without passing through a liquid phase to leave dried cells behind (Rey, 1999).

The removal of water from viable biological material in the frozen state (freeze-drying) provides another means of arresting the biological clock by withholding water and commencing it again by its addition (MacLellan and Day, 1995)

Although several drying strategies were tried in this project (Chapter 5), viable cells could not be recovered following complete dehydration, even though the cells were treated to let trehalose cross the cell membranes.

There were several possibilities why viable cells could not be obtained following drying or freeze drying of mammalian fibroblasts even in the presence of trehalose. Trehalose has been known as a major component in anhydrobiotic organisms (Crowe *et al.*, 1992), but other factors such as polyols (Puhlev, 2001), temperature and anaerobic atmosphere (Malik, 1992, 1994), oxygen radicals (Brawn and Fridovich, 1981) may affect the success of cell drying. Thus it may work if a lower vacuum pressure could be applied. The equipment used in this study could only draw a vacuum of 1 mTorr, while others

(Wolkers *et al.*, 2001) used 30 mTorr. The vacuum might not have been strong enough to efficiently sublimate the ice water and allowed the specimen to melt during drying.

Even if dried cells fail to recover their viability, alternative methods of utilising these cells may be applicable. Wakayama and Yanagimachi (1998) and Kusakabe *et al.* (2001) showed that freeze dried mouse spermatozoa retained their ability to fertilise mouse oocytes and produce normal offspring following ICSI. Those spermatozoa were considered non-viable after staining using Live/Dead staining. Similarly it should be possible to insert the nucleus of a dried cell into an oocyte even if the cell cytoplasm has been left non functional as a result of drying.

Although both spermatozoa and somatic cells (fibroblasts) remained non-viable after rehydration, drying provides a practical and widely used strategy for preserving biological materials such as foods, drugs, vaccines, seeds and embryos (*Artemia*). Research aimed at further developing drying protocols is therefore warranted.

7.4. Controversies in mammalian cell drying

There is currently a considerable amount of controversy surrounding dry storage. Some groups claim to have demonstrated that mammalian cells can be driedin the presence of trehalose (Guo *et al.*, 2000, Chen *et al*, 2001, Gordon *et al.*, 2001, Matsuo, 2001, Puhlev *et al.*, 2001 and Wolkers *et al* 2001). They introduced trehalose into the cells by gene transfection (Guo *et al.*, 2000), incubation in a trehalose solution (Chen *et al*, 2001, Gordon *et al.*, 2001, Matsuo, 2001, and Wolkers *et al* 2001) or with cryoprotectant (Puhlev *et al.*, 2001) (summarized in table 8.1).

One other group claims that trehalose only protects cells against osmotic stress, and cannot protect mammalian cells against drying (Tunnacliffe *et al.*, 2001), even though the cells were dried using freeze drying and stored under nitrogen. They showed that in the presence of trehalose cells survive after exposure to hyperosmotic condition.

The results of this study (chapters 5) agree with those of de Castro *et al* (2000), de Castro and Tunnacliffe (2000), and Tunnacliffe *et al.* (2001), in that trehalose is not sufficient to protect mammalian cells against drying. Trehalose might stabilise the

membrane but may fail to protect other cytosolic factors during dehydration, even though trehalose is thought to replace water molecules and thereby stabilise cell components during dehydration.

It may be important to consider the presence of other components such as antioxidants to protect dehydrated cells and it may be necessary to avoid permeable cryoprotectants even though some results showed that addition of 3% glycerol (Puhlev *et al.*, 2001) could improve the survival after drying.

Browne *et al.* (2002) have identified a gene that is upregulated in response to desiccation stress and whose encoded protein in the anhydrobiotic nematode *Aphelenchus avenae* shares sequence similarity with a late-embryonic gene that is induced in many plants when they are deprived of water. Their finding suggested that anhydrobiotic animals and plants may use similar protective strategies against dehydration, and provides a unifying insight into anhydrobiotic mechanism.

7.5. Concluding remarks

Drying biological material is being studied, but currently the results in this research field are open to controversy (see section 7.4.).

Currently, only mouse spermatozoa freeze-dried without adding any cryoprotectant or lyoprotectant have produced consistent results (Wakayama and Yanagimachi, 1998 and Kusakabe *et al*, 2001). Mouse spermatozoa can be dried and stored in dry state and remain able to fertilise oocytes. Our results in Chapter 3 showed that mouse spermatozoa could also be dried and stored under nitrogen and produce viable offspring. However this result was not repeatable in subsequent experiments. There is clearly a need for further research in refining protocols and defining the factors that contribute to success and failure.

Trehalose could be internalised into the cells by electroporation and protect fibroblast against snap freezing, but it failed to protects cells against drying. It is however possible that some survival would have been obtained if these cells had been dried in equipment delivering a higher vacuum, and stored under more suitable conditions such as under a vacuum.

Equipment was not available to determine the quantity of trehalose inside the cells in this study. Further research is required to determine the optimal intracellular trehalose concentration and if other components such as the genes that produced LEA protein in response to dehydration which have recently been found in both a nematode and plants (Browne et al., 2002) might be included in the strategy to protect mammalian cells against drying.

The mechanisms by which anhydrobiotic organisms survive drying are partially defined. Further investigations are required to improve our understanding of how to induce anhydrobiotic mechanisms in non-anhydrobiotic organisms such as mammalian cells.

Appendix Chapter 5

Appendix 5A1 (Fig 5.1). Viable cells after electroporation in different sugars dissolved in Milli-Q water at 500 and 975 μF capacitance.

	0.2 M Trehalose			0.3 M Trehalose			0.2 M Mannitol			0.3 M Mannitol			0.2 M Sucrose			0.3 M Sucrose		
500 μF																		
Voltage	N*	SEM	%	N*	SEM	%	N*	SEM	%	N*	SEM	%	N*	SEM	%	N*	SEM	%
50V	62	0.6	53.3	62	1.5	48.3	62	0.9	30.8	61	1.2	45.6	61	1.3	46.0	66	0.3	38.0
100V	62	1.2	53.1	61	1.2	47.4	58	0.7	37.9	62	0.6	34.0	62	0.3	40.7	61	0.9	36.0
150V	60	0.6	45.0	62	0.6	34.0	58	0.7	24.6	58	0.3	22.4	63	0.6	23.8	64	0.3	22.1
200V	59	0.9	32.0	60	0.9	23.2	68	0.7	6.1	62	0.7	7.7	53	0.3	7.7	62	0.3	8.2
250V	61	0.3	13.1	58	1.2	19.6	51	0.0	0.0	49	0.3	1.9	51	0.0	0.0	64	0.0	0.0
975 μF																		
50V	59	0.6	35.7	57	0.9	33.4	61	0.3	26.3	65	0.6	23.1	60	0.3	21.8	49	0.7	3.2
100V	57	0.3	33.3	56	0.3	30.6	62	0.7	16.3	66	0.6	13.9	60	0.3	11.6	53	0.6	10.9
150V	56	0.3	17.9	54	0.0	16.7	55	0.3	12.6	52	0.3	13.5	57	0.0	15.9	63	0.3	11.1
200V	57	0.3	14.0	54	0.3	12.9	50	0.6	5.8	43	0.0	0.0	47	0.7	3.7	51	0.6	5.4
250V	47	0.7	8.1	48	0.7	4.2	40	0.0	0.0	44	0.0	0.0	46	0.3	2.1	50	0.3	4.4

N*=The total number of cells (live and dead) that were assessed over the three replicates (The SEM was calculated on the three replicates). % expresses the average proportion of the cells that remained viable following the treatment.

(continued)

Appendix 5A2 (Fig. 5.1). Viable cells after electroporation in different sugars dissolved in media at 500 and 975 μF capacitance..

	0.2 M Trehalose			0.3 M Trehalose			0.2 M Mannitol			0.3 M Mannitol			0.2 M Sucrose			0.3 M Sucrose		
500 μF	N*	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%
500 μF in DMEM																		
100V	15.7	1.2	84.6	16.0	1.0	88.7	11.7	0.9	64.8	12.7	1.2	63.2	11.0	0.6	55.0	12.3	1.5	55.6
150V	13.7	0.7	66.1	14.7	0.3	74.9	11.0	0.6	55.0	11.7	0.3	50.0	12.0	0.6	52.2	11.3	0.3	50.0
200V	11.0	0.6	49.0	11.7	0.9	46.0	8.0	0.6	35.7	6.3	0.9	32.3	6.3	0.9	28.7	7.7	0.9	39.7
250V	9.3	0.3	41.8	9.7	0.9	43.7	5.0	0.6	22.1	5.3	0.3	25.8	6.0	0.6	26.8	5.7	0.3	26.7
975μF in DMEM																		
100V	14.0	0.6	67.8	12.7	1.2	57.2	11.7	0.3	45.5	12.3	0.3	51.6	12.0	0.6	60.2	12.3	0.9	61.1
150V	12.3	0.3	59.7	12.7	0.9	65.9	11.0	0.6	50.8	10.3	0.3	46.3	9.7	0.3	45.8	10.7	0.9	47.6
200V	7.7	0.9	40.9	9.3	0.3	44.6	2.7	0.3	12.2	6.0	0.6	29.2	7.7	0.3	36.0	5.3	1.3	25.4
250V	6.0	0.6	29.6	6.0	1.2	31.9	3.3	0.7	16.3	2.7	0.7	11.3	2.7	0.3	13.6	2.0	0.6	9.3
500μF in mHBS																		
100V	16.3	0.9	82.1	15.7	0.3	79.8	11.7	0.9	50.3	10.0	1.5	55.2	11.7	0.3	52.5	11.3	0.7	51.5
150V	11.3	0.3	50.1	10.7	0.9	68.2	7.7	0.3	43.7	9.0	0.6	42.3	12.7	0.3	66.0	11.0	0.6	54.1
200V	10.3	0.3	45.2	11.3	0.3	47.9	6.0	1.0	26.5	4.3	0.9	21.0	7.3	1.8	35.2	6.7	1.2	32.2
250V	8.0	2.0	35.1	10.0	1.5	40.8	3.0	0.6	14.7	2.3	0.3	11.9	3.0	0.6	15.1	4.0	1.2	22.0
975μF in mHBS																		
100V	12.0	0.6	52.9	12.3	1.2	57.0	10.7	1.5	53.8	9.7	1.9	46.0	10.7	0.9	57.4	9.0	0.6	47.3
150V	9.7	0.9	51.7	11.3	0.3	52.4	7.3	1.3	37.4	6.0	1.2	27.2	8.7	0.3	44.3	10.3	0.7	44.4
200V	8.7	0.7	42.7	8.7	0.3	44.3	5.3	0.3	25.1	3.3	0.7	17.8	4.3	1.2	22.0	5.7	0.3	30.9
250V	5.7	0.7	28.5	5.0	1.5	26.0	2.3	0.9	10.3	2.0	0.6	8.1	4.3	0.3	22.4	4.3	0.3	22.8

N*=The total number of cells (live and dead) that were assessed over the three replicates (The SEM was calculated on the three replicates). % expresses the average proportion of the cells that remained viable following the treatment.

Appendix 5B (Fig. 5.2). Proportion of non viable (dead), viable non-permeabilised and viable transiently permeabilised cells after a single electroporation pulse in different media at voltages between 100 and 350V at 500μF without trehalose.

Media	Voltage	Viable and Permeabilised		Dead		Viable non-permeabilised	
		%	SEM	%	SEM	%	SEM
DMEM	Not-EP	0.0	0.0	1.3	0.3	98.7	0.3
	100	7.2	2.9	9.1	2.5	83.7	1.5
	150	13.2	0.4	8.8	0.7	78.0	0.6
	200	19.5	3.2	26.3	3.5	54.2	4.7
	250	29.6	3.4	28.3	3.4	42.2	3.1
	300	14.2	4.0	60.0	4.5	16.3	7.6
	350	10.6	2.3	62.4	12.9	10.2	2.1
mHBS	Not-EP	0.0	0.0	0.7	0.7	99.3	0.7
	100	10.9	1.6	12.4	2.3	76.7	3.2
	150	15.7	1.1	10.7	2.6	73.6	2.7
	200	28.1	4.8	25.5	3.9	46.4	7.6
	250	30.5	4.3	31.2	1.6	38.3	5.6
	300	15.2	2.2	48.6	3.9	22.8	3.2
	350	14.6	4.7	54.7	6.2	22.6	1.6
Cytomix	Not-EP	0.0	0.0	1.7	0.3	98.3	0.3
	100	8.9	1.3	8.1	0.8	82.9	0.8
	150	22.7	6.7	8.7	2.0	68.6	6.0
	200	27.8	3.0	19.7	2.6	52.5	3.0
	250	40.6	4.0	26.8	3.8	32.7	5.3
	300	27.6	2.4	43.0	1.4	25.8	3.3
	350	29.1	0.6	51.9	2.2	20.9	2.6

Appendix 5C (Fig. 5.3). Proportion of all cells that were viable and transiently permeabilised cells by single electroporation pulse in various media, trehalose concentrations and voltages at 500 μF capacitance.

Trehalose (n.s)		0 M		0.1 M		0.2 M		0.3 M		0.4 M	
Media *)	Voltage*)	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
DMEM	Not-EP	0	0	0	0	0	0	0	0	0	0
	100V	3.6	0.1	2.4	0.1	16.5	1.5	6.5	0.8	8.0	2.1
	150V	14.2	0.4	26.2	1.3	16.7	1.3	19.9	2.4	13.1	3.5
	200V	24.7	0.7	43.5	2.2	50.2	4.0	46.8	5.5	30.5	8.2
	250V	38.3	1.1	42.4	2.7	39.6	2.8	38.7	4.6	27.4	7.3
	300V	33.1	0.9	32.9	1.7	42.0	3.0	34.2	4.0	18.2	6.2
	350V	8.0	0.2	15.7	0.8	12.8	0.9	10.7	1.3	8.6	2.9
mHBS	Not-EP	0	0	0	0	0	0	0	0	0	0
	100V	10.9	0.3	10.8	0.5	8.5	0.6	11.5	1.4	8.2	2.8
	150V	18.5	0.5	16.3	0.8	23.0	1.6	12.2	1.4	10.8	3.7
	200V	28.7	1.4	40.7	2.0	39.5	2.8	29.3	3.5	25.1	3.9
	250V	40.9	1.1	40.0	2.0	46.0	3.6	32.3	5.6	32.2	5.1
	300V	29.7	0.8	31.9	1.6	33.8	2.7	23.8	4.1	21.7	3.4
	350V	14.2	0.4	11.3	0.6	11.2	0.9	8.8	1.5	8.5	1.3

continued

Continued Appendix 5C.(Fig. 5.3). *Proportion of all cells that were viable and transiently* permeabilised cells by single electroporation pulse in various media, trehalose concentrations and voltages at 500 μF capacitance.

Trehalose (n.s)		0 M		0.1 M		0.2 M		0.3 M		0.4 M	
Media *)	Voltage*)	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
Cytomix	Not-EP	0	0	0	0	0	0	0	0	0	0
	100V	9.3	0.3	2.1	0.1	10.4	0.8	5.9	1.0	5.4	0.8
	150V	22.7	0.1	17.5	0.9	12.3	1.0	12.1	2.1	22.1	3.5
	200V	32.6	0.9	29.7	1.5	42.8	6.1	44.3	2.8	39.8	4.8
	250V	36.7	3.9	36.9	1.9	41.5	2.8	43.8	1.3	41.1	5.0
	300V	26.3	0.7	34.3	2.4	26.4	1.8	40.8	2.7	30.9	6.7
	350V	29.6	0.8	27.0	1.4	34.2	2.4	36.9	4.5	30.5	3.7

The cells were 99% viable before electroporation.; n.s. No significant effect between trehalose concentration. *) Significant effect ($P<0.01$) between voltages and media.

Appendix 5D (Fig. 5.3). Proportion of all cells that were viable and transiently permeabilised cells by single electroporation pulse in various media, trehalose concentrations and voltages at 500 μF capacitance.

Trehalose (n.s)		0 M		0.1 M		0.2 M		0.3 M		0.4 M	
Media *)	Voltage*)	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
DMEM	Not-EP	0	0	0	0	0	0	0	0	0	0
	100V	3.6	0.1	2.4	0.1	16.5	1.5	6.5	0.8	8.0	2.1
	150V	14.2	0.4	26.2	1.3	16.7	1.3	19.9	2.4	13.1	3.5
	200V	24.7	0.7	43.5	2.2	50.2	4.0	46.8	5.5	30.5	8.2
	250V	38.3	1.1	42.4	2.7	39.6	2.8	38.7	4.6	27.4	7.3
	300V	33.1	0.9	32.9	1.7	42.0	3.0	34.2	4.0	18.2	6.2
	350V	8.0	0.2	15.7	0.8	12.8	0.9	10.7	1.3	8.6	2.9
mHBS	Not-EP	0	0	0	0	0	0	0	0	0	0
	100V	10.9	0.3	10.8	0.5	8.5	0.6	11.5	1.4	8.2	2.8
	150V	18.5	0.5	16.3	0.8	23.0	1.6	12.2	1.4	10.8	3.7
	200V	28.7	1.4	40.7	2.0	39.5	2.8	29.3	3.5	25.1	3.9
	250V	40.9	1.1	40.0	2.0	46.0	3.6	32.3	5.6	32.2	5.1
	300V	29.7	0.8	31.9	1.6	33.8	2.7	23.8	4.1	21.7	3.4
	350V	14.2	0.4	11.3	0.6	11.2	0.9	8.8	1.5	8.5	1.3

continued

Continued Appendix 5D (Fig. 5.3). Proportion of all cells that were viable and transiently permeabilised cells by single electroporation pulse in various media, trehalose concentrations and voltages at 500 μF capacitance for Fig. 5.3.

Trehalose (n.s)		0 M		0.1 M		0.2 M		0.3 M		0.4 M	
Media *)	Voltage*)	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
Cytomix	Not-EP	0	0	0	0	0	0	0	0	0	0
	100V	9.3	0.3	2.1	0.1	10.4	0.8	5.9	1.0	5.4	0.8
	150V	22.7	0.1	17.5	0.9	12.3	1.0	12.1	2.1	22.1	3.5
	200V	32.6	0.9	29.7	1.5	42.8	6.1	44.3	2.8	39.8	4.8
	250V	36.7	3.9	36.9	1.9	41.5	2.8	43.8	1.3	41.1	5.0
	300V	26.3	0.7	34.3	2.4	26.4	1.8	40.8	2.7	30.9	6.7
	350V	29.6	0.8	27.0	1.4	34.2	2.4	36.9	4.5	30.5	3.7

The cells were 99% viable before electroporation.; n.s. No significant effect between trehalose concentration. *) Significant effect ($P<0.01$) between voltages and media.

Appendix 5D (Fig. 5.4). Number and proportion (%) of viable cells retaining intracellular PI following in vitro culture for 24 h after single pulse electroporation.

	0 M Trehalose			0.1 M Trehalose			0.2 M Trehalose			0.3 M Trehalose			0.4 M Trehalose		
	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%
DMEM															
Not-EP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
100V	3.7	0.3	23.6	6.7	0.7	26.0	4.7	1.2	29.1	4.7	0.9	25.2	4.0	1.0	23.7
150V	5.3	0.9	37.7	6.3	1.2	35.9	7.0	1.2	38.5	5.3	1.5	31.5	5.7	0.3	36.3
200V	6.7	1.9	53.3	10.0	1.0	60.8	9.0	2.1	61.3	8.3	2.0	51.2	7.0	1.0	63.0
250V	6.0	0.0	58.7	7.7	1.5	65.3	6.7	1.8	77.8	7.7	0.3	59.7	6.7	1.2	62.1
300V	2.7	0.9	71.1	3.7	0.3	88.9	3.3	0.3	68.3	3.3	0.9	79.2	4.7	1.2	71.5
350V	0.7	0.7	33.3	2.0	0.6	83.3	0.7	0.7	22.2	2.0	1.2	55.6	2.7	1.3	55.6
mHBS															
Not-EP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
100V	2.0	1.0	15.9	2.3	0.9	13.1	4.0	1.0	21.7	4.7	0.3	26.4	5.3	0.9	30.1
150V	2.7	1.2	14.8	4.7	2.2	23.2	6.0	1.0	29.9	4.3	0.3	29.6	6.0	1.0	33.6
200V	4.7	0.9	29.7	6.3	0.9	30.3	8.7	0.3	38.9	7.7	0.3	56.3	8.3	1.9	47.1
250V	8.3	2.3	43.7	6.7	0.9	45.0	6.7	1.7	38.6	10.0	2.0	49.3	6.7	1.2	49.1
300V	3.7	0.9	50.3	4.0	0.6	62.7	4.3	1.5	75.7	5.3	0.3	69.6	4.3	1.2	70.0
350V	1.7	0.9	47.2	1.7	0.9	53.3	1.7	1.2	22.2	1.7	0.9	55.6	2.3	1.2	58.3

(continued)

Continued Appendix 5D (Fig. 5.4). Number and proportion (%) of viable cells retaining intracellular PI following in vitro culture for 24 h after single pulse electroporation.

	0 M Trehalose			0.1 M Trehalose			0.2 M Trehalose			0.3 M Trehalose			0.4 M Trehalose		
	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%
Cytomix															
Not-EP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
100V	4.0	1.2	21.5	5.0	1.5	29.8	5.3	1.9	29.4	5.7	1.5	29.3	4.7	1.2	27.1
150V	6.3	1.7	32.1	5.7	0.7	39.7	8.3	1.5	40.4	8.0	1.5	36.5	6.0	1.2	31.6
200V	7.3	1.9	38.4	9.7	0.3	47.6	8.0	1.5	41.0	8.7	0.3	47.7	8.0	0.6	40.8
250V	11.7	2.3	58.2	11.3	1.2	54.3	12.3	1.5	57.8	12.3	0.9	58.7	12.7	0.7	58.9
300V	9.3	2.2	55.9	8.7	2.6	49.4	9.0	0.6	53.7	7.3	1.2	50.0	7.7	0.9	52.5
350V	6.3	1.5	56.5	8.3	2.0	54.7	6.7	1.3	59.8	8.3	0.7	68.3	8.0	0.6	63.8

Appendix 5E (Fig. 5.7). Cell viability after electroporation (1, 5 or 10 pulses) given at 5-second intervals.

Voltages and Pulses	Live (TB-)			Dead (TB+)			Viable and Permeable		
	N	SEM	%	N	SEM	%	N	SEM	%
Not-EP	101.0	6.2	99.3	0.7	0.3	0.7	0.0	0.0	0.0
50Vx1	74.0	12.7	83.8	14.3	1.2	16.2	11.7	2.2	13.2
50Vx5	73.3	15.1	72.4	28.0	2.1	27.6	14.3	1.7	14.1
50Vx10	83.3	9.9	71.0	34.0	7.0	29.0	15.7	0.9	13.4
100Vx1	90.7	20.0	82.7	19.0	3.1	17.3	16.3	2.0	14.9
100Vx5	75.7	7.3	73.2	27.7	4.7	26.8	15.0	4.5	14.5
100Vx10	62.0	10.3	74.1	21.7	2.9	25.9	22.3	1.3	26.7
150Vx1	82.7	5.8	77.7	23.7	4.3	22.3	13.0	1.5	12.2
150Vx5	63.0	8.1	74.1	22.0	3.5	25.9	11.0	2.6	12.9
150Vx10	35.7	9.3	46.9	40.3	7.9	53.1	10.3	1.5	13.6
200Vx1	79.3	11.9	77.0	23.7	1.3	23.0	18.0	2.6	17.5
200Vx5	36.3	3.0	41.6	51.0	2.5	58.4	19.0	3.5	21.8
200Vx10	16.3	3.0	30.2	37.7	11.6	69.8	6.7	1.8	12.3
250Vx1	84.0	8.5	80.0	21.0	1.7	20.0	45.0	6.7	42.9
250Vx5	65.3	13.3	52.1	60.0	1.0	47.9	15.7	0.3	12.5
250Vx10	9.7	3.3	27.6	25.3	2.6	72.4	5.7	1.2	16.2

Appendix 5F (Fig. 5.7). Cell viability after electroporation (1, 5 or 10 pulses) given at 3-min intervals.

Voltages and Pulses	Live (TB-)			Dead (TB+)			Viable and Permeable		
	N	SEM	%	N	SEM	%	N	SEM	%
Not-EP	101.0	6.2	99.3	0.7	0.3	0.7	0.0	0.0	0.0
50Vx1	123.7	18.9	87.1	18.3	1.5	12.9	9.0	2.3	6.3
50Vx5	97.3	11.5	86.9	14.7	1.7	37.3	10.0	2.5	8.9
50Vx10	56.3	14.7	69.8	24.3	3.9	44.0	6.7	1.8	8.3
100Vx1	105.3	3.8	87.3	15.3	2.8	41.1	6.7	2.6	5.5
100Vx5	105.0	15.7	81.4	24.0	4.9	43.1	7.7	2.9	5.9
100Vx10	68.7	3.5	73.3	25.0	2.6	33.8	24.0	16.7	25.6
150Vx1	108.3	7.5	82.3	23.3	4.5	37.6	15.3	2.3	11.6
150Vx5	83.0	4.2	68.0	39.0	1.7	41.3	16.3	1.5	13.4
150Vx10	59.3	4.7	48.9	62.0	2.1	44.0	17.0	5.2	14.0
200Vx1	118.7	5.5	83.2	24.0	4.0	35.6	19.3	7.3	13.6
200Vx5	61.3	5.3	64.6	33.7	8.1	40.6	15.7	4.3	16.5
200Vx10	59.7	7.3	57.2	44.7	7.0	42.7	15.3	1.8	14.7
250Vx1	133.3	7.4	82.0	29.3	4.4	24.4	61.3	6.8	37.7
250Vx5	60.0	4.9	71.4	24.0	4.0	37.7	15.7	1.3	18.7
250Vx10	48.7	0.3	57.7	35.7	3.3	43.5	10.7	1.9	12.6

Appendix 5G (fig. 5.7). Cell viability after electroporation (1, 5 or 10 pulses) given at 6 min intervals.

Voltages and Pulses	Live (TB-)			Dead (TB+)			Viable and Permeable		
	N	SEM	%	N	SEM	%	N	SEM	%
Not-EP	101.0	6.2	99.3	0.7	0.3	0.7	0.0	0.0	0.0
50Vx1	61.0	4.9	83.2	12.3	2.6	16.8	8.7	0.9	11.8
50Vx5	58.3	9.5	76.8	17.7	4.9	23.2	11.0	2.6	14.5
50Vx10	74.7	7.2	74.9	25.0	5.2	25.1	17.0	0.6	17.1
100Vx1	62.7	8.7	76.1	19.7	3.8	23.9	9.0	2.6	10.9
100Vx5	58.7	8.2	71.3	23.7	1.5	28.7	11.7	1.5	14.2
100Vx10	48.7	8.8	62.9	28.7	7.3	37.1	12.7	1.2	16.4
150Vx1	64.7	7.7	74.9	21.7	3.2	25.1	14.3	0.7	16.6
150Vx5	52.0	5.5	67.0	25.7	2.3	33.0	11.7	0.3	15.0
150Vx10	33.7	6.1	55.8	26.7	4.7	44.2	12.0	1.2	19.9
200Vx1	58.0	1.2	75.0	19.3	3.0	25.0	20.3	2.0	26.3
200Vx5	40.7	6.8	48.6	43.0	7.0	51.4	19.0	3.0	22.7
200Vx10	20.7	5.9	40.0	31.0	6.1	60.0	12.3	2.3	23.9
250Vx1	73.0	10.1	74.0	25.7	3.2	26.0	37.3	0.3	37.8
250Vx5	29.3	7.8	35.9	52.3	5.2	64.1	15.0	0.0	18.4
250Vx10	16.0	2.0	32.7	33.0	1.0	67.3	10.7	0.3	21.8

Appendix 5H (Fig. 5.8). Viability of non frozen cells following electroporation with a single 250V pulse in solutions with or without trehalose (percentage of cells excluding trypan-blue).

Time and Treatment	Immediately after EP and CP				30 min after EP and CP			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
	% Viable	SEM	% Viable	SEM	% Viable	SEM	% Viable	SEM
No-cryoprotectant	69.8	5.0	92.7	2.8	81.4	1.2	92.0	1.2
0.8% Glycerol	78.9	3.4	88.6	3.4	75.1	3.7	81.9	2.9
1% DMSO	73.8	3.6	84.1	0.5	67.6	2.9	81.9	2.9
8% Glycerol	77.1	2.3	91.2	1.3	69.1	4.3	85.9	0.4
10% DMSO	73.2	5.2	92.8	1.3	58.4	5.6	74.5	6.7

Appendix 5I (Fig. 5.9). Viability of non electroporated cells after freezing ASAP or 30 min later with and without trehalose.

	Non frozen		Snap Frozen		Slow cooled	
	No EP - No Trehalose and used ASAP					
Media	% Viable	SEM	% Viable	SEM	% Viable	SEM
No CPA	90.0	2.6	0.0	0.0	0.0	0.0
1% DMSO	91.1	1.8	7.1	2.1	3.4	2.1
8% Glycerol	83.5	0.3	81.7	3.4	83.0	3.0
10%DMSO	82.5	0.8	80.6	2.7	88.4	2.9
	NoEP containing 0.3 M Trehalose and used ASAP					
No CPA	94.0	3.6	8.6	2.0	3.0	1.7
0.8% Glycerol	97.1	1.6	9.5	3.1	8.1	5.0
1% DMSO	91.4	2.9	8.4	2.9	11.2	1.3
8% Glycerol	93.7	1.7	90.9	3.0	90.6	1.2
10%DMSO	88.9	2.6	90.8	3.3	91.2	0.8
	No EP - No Trehalose and used 30 min later					
No CPA	97.3	1.6	0.0	0.0	0.0	0.0
0.8% Glycerol	86.5	0.6	4.3	0.6	5.9	1.5
1% DMSO	89.0	2.4	6.3	1.1	8.3	1.4
8% Glycerol	85.2	2.0	85.0	1.1	84.0	0.9
10%DMSO	88.5	0.5	88.5	1.0	85.2	1.6
	No EP - containing 0.3 M Trehalose and used 30 min later					
No CPA	98.8	1.2	2.4	1.2	2.8	2.8
0.8% Glycerol	89.8	1.7	3.6	2.2	2.5	2.5
1% DMSO	88.7	1.8	6.1	1.1	2.7	1.3
8% Glycerol	81.2	1.9	85.7	2.3	90.6	3.4
10%DMSO	83.2	1.1	87.5	3.9	84.3	3.5

Appendix 5J (Figs. 5.10; 5.11; 5.12; 5.13; 5.14). Effect of Slow-cooling (1°/min to -80°C) and storage in LN2 on the viability of non-electroporated cells (percentage of cells exclude trypan-blue)..

Time and Treatment	Immediately after CP addition				30 min after CP addition			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
	% Viable	SEM	% Viable	SEM	% Viable	SEM	% Viable	SEM
No-cryoprotectant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.8% Glycerol	3.9	2.0	1.7	1.7	1.7	1.7	4.4	2.2
1% DMSO	31.3	3.4	21.7	5.8	26.1	7.6	27.6	3.9
8% Glycerol	56.4	3.0	51.6	2.9	62.6	3.8	65.5	1.3
10% DMSO	70.8	2.9	75.2	3.1	59.0	1.9	75.6	0.2

Appendix 5K. Effect of snap freezing of non-electroporated cells on cell viability (percentage of cells exclude trypan-blue).

Time and Trehalose concentration	Immediately after CP addition				30 min after CP addition			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
Cryoprotectants	% Viable	SEM	% Viable	SEM	% Viable	SEM	% Viable	SEM
No-cryoprotectant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.8% Glycerol	3.9	2.0	1.7	1.7	1.7	1.7	4.4	2.2
1% DMSO	31.3	3.4	21.7	5.8	26.1	7.6	27.6	3.9
8% Glycerol	56.4	3.0	51.6	2.9	62.6	3.8	65.5	1.3
10% DMSO	70.8	2.9	75.2	3.1	59.0	1.9	75.6	0.2

Appendix 5L (Figs. 5.11; 5.12; 5.13; 5.14). Effect of snap freezing after electroporation with a single 250V pulse on cell viability (percentage of cells excluding trypan-blue).

Time and Trehalose concentration	Immediately after EP and CP				30 min after EP and CP			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
Cryoprotectants	% Viable	SEM	% Viable	SEM	% Viable	SEM	% Viable	SEM
No-cryoprotectant	0.00	0.00	20.12	0.06	2.78	0.03	28.57	0.05
0.8% Glycerol	6.91	0.00	23.06	0.01	3.54	0.02	40.72	0.03
1% DMSO	15.34	0.05	39.97	0.01	1.55	0.01	31.04	0.03
8% Glycerol	21.57	0.02	36.77	0.01	16.83	0.05	40.74	0.02
10% DMSO	17.63	0.03	48.43	0.05	18.40	0.03	41.90	0.04

Appendix 5M (Fig. 5.10; 5.11; 5.12; 5.13; 5.14). Effect of Slow-cooling (1°/min to -80°C) and storage in LN2 after electroporation with a single 250V pulse on cell viability (percentage of cells exclude trypan-blue).

Time and Treatment	Immediately after EP and CP				30 min after EP and CP			
	0M Trehalose		0.3M Trehalose		0M Trehalose		0.3M Trehalose	
	% Viable	SEM	% Viable	SEM	% Viable	SEM	% Viable	SEM
No-cryoprotectant	0.00	0.00	23.96	0.07	0.00	0.00	68.84	0.01
0.8% Glycerol	3.02	0.02	39.88	0.08	6.15	0.03	72.91	0.05
1% DMSO	16.28	0.01	30.43	0.08	49.74	0.03	68.31	0.04
8% Glycerol	41.38	0.04	71.95	0.01	39.31	0.04	82.66	0.05
10% DMSO	48.78	0.04	64.13	0.02	69.04	0.01	65.90	0.02

Appendix 5N (Fig. 5.15). The number of cells plating after 24 h in culture of non electroporated cells with a single 250V pulse with and without trehalose and used immediately or after 30 minutes (average of 3 replicates).

Trehalose and time	Cryoprotectant added	Non Frozen		Slow Cooled		Snap Frozen	
		N	SEM	N	SEM	N	SEM
No trehalose and used immediately	No-cryoprotectant	45.33	6.57	0.00	0.00	0.00	0.00
	0.8% Glycerol	40.33	5.21	4.33	1.20	3.33	0.88
	1% DMSO	48.00	10.15	4.00	0.58	2.00	1.15
	8% Glycerol	42.67	10.09	31.00	7.55	26.33	4.37
	10% DMSO	43.00	8.72	31.67	5.46	25.67	5.49
No trehalose and used 30 minutes later	No-cryoprotectant	35.33	8.41	0.00	0.00	0.00	0.00
	0.8% Glycerol	33.00	6.11	3.33	1.33	3.33	1.20
	1% DMSO	29.00	5.13	3.67	1.20	3.00	0.58
	8% Glycerol	24.00	5.51	18.00	3.51	16.00	2.52
	10% DMSO	31.33	6.89	21.67	5.24	19.33	0.88

Continued next page
*) =from 2 replicates.

Appendix 5O (Fig. 5.16; 5.17; 5.18; 5.19). The number of cells plating after 24 h in culture after electroporation with a single 250V pulse with and without trehalose and used immediately or after 30 minutes (average of 3 replicates).

Trehalose and time	Cryoprotectant added	Non Frozen		Slow Cooled		Snap Frozen	
		N	SEM	N	SEM	N	SEM
No trehalose and used immediately	No-cryoprotectant	49.33	10.11	0.00	0.00	0.00	0.00
	0.8% Glycerol	44.33	4.84	4.33	2.03	3.33	0.33
	1% DMSO	52.33	9.40	5.00	1.53	4.33	1.76
	8% Glycerol	49.33	7.97	22.33	2.33	35.00	7.94
	10% DMSO	42.33	5.90	22.67	2.19	29.00	4.62
No trehalose and used 30 minutes later	No-cryoprotectant	35.67	5.49	0.00	0.00	0.00	0.00
	0.8% Glycerol	18.00	2.31	5.67	1.20	4.00	0.58
	1% DMSO	19.67	1.76	3.67	1.33	2.00	0.58
	8% Glycerol	19.00	2.89	11.33	1.76	9.67	1.20
	10% DMSO	19.33	2.73	12.33	1.76	9.00	0.00

Continued next page
*) =from 2 replicates.

Continued Appendix 5O (Fig. 5.16; 5.17; 5.18; 5.19). The number of cells plating after 24 h in culture after electroporation with a single 250V pulse with and without trehalose and used immediately or after 30 minutes (average of 3 replicates).

0.3 trehalose and used immediately	No-cryoprotectant	43.00	5.51	11.00	2.08	13.00	1.53
	0.8% Glycerol	40.33	4.41	13.00	1.00	12.67	0.33
	1% DMSO	40.00	1.53	8.67	1.86	11.33	1.76
	8% Glycerol	42.00	4.73	19.33	1.86	32.00	4.62
	10% DMSO	38.33	2.03	22.33	2.40	52.50*)	10.50
0.3 trehalose and used 30 minutes later	No-cryoprotectant	34.67	3.53	12.67	1.76	7.67	0.88
	0.8% Glycerol	33.33	4.41	13.00	1.73	6.33	0.88
	1% DMSO	34.00	2.52	13.33	1.33	12.67	0.67
	8% Glycerol	32.67	0.67	21.00	2.08	16.67	1.20
	10% DMSO	33.00	2.08	20.67	1.20	15.33	1.20

Appendix Chapter 6

Appendix 6.1A. (Figs. 6.2 and 6.3). Proportion of viable , dead, and viable permeable cells after cooling at 1°C/min from +25 C to -25°C

Media and Trehalose (M)	Temperature	Cooling 1°C/min from +25°C to -25°C.			Snap freezing					
					ASAP			30 min later		
		Live	Dead	VP	Live	Dead	VP	Live	Dead	VP
FBS		%	%	%	%	%	%	%	%	%
0	25°C	100	0	0	6	94	2.4	6.1	93.9	2.4
0	15°C	96.4	3.6	0	8	92	4	1.6	98.4	1.6
0	5°C	96.9	3.1	0	8.3	91.7	2.8	0	100	0
0	-5°C	84.7	15.3	0	7.6	92.4	1.3	2.1	97.9	2.1
0	-15°C	62.5	37.5	0	0	100	0	2.8	97.2	1.4
0	-25°C	46.5	53.5	18.6	0	100	0	13.3	86.7	0
0.3	25°C	100	0	0	18.7	81.3	2.8	6.4	93.6	2.6
0.3	15°C	97.2	2.8	0	10.7	89.3	2.7	2.4	97.6	1.2
0.3	5°C	88.5	11.5	0	17.4	82.6	2.9	7	93	1.8
0.3	-5°C	61.9	38.1	7.2	19.7	80.3	3.3	13.2	86.8	5.9
0.3	-15°C	60.3	39.7	13.2	20.3	79.7	5.8	8.8	91.2	2.9
0.3	-25°C	58	42	6.2	15	85	2.5	4.1	95.9	1.4
DMEM										
0	25°C	97.3	2.7	0	7.3	92.7	0	0	100	0
0	15°C	93.2	6.8	0	11.3	88.7	0	2.9	97.1	0
0	5°C	83.3	16.7	0	9.5	90.5	0	7.8	92.2	2
0	-5°C	41.2	58.8	5.9	7.7	92.3	1.9	3.4	96.6	0
0	-15°C	0	100	0	0	100	0	0	100	0
0	-25°C	0	100	0	0	100	0	0	100	0
0.3	25°C	98	2	0	14.7	85.3	2.9	5	95	1.7
0.3	15°C	98.6	1.4	0	12.8	87.2	5.1	4.6	95.4	1.5
0.3	5°C	81.6	18.4	2.6	16.5	83.5	3.5	9.3	90.7	2.7
0.3	-5°C	53.6	46.4	7.1	10.2	89.8	3.4	4.2	95.8	4.2
0.3	-15°C	48.6	51.4	0	12	88	2	10.2	89.8	8.2
0.3	-25°C	29.3	70.7	5.3	3.5	96.5	1.8	4.8	95.2	3.2
0.3	-25°C	51.6	48.4	2.2	15.6	84.4	6.5	8.8	91.3	6.3

VP= viable permeabilised

Continued.

Continued Appendix 6.1A. (Figs. 6.2 and 6.3). Proportion of viable , dead, and viable permeable cells after cooling at 1°C/min from +25 C to -25°C.

Media and Trehalose (M)	Tempe rature	Cooling 1°C/min from +25°C to -25°C.			Snap freezing					
					ASAP			30 min later		
		Live	Dead	VP	Live	Dead	VP	Live	Dead	VP
Cytomix										
0	25°C	97	3	0	0	100	0	0	100	0
0	15°C	98.7	1.3	0	0	100	0	0	100	0
0	5°C	86.9	13.1	0	0	100	0	1.5	98.5	1.5
0	-5°C	62.3	37.7	1.4	0	100	0	3.6	96.4	3.6
0	-15°C	29.2	70.8	18.1	3.8	96.2	1.9	0	100	0
0	-25°C	0	100	0	0	100	0	0	100	0
0.3	25°C	98.2	1.8	0	11.6	88.4	2.9	10.9	89.1	5.5
0.3	15°C	98.4	1.6	0	15.2	84.8	1.5	4.6	95.4	1.5
0.3	5°C	100	0	0	13.5	86.5	2.7	12.5	87.5	3.1
0.3	-5°C	87.7	12.3	0	23.7	76.3	3.4	9.7	90.3	4.2
0.3	-15°C	63.3	36.7	5.6	15.2	84.8	5.4	8.5	91.5	8.5
0.3	-25°C	51.6	48.4	2.2	15.6	84.4	6.5	8.8	91.3	6.3
mHBS										
0	25°C	81.5	18.5	0	0	100	0	0	100	0
0	15°C	64.3	35.7	1.4	0	100	0	0	100	0
0	5°C	35.2	64.8	8.5	0	100	0	0	100	0
0	-5°C	26	74	7.8	0	100	0	0	100	0
0	-15°C	0	100	0	0	100	0	0	100	0
0	-25°C	0	100	0	0	100	0	0	100	0
0.3	25°C	85.7	14.3	0	0	100	0	0	100	0
0.3	15°C	85.3	14.7	0	13	87	0	7.1	92.9	0
0.3	5°C	52.9	47.1	4.3	12.1	87.9	0	7.3	92.7	0
0.3	-5°C	29	71	8.7	8.9	91.1	0	2.9	97.1	0
0.3	-15°C	11.1	88.9	5.6	0	100	0	3.6	96.4	0
0.3	-25°C	0	100	0	0	100	0	0	100	0

Appendix 6.1B (Fig 6.4). The number of plated cells after thawing of re-frozen ASAP and 30 min after equilibration in first solution.

	+25°C		+15°C		+5°C		-5°C		-15°C		-25°C	
ASAP	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM
FBS 0T	5.3	1.3	9.7	4.2	6.3	0.9	8.7	1.8	0.0	0.0	0.0	0.0
FBS 0.3 T	8.7	0.3	12.3	1.9	12.7	1.2	12.0	1.7	11.3	2.7	7.7	1.3
DMEM 0T	3.7	0.3	11.0	1.2	8.7	1.5	5.3	1.2	0.0	0.0	0.0	0.0
DMEM 0.3T	5.0	0.6	10.7	2.2	10.3	1.2	10.3	2.6	7.7	0.9	1.3	0.7
Cytomix 0T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7	1.8	0.0	0.0
Cytomix 0.3 T	2.7	0.3	11.7	1.5	11.7	1.3	9.7	1.9	6.3	0.3	9.0	1.7
mHBS 0T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
mHBS 0.3T	0.0	0.0	9.0	2.1	3.7	1.9	8.3	1.5	0.0	0.0	0.0	0.0
30 min												
FBS 0T	5.0	0.6	0.0	0.0	1.0	0.6	1.7	0.3	0.3	0.3	2.7	1.8
FBS 0.3 T	7.0	0.6	1.7	0.3	5.0	0.6	2.7	0.3	3.3	0.9	2.7	0.9
DMEM 0T	0.0	0.0	0.0	0.0	2.0	1.5	1.7	0.3	0.0	0.0	0.0	0.0
DMEM 0.3T	3.0	0.6	3.3	0.9	4.3	2.4	3.3	0.3	2.7	0.3	3.0	0.6
Cytomix 0T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.7	0.0	0.0
Cytomix 0.3 T	4.3	0.7	2.3	1.2	3.7	0.7	2.3	0.3	2.0	0.6	3.0	0.6
mHBS 0T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
mHBS 0.3T	0.0	0.0	4.3	2.3	2.3	1.2	1.3	0.9	0.7	0.7	0.0	0.0

Appendix 6.2A. (Fig. 6.6). Cell viability after equilibration in the first solution without trehalose)

	Viable		Non Viable		Viable permeabilised	
RT 5 min in FBS						
Media	%	SEM	%	SEM	%	SEM
20% DMSO	89.6	3.5	10.4	3.5	2.0	1.0
10% D+10%EG	88.3	1.5	11.7	1.5	2.1	1.4
10%DMSO	90.2	3.1	9.8	3.1	1.3	1.3
10% EG	88.1	3.3	11.9	3.3	1.4	1.4
RT 5 min in Cytomix						
20% DMSO	89.3	1.0	10.7	1.0	1.7	1.0
10% D+10%EG	92.7	0.6	7.3	0.6	3.7	0.6
10%DMSO	89.7	0.9	10.4	0.9	1.7	1.1
10% EG	92.3	0.5	7.7	0.5	2.2	0.3
RT 10 min in FBS						
20% DMSO	84.3	4.3	15.7	4.3	4.0	1.6
10% D+10%EG	85.3	2.3	14.7	2.3	4.8	0.7
10%DMSO	86.5	1.6	13.5	1.6	3.5	1.0
10% EG	88.2	2.6	11.8	2.6	1.9	1.0
RT 10 min in Cytomix						
20% DMSO	84.0	2.5	16.0	2.5	5.3	0.8
10% D+10%EG	88.1	1.5	11.9	1.5	2.9	0.5
10%DMSO	87.5	1.3	12.5	1.3	4.6	1.6
10% EG	89.8	3.0	10.2	3.0	3.0	0.2
Slow cooling to -25C in FBS						
20% DMSO	85.7	2.0	14.3	2.0	31.5	5.8
10% D+10%EG	85.7	0.4	14.3	0.4	43.6	2.3
10%DMSO	91.9	1.3	8.1	1.3	47.6	11.0
10% EG	91.4	1.8	8.6	1.8	36.9	6.7
Slow cooling to -25C in Cytomix						
20% DMSO	85.2	0.8	14.8	0.8	42.5	10.8
10% D+10%EG	83.7	1.7	16.3	1.7	38.2	6.2
10%DMSO	86.3	1.9	13.7	1.9	42.0	2.6
10% EG	84.4	2.8	15.6	2.8	44.0	7.8

Appendix 6.2B. (Fig. 6.7). Cell viability after equilibration in the first solution containing 0.6 M trehalose.

	Viable		Non Viable		Viable permeabilised	
RT 5 min in FBS containing 0.6 T						
Media	%	SEM	%	SEM	%	SEM
20% DMSO	89.3	3.4	10.9	3.9	6.2	4.7
10% D+10%EG	88.6	1.2	11.7	1.0	2.4	1.6
10%DMSO	90.5	2.9	9.2	2.9	1.4	1.4
10% EG	88.7	3.3	13.1	4.2	2.9	1.5
RT 5 min in Cytomix containing 0.6 T						
20% DMSO	88.0	1.6	12.4	1.7	2.1	0.4
10% D+10%EG	91.1	1.0	8.5	1.0	5.0	1.0
10%DMSO	87.6	1.7	13.1	2.1	3.8	1.2
10% EG	92.6	0.8	8.1	0.9	3.5	1.0
RT 10 min in FBS containing 0.6 T						
20% DMSO	85.2	2.8	15.5	2.9	1.0	1.0
10% D+10%EG	88.3	2.7	11.7	3.0	4.3	3.2
10%DMSO	88.2	1.0	11.9	1.0	1.4	1.4
10% EG	86.8	4.2	15.7	5.7	5.4	4.5
RT 10 min in Cytomix containing 0.6 T						
20% DMSO	86.1	3.6	14.9	4.7	3.3	1.7
10% D+10%EG	84.5	2.7	15.5	2.9	2.8	1.4
10%DMSO	86.4	1.8	13.5	1.6	3.4	1.2
10% EG	87.9	3.1	14.0	3.9	1.4	1.4
Slow cooling to -25C in FBS containing 0.6 T						
20% DMSO	85.6	1.6	15.0	2.2	21.8	6.5
10% D+10%EG	87.3	2.1	13.3	2.3	14.9	2.1
10%DMSO	90.3	2.6	9.9	2.8	16.7	2.9
10% EG	90.2	0.1	10.8	0.2	20.6	4.3
Slow cooling to -25C in Cytomix containing 0.6 T						
20% DMSO	84.4	1.6	15.7	2.1	10.5	3.9
10% D+10%EG	85.0	1.4	17.0	2.0	11.3	3.8
10%DMSO	88.8	2.6	11.1	2.8	21.9	6.0
10% EG	87.6	3.7	14.5	4.7	17.4	7.4

Appendix 6.2C (Fig. 6.8). Proportion of viable cells after equilibration in both solution 1 not containing trehalose and in solution 2 at different times and temperatures

1st Solution	FBS without trehalose																	
	5 min at RT						10 min at RT						Slow cooling 1°C/min to -25					
2 nd solution	10 min at RT			60 min at RT			10 min at RT			60 min at RT			10 min at RT			60 min at RT		
	FBS without Trehalose																	
CPA	N	SEM	%	N	SEM	%	N	SEM	%		SEM	%	N	SEM	%	N	SEM	%
20 DMSO	42.33	9.39	82.2	65.67	11.55	87.2	25.86	13.45	89.8	45.00	10.58	91.0	146.67	25.86	98.8	47.33	2.40	87.6
DMSO/EG	49.00	2.52	79.1	102.33	13.37	98.2	25.76	18.98	87.9	42.67	8.51	89.0	123.00	13.05	99.2	49.67	4.70	87.6
10DMSO	20.33	1.67	93.6	123.33	9.91	95.1	11.00	7.62	84.1	53.00	2.08	90.5	95.33	21.36	91.5	31.00	2.89	88.2
10EG	19.33	0.88	100.0	130.00	4.00	98.3	10.11	7.53	91.5	41.00	8.08	88.8	83.33	2.67	94.9	70.33	4.26	97.4
FBS with 0.3 M Trehalose																		
20 DMSO	25.00	1.53	83.8	64.00	15.53	78.6	13.26	9.58	85.8	44.33	8.01	94.2	41.33	4.98	71.3	45.33	4.06	96.8
DMSO/EG	27.67	5.36	76.9	30.00	10.69	64.2	16.52	9.10	88.7	41.67	12.02	85.1	31.67	0.88	76.7	47.33	2.40	88.8
10DMSO	15.67	4.70	88.9	79.00	8.62	88.7	10.18	4.48	82.4	51.33	2.33	92.7	63.67	7.54	85.0	37.33	3.84	90.7
10EG	16.00	1.53	89.3	112.33	5.36	88.2	8.76	5.91	85.3	51.00	3.51	90.5	93.33	2.67	96.4	149.33	9.96	97.7
Cytomix without Trehalose																		
20 DMSO	141.33	24.33	93.5	49.00	12.12	89.9	82.83	47.77	90.0	45.00	7.00	87.3	161.00	41.39	99.1	46.33	4.33	92.7
DMSO/EG	85.33	8.35	92.2	40.33	8.09	85.9	46.84	31.43	92.7	45.67	7.36	87.9	70.00	4.73	96.6	39.33	7.84	91.7
10DMSO	65.33	12.44	92.8	36.00	3.61	95.3	38.89	21.59	92.8	42.00	10.21	88.7	86.00	5.13	98.4	43.33	10.71	92.7
10EG	95.00	15.31	94.3	49.67	13.64	90.7	55.15	32.53	92.4	43.00	9.29	89.7	113.33	16.29	96.5	37.33	7.42	90.0
Cytomix with 0.3 M Trehalose																		
20 DMSO	22.33	3.18	84.3	24.67	4.33	87.1	12.76	7.82	88.6	33.67	4.67	84.2	26.00	5.29	70.9	68.00	10.21	71.3
DMSO/EG	33.33	6.39	87.1	37.67	7.13	91.9	19.86	11.00	89.5	35.67	3.93	89.8	31.33	4.06	55.5	15.67	0.67	70.0
10DMSO	36.67	8.97	88.2	40.00	13.32	89.9	22.82	11.31	86.6	48.00	5.13	92.4	32.67	2.67	66.5	90.00	3.06	83.5
10EG	43.67	6.94	88.1	36.67	5.36	88.1	25.30	15.0	95.0	45.67	3.28	92.0	39.67	4.18	98.2	100.67	12.35	87.9

(Continued)

Continued appendix 6.2C (Fig. 6.8). Proportion of viable cells after equilibration in both solution 1 not containing trehalose and in solution 2 at different times and temperatures

1st Solution	Cytomix without trehalose																	
	5 min at RT						10 min at RT						Slow cooling 1°C/min to -25					
2 nd solution	10 min at RT			60 min at RT			10 min at RT			60 min at RT			10 min at RT			60 min at RT		
	FBS without Trehalose																	
CPA	N	SEM	%	N	SEM	%	N	SEM	%		SEM	%	N	SEM	%	N	SEM	%
20 DMSO	23.33	0.67	100.0	47.67	6.77	97.4	48.67	3.67	86.2	33.00	5.03	82.6	21.00	1.73	78.4	13.33	0.33	87.7
DMSO/EG	44.00	2.65	98.5	53.33	6.36	97.3	25.00	3.51	94.7	22.67	2.40	97.6	12.67	2.40	94.3	16.00	2.08	96.5
10DMSO	50.00	1.15	100.0	66.00	7.09	98.9	42.00	3.46	98.5	44.33	9.49	99.0	15.33	2.19	93.7	25.33	3.38	96.1
10EG	34.33	3.84	100.0	40.67	3.84	96.6	37.00	7.23	95.6	41.67	6.39	96.3	17.33	1.20	92.7	11.67	1.86	83.9
	FBS containing 0.3 M Trehalose																	
20 DMSO	19.67	2.73	92.0	32.00	3.06	95.0	33.00	4.16	82.7	48.33	6.89	84.1	28.00	4.58	82.5	10.00	1.53	87.3
DMSO/EG	25.33	5.78	73.4	36.33	5.70	93.1	22.33	3.84	59.6	23.00	4.04	61.8	14.67	1.45	77.6	13.33	1.45	79.8
10DMSO	34.00	3.79	87.7	58.00	7.21	99.3	31.33	5.84	84.4	42.67	5.90	87.9	18.67	8.76	60.3	8.00	0.58	72.2
10EG	35.00	2.08	84.0	27.67	3.71	90.8	26.00	5.13	80.1	28.00	5.03	78.3	20.00	3.46	87.4	12.33	1.45	75.8
	Cytomix without Trehalose																	
20 DMSO	24.00	12.17	62.1	26.67	1.33	66.7	52.33	7.88	72.4	28.33	2.33	71.3	6.33	1.86	26.1	28.33	6.89	88.2
DMSO/EG	29.67	8.99	83.8	13.00	2.52	73.7	36.67	6.89	71.9	14.67	3.33	74.3	8.67	1.76	50.1	5.33	1.45	39.0
10DMSO	31.00	7.64	85.3	50.00	8.72	95.7	14.00	2.65	77.3	16.00	3.51	81.9	12.33	1.20	85.3	5.33	0.88	79.6
10EG	29.00	4.04	87.5	27.67	2.91	78.6	23.33	7.06	74.0	22.33	6.94	70.1	12.67	2.73	75.0	2.67	0.67	47.7
	Cytomix containing 0.3 M Trehalose																	
20 DMSO	23.00	4.73	93.9	64.33	1.86	95.2	29.67	4.06	96.5	26.33	0.33	88.0	24.33	5.78	81.8	1.00	0.58	23.3
DMSO/EG	23.33	3.18	94.6	56.00	3.06	92.7	28.00	4.62	100.0	36.67	15.39	96.7	30.00	3.61	93.9	22.67	0.67	94.4
10DMSO	33.00	6.08	96.6	71.00	9.07	96.9	32.00	8.08	99.3	23.00	3.79	96.5	42.67	5.36	93.6	28.33	3.18	97.5
10EG	28.67	3.84	99.0	67.67	7.22	96.9	41.00	2.52	95.3	36.33	3.71	94.1	52.33	7.26	95.9	24.33	1.86	91.1

Appendix 6.2 D (Fig. 6.9). Proportion of viable cells after equilibration in both solution 1 containing 0.6 trehalose and in solution 2 at different times and temperatures

CPA Concentration	20% DMSO		10% D+10%EG		10%DMSO		10%EG	
Treatments	FCS 0.6 T into FCS							
5O10LNoT	77.9	6.1	92.2	3.0	88.5	3.5	88.9	1.4
5O10L03T	82.2	2.7	93.6	3.6	86.6	0.8	86.9	6.2
5O60LNoT	86.3	6.9	94.0	1.6	87.3	6.9	86.3	4.6
5O60L03T	84.7	1.8	90.2	4.5	88.6	4.6	88.2	3.4
1O10LNoT	94.5	2.7	98.5	0.8	84.1	6.8	88.7	6.0
1O10L03T	79.6	7.8	78.1	3.8	82.4	2.0	92.8	5.8
1O60LNoT	95.8	1.5	92.3	2.3	90.5	3.0	95.7	1.3
1O60L03T	93.4	0.9	86.2	3.0	92.7	1.1	86.6	3.5
-25C10LNoT	81.8	2.4	86.9	2.4	85.2	2.5	85.6	1.9
-25O10L03T	84.9	4.5	89.4	1.5	80.5	3.5	85.1	2.8
-25O60LNoT	85.1	4.6	86.1	1.3	85.9	1.7	86.9	1.9
-25O60L03T	89.0	2.1	84.6	2.2	86.5	1.1	85.6	3.2
	Cytomix 0.6 T into FCS							
5O10LNoT	88.8	2.7	85.9	1.8	86.1	1.8	86.4	0.5
5O10L03T	85.3	5.4	87.0	1.4	87.7	2.0	89.0	2.0
5O60LNoT	85.4	3.3	85.8	3.8	80.9	3.6	86.7	1.1
5O60L03T	89.6	4.2	87.8	5.1	89.4	1.4	86.2	1.7
1O10LNoT	91.5	4.6	81.6	7.5	89.3	2.5	82.5	1.4
1O10L03T	97.1	1.5	86.8	4.9	81.6	2.1	84.9	4.5
1O60LNoT	83.1	12.4	92.0	1.9	87.5	3.1	89.1	2.6
1O60L03T	85.4	2.2	76.3	8.5	85.9	3.4	88.3	3.8
-25C10LNoT	91.0	4.7	84.4	4.1	86.7	4.3	90.5	1.5
-25O10L03T	90.8	1.1	89.7	2.2	83.5	4.4	85.4	0.9
-25O60LNoT	92.2	5.9	84.6	3.0	88.1	3.6	83.2	2.8
-25O60L03T	93.8	3.4	83.1	5.2	84.8	1.3	88.9	2.8

Continued Appendix 6.2D (Fig. 6.9) Proportion of viable cells after equilibration in both solution 1 containing 0.6 trehalose and in solution 2 at different times and temperatures

	FCS 0.6 T into Cytomix							
5O10LNoT	68.9	2.7	69.2	1.5	60.5	5.5	59.7	3.6
5O10L03T	55.6	5.6	71.9	2.2	77.7	5.2	62.8	6.9
5O60LNoT	48.6	9.7	55.6	2.1	56.5	3.3	55.8	3.6
5O60L03T	59.0	8.2	51.1	8.3	58.7	3.4	59.3	6.4
10O10LNoT	46.3	2.3	53.5	6.5	60.3	2.6	47.4	0.6
10O10L03T	60.5	5.1	61.7	6.4	62.4	7.5	55.7	0.8
10O60LNoT	57.0	1.0	49.0	3.6	50.2	3.5	43.1	1.8
10O60L0.3T	38.7	3.0	38.7	2.1	44.6	3.4	36.6	2.5
-25O10LNoT	53.3	5.3	54.1	6.4	58.9	7.5	45.4	3.3
-25O10L03T	57.2	2.8	53.1	5.9	46.6	2.8	49.0	3.3
-25O60LNoT	43.0	8.4	38.8	8.8	32.5	1.5	35.1	7.9
-25O60LT03T	60.1	5.4	53.9	5.5	50.6	2.7	54.3	1.0
	Cytomix 0.6 T into Cytomix							
5O10LNoT	73.8	7.4	67.5	4.5	65.4	0.0	68.2	3.5
5O10L03T	60.3	4.6	51.1	0.8	51.6	3.4	52.7	6.5
5O60LNoT	65.3	2.5	54.3	9.2	66.5	5.4	68.8	2.4
5O60L03T	59.7	1.9	34.0	7.7	51.3	0.3	55.3	4.0
10O10LNoT	68.6	4.6	70.5	8.3	76.2	0.9	78.9	5.6
10O10L03T	50.5	3.5	34.1	6.0	35.7	1.3	42.4	5.2
10O60LNoT	69.6	3.4	60.9	6.8	70.9	3.6	68.3	9.8
10O60L0.3T	47.9	2.0	51.0	3.9	53.7	1.1	45.7	2.8
-25O10LNoT	79.2	4.0	60.4	6.9	74.5	2.2	75.8	5.7
-25O10L03T	44.1	5.1	26.9	6.8	40.0	1.1	39.6	4.8
-25O60LNoT	72.8	4.4	57.6	3.1	71.0	1.9	72.6	2.4
-25O60LT03T	69.5	0.9	52.4	10.8	65.5	1.6	65.8	2.3

Appendix 6.2E (Fig. 6.10) Cells surviving (TB-) snap freezing in solution 2 in relation to the equilibration protocols, and the cryoprotectant type and concentration in solution 1 without trehalose (Cytomix or FBS).

Treatments	20% DMSO		10% D+10% EG		10% DMSO		10% EG	
Cytomix 0T to FBS								
	%	SEM	%	SEM	%	SEM	%	SEM
5O10LNoT	6.6	3.5	6.7	0.5	4.1	0.6	7.4	2.3
5O10L03T	18.8	4.5	14.7	2.7	18.1	3.7	22.8	4.3
5O60LNoT	8.8	0.2	8.1	1.9	7.8	2	11	0.8
5O60L03T	11.9	1.4	10.5	2.9	9.9	1.5	9.5	2.3
10O10NoT	5.1	2.6	5.9	0.9	5.9	1.9	4.8	1.4
10O1003T	4.4	2.3	6.3	1.2	4.8	2.4	6.7	1.1
10O60LNoT	2.6	2.6	5.4	0.2	3.7	1	8.8	1.8
10O60L03T	7.5	1.4	6.4	0.7	6.7	1.9	4.1	0.8
-25O10LNoT	4.3	2.2	7.5	1.1	5.3	3	7.3	2.3
-25O10L03T	9.3	3.2	7.7	3.9	12.1	3.6	7.6	2
-25O60LNoT	5.2	3.4	2	1	4.9	2.6	3.4	1.8
-25O60L03T	6.5	0.7	3.6	2.6	7.7	1.8	5.1	0.9
FBS 0T to FBS								
5O10LNoT	12.8	3.8	16.6	1.9	14.3	2.5	16.7	1.3
5O10L03T	22.3	0.9	26.7	2	20.7	1.9	21.9	0.3
5O60LNoT	20.4	3.2	24.5	2	31.3	1.5	26.1	1.4
5O60L03T	11.9	2.5	13.6	2.9	13.8	4.3	13.3	1
10O10NoT	10.6	1.7	14	1.5	12.2	0.9	11.9	2.4
10O1003T	7.5	1.1	9	2.3	7.1	2.3	7.1	2.3
10O60LNoT	6.1	2.2	7.2	0.7	4.4	3.1	4.4	2.2
10O60L03T	8.6	0.6	7.8	2.2	8.3	1.1	8.6	1.4
-25O10LNoT	9.5	0.6	7.4	0.6	3.5	2.1	7.6	2.3
-25O10L03T	7.8	2.1	9.2	1.2	7.7	3	9.9	2.1
-25O60LNoT	6.7	1.3	5.2	3	5.8	3.2	4.6	0.6
-25O60L03T	5.7	1.5	7.5	1.8	8.1	2.2	6.6	1.3
Cytomix 0T to Cytomix								
5O10LNoT	0	0	0	0	0	0	0	0
5O10L03T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5O60LNoT	3.8	1.2	5.3	1.2	8.3	1.1	1.1	0.9
5O60L03T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10O10NoT	2.9	1.1	1.8	1.6	2.9	1.2	1.9	1.1
10O1003T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10O60LNoT	2.7	1.6	2.5	1.2	1.6	1.5	1.9	1.1
10O60L03T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-25O10LNoT	4.1	1.6	2.0	1.6	6.7	1.5	4.0	1.6
-25O10L03T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-25O60LNoT	9.3	1.5	7.7	1.5	12.1	1.8	7.6	1.8
-25O60L03T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Continued

Continued Appendix 6.2E (Fig. 6.10). Cells surviving (TB-) snap freezing in solution 2 in relation to the equilibration protocols, and the cryoprotectant type and concentration in solution 1 without trehalose (Cytomix or FBS).

Treatments	20% DMSO		10% D+10% EG		10% DMSO		10% EG	
FBS 0T to Cytomix								
5O10LNoT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5O10L03T	4.2	1.2	4.6	1.1	4.5	1.2	5.4	1.2
5O60LNoT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5O60L03T	0.8	0.7	1.9	1.2	1.8	1.1	2.7	1.2
10O10NoT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10O1003T	2.9	1.0	3.9	1.6	1.8	1.1	3.3	1.2
10O60LNoT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10O60L03T	3.5	1.2	4.8	1.4	2.1	1.2	4.0	1.3
-25O10LNoT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-25O10L03T	7.8	1.3	9.2	1.2	7.7	2.1	9.9	2.1
-25O60LNoT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-25O60L03T	1.9	1.2	3.0	1.5	3.5	1.0	4.5	1.2

Appendix 6.2F (Fig. 6.11). Cells surviving (TB-) snap freezing in solution 2 in relation to the equilibration protocols, and the cryoprotectant type and concentration in solution 1 containing trehalose (Cytomix or FBS).

Treatments	20% DMSO		10% D+10% EG		10% DMSO		10% EG	
Cytomix 0.6 T to FBS								
	%	SEM	%	SEM	%	SEM	%	SEM
5010LNoT	7.8	2.9	5.4	1.1	5.3	1.2	7.4	2.9
5O10L03T	17.5	3.9	13.4	2.7	19.3	4.3	22.8	4.9
5O60LNoT	8.8	1.2	6.8	1.9	9	2.6	11	1.4
5O60L03T	11.9	0.8	9.2	2.9	11.1	2.1	8.2	2.9
10O10NoT	5.1	2	7.1	0.9	7.1	2.5	3.5	2
10O1003T	3.1	1.7	7.5	0.6	6	1	5.4	1.7
10O60LNoT	2.3	1.2	6.6	1.1	3.7	1.6	7.5	1.2
10O60L03T	6.6	0.8	7.6	0.9	6.7	2.5	2.8	0.2
-25O10LNoT	3.4	1.8	8.7	0.5	4	3.6	7.3	1.7
-25O10L03T	8.4	2.1	6.5	3.3	10.8	3.6	7.6	1.4
-25O60LNoT	4.3	2.6	2.6	0.4	3.6	2.3	3.4	1.2
-25O60L03T	5.6	1.1	4.5	2.6	6.4	2.1	5.1	0.3
FBS 0.6 T to FBS								
5010LNoT	14.4	3.5	18.2	2.3	13.1	2.2	17.5	1.7
5O10L03T	21.1	1.3	28.3	2.4	19.5	1.6	22.7	0.7
5O60LNoT	19.2	3.6	26.1	2.4	30.1	1.2	26.9	1.8
5O60L03T	10.7	2.9	15.2	3.3	12.6	4	14.1	1.4
10O10NoT	9.4	2.1	15.6	1.9	11	0.6	12.7	2.8
10O1003T	6.3	1.5	9	2.7	7.9	2.7	7.1	2.7
10O60LNoT	4.9	2.6	7.2	1.1	5.2	3.5	4.4	2.6
10O60L03T	7.4	1	6.6	2.6	9.1	1.5	10.2	1.8
-25O10LNoT	10.3	0.3	6.2	1	4.3	2.5	9.2	2.7
-25O10L03T	8.6	1.8	8	1.6	8.5	3.4	11.5	2.5
-25O60LNoT	7.5	1	4	3.4	6.6	3.6	6.2	1
-25O60L03T	6.5	1.2	6.3	2.2	8.9	2.6	8.2	1.7
Cytomix 0.6 T to Cytomix								
5010LNoT	0	0	0	0	0	0	0	0
5O10L03T	3	1	2.1	1.1	5.2	1.2	2.2	0.1
5O60LNoT	0	0	0	0	0	0	0	0
5O60L03T	2.7	0.9	2.1	0.9	2.1	1.5	2.2	0.3
10O10NoT	0	0	0	0	0	0	0	0
10O1003T	2.5	1.4	2.2	0.9	0.8	1.9	2.2	0.3
10O60LNoT	0	0	0	0	0	0	0	0
10O60L03T	2	1	3.2	1.4	5.9	2.1	5.1	1.6
-25O10LNoT	0	0	0	0	0	0	0	0
-25O10L03T	6.2	0.9	7.7	1.3	11.3	2.6	7.4	1.8
-25O60LNoT	0	0	0	0	0	0	0	0
-25O60L03T	3.3	0.9	1.2	0.6	4.6	1.9	4.9	1.5

Continued

Continued appendix 6.2F (Fig. 6.11). Cells surviving (TB-) snap freezing in solution 2 in relation to the equilibration protocols, and the cryoprotectant type and concentration in solution 1 containing trehalose (Cytomix or FBS).

Treatments	20% DMSO		10% D+10% EG		10% DMSO		10% EG	
FBS 0.6 T to Cytomix								
5O10LNoT	0	0	0	0	0	0	0	0
5O10L03T	4.8	1	4.9	0.8	4.2	0.9	5	0.9
5O60LNoT	0	0	0	0	0	0	0	0
5O60L03T	1.4	0.5	2.2	0.9	1.5	0.8	2.3	0.9
10O10NoT	0	0	0	0	0	0	0	0
10O1003T	3.5	0.8	4.2	1.3	1.5	0.8	2.9	0.9
10O60LNoT	0	0	0	0	0	0	0	0
10O60L03T	4.1	1	5.1	1.1	1.8	0.9	3.6	1
-25O10LNoT	0	0	0	0	0	0	0	0
-25O10L03T	8.4	1.1	9.5	0.9	7.4	1.8	9.5	1.8
-25O60LNoT	0	0	0	0	0	0	0	0
-25O60L03T	2.5	1	3.3	1.2	3.2	0.7	4.1	0.9

Appendix 6.2G (Fig. 6.12). The number of plated cells observed in 96-well TC-dish after incubation in solution 1 and 2 then snap frozen.

Cryorotectants	20% DMSO		10% D+10% EG		10% DMSO		10% EG	
Cytomix 0T to FBS								
Treatments	%	SEM	%	SEM	%	SEM	%	SEM
5010LNoT	4.7	0.9	4.0	1.5	4.7	0.7	4.7	1.2
5O10L03T	6.3	0.9	6.3	0.7	6.3	1.2	6.0	1.2
5O60LNoT	4.3	1.9	5.7	1.5	5.7	0.9	6.7	1.9
5O60L03T	8.0	1.5	8.3	1.2	6.0	1.5	5.7	1.8
10O10NoT	6.3	0.9	8.7	3.2	6.3	1.3	6.0	1.2
10O1003T	6.3	0.9	7.7	2.2	6.3	0.9	7.0	0.6
10O60LNoT	7.3	0.7	5.0	2.1	7.3	1.5	7.0	1.0
10O60L03T	10.7	2.4	8.3	0.9	9.3	2.3	7.3	1.2
-25O10LNoT	5.7	0.9	8.0	2.1	5.0	1.5	6.0	0.6
-25O10L03T	8.3	2.0	10.7	2.4	8.7	2.0	10.3	2.2
-25O60LNoT	6.3	0.7	7.0	2.6	5.0	2.1	6.7	1.5
-25O60L03T	9.0	1.2	8.3	0.3	12.7	3.5	8.3	1.2
FBS 0 T to FBS								
5010LNoT	11.0	1.2	11.0	2.5	11.7	1.5	10.3	3.2
5O10L03T	9.7	1.2	8.3	0.3	9.0	0.6	11.3	1.2
5O60LNoT	7.7	0.9	9.3	2.2	7.3	0.9	8.3	0.3
5O60L03T	9.0	1.2	10.0	1.5	9.0	2.1	10.3	2.7
10O10NoT	13.7	2.8	12.0	2.5	14.0	3.6	8.7	1.5
10O1003T	10.3	1.2	9.0	1.7	7.3	0.7	7.7	0.9
10O60LNoT	7.0	0.6	7.7	0.3	8.7	0.3	7.7	0.3
10O60L03T	9.3	2.4	8.3	0.9	10.0	1.0	10.7	1.7
-25O10LNoT	7.0	1.0	7.7	1.7	8.0	2.3	11.3	1.7
-25O10L03T	12.7	1.2	10.7	0.9	11.3	1.3	8.3	2.3
-25O60LNoT	11.3	1.7	9.7	1.8	10.0	1.2	8.0	1.2
-25O60L03T	11.0	1.5	9.7	1.2	9.0	0.6	11.0	1.5
Cytomix 0.6 T to FBS								
5010LNoT	18.0	4.0	15.7	0.7	14.0	2.3	15.3	0.9
5O10L03T	17.3	2.4	21.3	3.8	22.0	0.6	19.7	1.2
5O60LNoT	12.0	0.6	11.7	0.9	13.3	0.9	12.3	2.0
5O60L03T	11.0	1.0	11.0	1.5	12.0	2.0	12.0	2.1
10O10NoT	13.0	1.5	11.0	2.1	10.3	1.2	10.7	1.3
10O1003T	12.0	3.0	10.7	0.9	8.3	0.9	7.3	0.9
10O60LNoT	7.7	0.9	7.0	0.6	7.0	1.5	6.3	1.5
10O60L03T	10.7	1.2	11.7	2.0	9.7	1.2	10.3	0.7
-25O10LNoT	12.3	1.5	8.0	0.6	4.0	1.2	8.7	1.8
-25O10L03T	9.0	0.6	10.7	0.9	10.0	1.2	12.0	1.2
-25O60LNoT	8.0	0.6	5.3	1.5	7.0	1.0	7.3	1.2
-25O60L03T	7.0	0.6	9.3	0.9	8.3	1.5	8.7	0.9

Continued

Continued appendix 6.2G (Fig. 6.12). The number of plated cells observed in 96-well TC-dish after incubation in solution 1 and 2 then snap frozen.

Treatments	20% DMSO		10% D+10% EG		10% DMSO		10% EG	
FBS 0.6 T to FBS								
5O10LNoT	12.3	2.6	11.3	0.9	13.7	0.9	13.7	1.5
5O10L03T	13.0	3.0	12.0	0.6	13.0	2.6	14.3	0.9
5O60LNoT	7.7	0.9	7.0	1.0	6.3	1.2	9.7	0.9
5O60L03T	8.7	1.8	10.3	0.3	8.7	0.7	6.7	1.8
10O10NoT	5.0	0.6	5.0	0.6	6.7	0.7	6.7	0.9
10O1003T	7.0	1.5	10.3	0.9	12.3	0.3	10.7	1.7
10O60LNoT	4.7	0.9	5.0	0.6	7.0	1.0	8.7	0.3
10O60L03T	8.0	0.6	7.0	0.6	7.0	0.6	6.0	0.6
-25O10LNoT	5.0	0.6	8.0	0.6	5.3	0.3	7.0	0.6
-25O10L03T	9.3	0.3	9.7	1.2	12.3	0.9	9.7	0.9
-25O60LNoT	6.3	1.3	4.0	1.2	5.3	1.5	4.0	0.6
-25O60L03T	7.0	0.6	6.3	1.2	7.3	1.2	6.3	0.7

Appendix 6.3A. (Fig. 6.13) Viability and permeability of non-frozen, diluted cells.

	Viable			Dead			Viable permeable		
Non Frozen	N*)	SEM	%	N	SEM	%	N	SEM	%
Control**)	46.3	3.0	97.2	1.3	0.3	2.8	0.0	0.0	0.0
DF	24.3	4.4	94.8	1.3	0.3	5.2	1.0	0.0	3.9
DFT	26.7	2.7	95.2	1.3	0.3	4.8	0.3	0.3	1.2
DFTA1	30.7	4.3	92.9	2.3	0.3	7.1	1.0	0.0	3.0
DFTA5	25.3	4.7	91.6	2.3	0.3	8.4	1.7	0.3	6.0
DFTG10	26.3	2.8	96.3	1.0	0.6	3.7	0.3	0.3	1.2
DFTEG	27.3	5.0	98.8	0.3	0.3	1.2	1.0	0.6	3.6

*) Average from 3 replicates

**) Non-diluted

Appendix 6.3B (Fig. 6.14). Cell characteristics after conventional slow cooling and different thawing methods.

Freezing solution	Viable (TB-)			Dead (TB+)			Viable permeable (TB-; PI+)		
	N	SEM	%	N	SEM	%	N	SEM	%
Thawing method: 37°C 5 min[a)]									
DF	22.0	1.5	95.7	1.0	0.6	4.3	18.0	1.5	78.3
DFT	27.0	3.1	94.2	1.7	0.3	5.8	20.3	4.3	70.9
DFTA1	23.7	1.3	83.5	4.7	0.3	16.5	13.7	2.3	48.2
DFTA5	15.0	1.7	59.2	10.3	0.9	40.8	11.3	2.4	44.7
DFTG10	26.7	2.2	93.0	2.0	0.0	7.0	20.3	1.5	70.9
DFTEG	24.7	1.5	92.5	2.0	0.6	7.5	18.3	0.7	68.7
Thawing method: RT 15 min[a)]									
DF	21.7	0.7	89.0	2.7	0.3	11.0	18.0	2.3	74.0
DFT	23.0	0.6	86.2	3.7	0.7	13.8	16.3	1.2	61.2
DFTA1	22.0	3.1	84.6	4.0	1.2	15.4	14.7	1.5	56.4
DFTA5	15.0	2.6	55.6	12.0	1.7	44.4	11.7	2.3	43.2
DFTG10	20.3	0.7	85.9	3.3	0.7	14.1	19.0	0.6	80.3
DFTEG	22.3	1.7	87.0	3.3	0.7	13.0	17.0	1.2	66.2
Thawing method: 0°C 30 min[b)]									
DF	17.3	1.9	63.4	10.0	0.6	36.6	13.3	1.9	48.8
DFT	19.7	1.2	67.8	9.3	0.3	32.2	16.3	0.3	56.3
DFTA1	17.3	1.8	57.1	13.0	0.6	42.9	11.7	2.6	38.5
DFTA5	13.7	1.9	47.7	15.0	0.6	52.3	10.3	2.2	36.0
DFTG10	18.7	1.5	59.6	12.7	0.7	40.4	12.3	0.9	39.4
DFTEG	18.7	1.9	61.5	11.7	0.3	38.5	12.7	0.7	41.8

Different subscripts indicate a significant difference ($P<0.01$)

Appendix 6.3C (Fig. 6.15). Cell plating by control non-frozen cells as compared to conventionally slow cooled cells thawed by each of three different thawing protocols.

Treatments	Non Frozen[a]		Thawed at 37°C[a]		Thawed at RT[b]		Thawed at 0°C[c]	
	N	SEM	N	SEM	N	SEM	N	SEM
DF	21.7	0.3	20.0	0.6	15.0	1.5	12.7	0.7
DFT	22.3	1.9	21.3	1.2	14.7	0.9	12.7	0.7
DFTA1	20.3	0.9	20.3	1.3	14.0	1.2	13.0	0.6
DFTA5	19.3	2.0	12.3	1.5	12.0	1.2	10.7	0.9
DFTG10	18.7	1.8	16.7	1.2	14.7	0.3	11.3	0.9
DFTEG	18.7	2.6	17.3	0.7	13.7	1.2	10.7	0.9

5.1.1.1 Different superscripts are significantly different (P<0.01)

Appendix 6.3D (Fig. 6.16). Viability and permeability of cells that were re-frozen (snap frozen) in Cytomix containing 0.3M Trehalose after an initial slow freeze showing an effect of the first thawing procedure.

Solution	Viable (TB-)			Dead (TB+)			Viable permeable (TB-; PI+)		
	N	SEM	%	N	SEM	%	N	SEM	%
Thawing method: 37°C 5 min[a]									
DF	11.3	1.8	35.8	20.3	0.9	64.2	11.3	1.8	35.8
DFT	12.3	1.2	37.8	20.3	0.7	62.2	12.3	1.2	37.8
DFTA1	7.0	1.5	23.9	22.3	1.8	76.1	7.0	1.5	23.9
DFTA5	4.7	1.8	15.6	25.3	2.3	84.4	4.7	1.8	15.6
DFTG10	0.0	0.0	0.0	25.7	2.7	100.0	0.0	0.0	0.0
DFTEG	5.7	1.2	19.8	23.0	1.2	80.2	5.7	1.2	19.8
Thawing method: RT 5 min[a]									
DF	9.3	0.3	29.2	22.7	0.3	70.8	9.3	0.3	29.2
DFT	13.0	0.6	38.6	20.7	0.3	61.4	13.0	0.6	38.6
DFTA1	6.7	0.7	22.7	22.7	2.0	77.3	6.7	0.7	22.7
DFTA5	3.3	1.8	12.3	23.7	3.0	87.7	3.3	1.8	12.3
DFTG10	0.7	0.3	2.5	26.0	3.1	97.5	0.7	0.3	2.5
DFTEG	4.7	0.7	16.7	23.3	0.7	83.3	4.7	0.7	16.7
Thawing method: 0°C 30 min[b]									
DF	6.7	1.2	20.8	25.3	2.3	79.2	6.7	1.2	20.8
DFT	8.0	1.2	28.2	20.3	1.3	71.8	8.0	1.2	28.2
DFTA1	5.0	0.6	17.4	23.7	0.3	82.6	5.0	0.6	17.4
DFTA5	1.7	0.9	6.3	24.7	0.9	93.7	1.7	0.9	6.3
DFTG10	0.3	0.3	1.3	25.3	0.9	98.7	0.3	0.3	1.3
DFTEG	2.3	1.3	8.3	25.7	2.0	91.7	2.3	1.3	8.3

Different superscripts are significantly different ($P<0.01$)

Appendix 6.3E (Fig. 6.17). Cells re-frozen using slow cooling in Cytomix containing 0.3M Trehalose in relation to the initial thawing protocol.

	Viable (TB-)			Dead (TB+)			Viable and permeable (TB-; PI+)		
	N	SEM	%	N	SEM	%	N	SEM	%
Thawing method: 37°C 5 min[a]									
DF	8.7	0.3	27.4	23.0	1.5	72.6	8.7	0.3	27.4
DFT	9.3	0.9	30.4	21.3	0.3	69.6	9.3	0.9	30.4
DFTA1	5.0	1.7	16.9	24.7	0.3	83.1	5.0	1.7	16.9
DFTA5	2.7	0.7	10.1	23.7	0.7	89.9	2.7	0.7	10.1
DFTG10	1.0	0.6	3.8	25.3	0.7	96.2	1.0	0.6	3.8
DFTEG	3.7	0.9	12.5	25.7	0.9	87.5	3.7	0.9	12.5
Thawing method: RT 5 min[a]									
DF	7.3	1.9	24.2	23.0	1.7	75.8	7.3	1.9	24.2
DFT	8.0	1.2	26.1	22.7	0.9	73.9	8.0	1.2	26.1
DFTA1	4.3	0.3	15.1	24.3	0.7	84.9	4.3	0.3	15.1
DFTA5	2.0	1.2	8.3	22.0	2.1	91.7	2.0	1.2	8.3
DFTG10	0.7	0.7	2.9	22.0	1.5	97.1	0.7	0.7	2.9
DFTEG	3.0	0.6	11.2	23.7	1.5	88.8	3.0	0.6	11.2
Thawing method: 0°C 30 min[a]									
DF	7.0	1.0	23.9	22.3	1.3	76.1	7.0	1.0	23.9
DFT	9.0	0.6	27.6	23.7	2.3	72.4	9.0	0.6	27.6
DFTA1	4.7	0.9	15.7	25.0	1.7	84.3	4.7	0.9	15.7
DFTA5	3.0	0.6	10.3	26.0	0.0	89.7	3.0	0.6	10.3
DFTG10	0.7	0.3	2.7	24.0	1.5	97.3	0.7	0.3	2.7
DFTEG	2.7	0.7	10.8	22.0	1.0	89.2	2.7	0.7	10.8

Different superscript is significant different ($P<0.01$)

Appendix 6.3F (Fig. 6.18). Plating by cells conventionally frozen cells after re-freezing in Cytomix containing 0.3M Trehalose, showing an effect of the thawing protocol used after the first freeze.

From cells	Thawed at 37°C		Thawed at RT		Thawed at 0°C	
Snap frozen	N	SEM	N	SEM	N	SEM
DF	7.0	1.5	3.7	0.7	1.0	0.6
DFT	9.3	0.9	4.7	0.7	1.7	0.3
DFTA1	11.0	0.6	1.7	0.3	1.0	0.6
DFTA5	1.7	0.9	1.0	0.6	0.0	0.0
DFTG10	0.0	0.0	0.0	0.0	0.0	0.0
DFTEG	5.7	0.9	1.0	0.6	0.0	0.0
Slow cooled						
DF	6.0	1.5	5.0	0.6	2.3	0.3
DFT	8.0	1.2	5.3	0.3	2.3	0.3
DFTA1	1.7	0.9	2.3	1.2	2.0	0.6
DFTA5	2.7	0.7	0.7	0.7	0.0	0.0
DFTG10	0.0	0.0	0.0	0.0	0.0	0.0
DFTEG	1.0	0.6	0.7	0.3	0.3	0.3

Appendix 6.4A (Fig. 6.20). Number and proportion of live, dead and viable permeable cells after slow cooling at 1°C/min.

	N	SEM	%	N	SEM	%	N	SEM	%	N	SEM	%
RT	49.7	4.5	99.4	0.3	0.3	0.6	0.3	0.3	0.6	0.0	0.0	0.0
0°C	62.0	8.5	91.5	5.3	1.5	8.5	5.3	1.5	8.5	0.0	0.0	0.0
-10°C	49.3	6.8	86.1	7.7	0.9	13.9	9.0	1.7	16.1	1.3	0.9	2.3
-20°C	44.3	5.0	86.1	7.0	1.0	13.9	19.7	2.7	40.0	1.7	0.9	26.1
-30°C	54.7	7.8	89.0	6.7	0.7	11.0	26.0	1.2	44.5	6.0	1.2	33.5
-40°C	52.0	6.1	89.6	5.7	1.8	10.4	23.7	1.3	41.5	5.3	1.9	31.1
-50°C	51.0	3.8	86.1	8.3	1.9	13.9	24.7	0.3	42.0	5.3	1.9	28.1
-60°C	58.3	7.5	90.2	6.3	0.9	9.8	25.7	2.0	40.2	10.7	1.9	30.5
-70°C	44.7	5.9	87.2	6.7	1.3	12.8	26.3	0.9	53.4	9.3	1.5	40.5
-80°C	55.0	4.0	89.5	6.3	0.9	10.5	26.7	1.2	43.9	10.3	0.7	33.3
LN	47.7	4.3	88.8	6.0	1.2	11.2	27.7	2.2	52.9	10.0	1.7	41.7

Appendix 6.4B (Fig. 6.21). Effect of the temperature to which cells were cooled and the trehalose concentration on the proportion of viable, dead and viable permeable cells after cooling in Cytomix.

	Live			Dead			Viable permeable		
Temp.	N	SEM	%	N	SEM	%	N	SEM	%
0 M T									
RT	29.0	4.7	90.7	3.0	0.6	9.3	0.0	0.0	0.0
0°C	27.7	1.5	86.5	4.3	0.9	13.5	0.3	0.3	1.0
-10°C	35.0	7.0	88.9	4.0	0.6	11.1	1.7	0.3	4.2
-20°C	35.0	5.7	80.1	8.3	0.3	19.9	5.0	1.0	11.6
-30°C	36.0	6.7	83.6	7.7	2.6	16.4	7.7	0.9	20.2
-40°C	34.0	2.5	82.5	7.3	1.2	17.5	8.7	1.8	21.9
-50°C	34.3	1.7	82.3	7.3	0.7	17.7	8.3	1.3	20.0
-60°C	42.7	8.2	83.6	7.7	3.5	16.5	11.3	3.2	22.6
-70°C	32.7	4.6	80.7	8.0	2.0	19.3	9.0	2.3	24.2
-80°C	35.3	4.3	83.6	6.7	0.7	16.4	10.0	1.0	24.2
LN	35.3	8.7	83.8	6.3	0.9	16.2	10.0	0.6	26.8
0.3 M T									
RT	29.3	3.2	2.2	0.7	0.7	1.2	0.0	0.0	0.0
0°C	33.3	1.3	0.7	2.7	1.5	0.6	0.0	0.0	0.0
-10°C	32.7	5.0	5.2	4.3	1.2	1.0	1.7	1.7	6.2
-20°C	32.0	3.0	3.4	8.7	1.5	0.9	5.7	0.9	14.4
-30°C	35.0	3.5	2.0	10.3	0.9	0.3	8.7	0.3	19.5
-40°C	35.7	2.7	5.2	7.0	0.6	0.9	9.0	0.6	21.1
-50°C	31.7	1.8	1.8	7.3	0.9	0.9	10.0	2.3	26.5
-60°C	37.7	2.3	2.8	8.3	1.2	0.6	11.3	1.2	25.2
-70°C	33.3	2.6	1.7	9.0	0.6	1.2	8.0	0.0	19.0
-80°C	32.7	4.4	2.9	4.7	1.8	1.9	13.0	1.5	35.3
LN	30.3	3.5	2.4	4.3	0.9	1.2	12.7	1.3	36.6

Continued.

Continued Appendix 6.4B. Effect of the temperature to which cells were cooled and the trehalose concentration on the proportion of viable, dead and viable permeable cells after cooling in Cytomix.

0.5 M T									
RT	30.7	2.2	88.3	4.0	1.2	11.7	0.0	0.0	0.0
0°C	32.7	0.7	89.1	4.0	0.6	10.9	0.0	0.0	0.0
-10°C	31.7	5.2	88.9	4.0	1.0	11.1	2.3	1.3	7.9
-20°C	28.3	3.4	74.8	9.3	0.9	25.2	9.7	3.5	26.9
-30°C	37.0	2.0	79.8	9.3	0.3	20.2	9.3	0.9	20.1
-40°C	32.7	5.2	82.1	6.7	0.9	17.9	10.0	1.7	25.1
-50°C	28.3	1.8	78.6	7.7	0.9	21.4	9.3	1.2	25.8
-60°C	36.3	2.8	80.1	9.0	0.6	19.9	11.3	0.3	25.3
-70°C	34.7	1.7	77.7	10.0	1.2	22.3	7.7	0.9	17.5
-80°C	31.0	2.9	85.2	5.3	1.9	14.8	12.3	2.7	34.0
LN	33.7	2.4	85.2	6.0	1.2	14.8	11.7	2.0	30.8
1.0 M T									
RT	20.3	0.7	93.9	1.3	0.7	6.1	0.0	0.0	0.0
0°C	22.7	1.2	83.0	4.7	1.3	17.0	0.0	0.0	0.0
-10°C	21.7	3.8	85.1	3.7	0.3	14.9	3.0	1.0	13.6
-20°C	20.3	1.9	81.0	4.7	0.3	19.0	13.7	4.3	56.7
-30°C	21.7	2.8	78.1	6.0	0.6	21.9	9.0	3.1	30.5
-40°C	19.3	2.4	81.3	4.3	0.3	18.7	11.3	1.3	47.8
-50°C	25.7	2.9	81.6	5.7	0.3	18.4	10.7	1.5	33.8
-60°C	21.3	1.5	74.4	7.3	0.9	25.6	12.0	1.5	42.4
-70°C	22.7	1.3	77.4	6.7	1.3	22.6	11.0	1.0	37.8
-80°C	20.3	1.3	78.3	5.7	0.9	21.7	10.0	1.2	38.5
LN	24.0	1.5	79.9	6.0	0.0	20.1	10.7	1.8	35.2

Appendix 6.4C (Fig. 6.22). Effect of the temperature to which cells were cooled and the trehalose concentration on the proportion of viable, dead and viable permeable cells after cooling in FBS

	Live			Dead			Viable permeable		
Temp.	N	SEM	%	N	SEM	%	N	SEM	%
O M T									
RT	26.3	2.7	98.5	0.3	0.3	1.5	0.0	0.0	0.0
0°C	28.3	2.4	95.6	1.3	0.7	4.4	0.3	0.3	1.2
-10°C	25.0	0.6	89.3	3.0	0.6	10.7	2.7	0.3	9.5
-20°C	33.3	3.7	83.2	6.7	0.7	16.8	9.7	1.9	25.6
-30°C	35.0	3.0	85.1	6.0	1.2	14.9	8.3	2.2	20.3
-40°C	37.0	3.2	80.1	9.3	2.0	19.9	7.0	1.5	15.9
-50°C	39.3	3.8	83.8	7.7	1.3	16.2	9.3	3.4	21.5
-60°C	29.7	0.7	80.6	7.3	1.8	19.4	11.7	1.5	31.5
-70°C	34.3	2.9	79.0	9.0	0.6	21.0	8.3	2.8	20.2
-80°C	35.3	3.8	78.9	9.3	0.9	21.1	7.7	0.9	17.1
LN	34.3	4.1	79.6	8.7	0.3	20.4	7.7	2.2	18.3
0.3 M T									
RT	33.7	3.0	96.4	1.3	0.9	3.6	0.0	0.0	0.0
0°C	28.3	2.8	94.0	2.0	1.2	6.0	0.0	0.0	0.0
-10°C	34.3	1.8	87.3	5.0	0.6	12.7	2.3	0.9	5.9
-20°C	37.7	2.2	82.8	8.0	1.7	17.2	7.3	1.2	16.6
-30°C	32.3	1.7	76.4	10.0	1.5	23.6	9.0	2.5	21.3
-40°C	32.7	2.4	82.4	7.0	1.0	17.6	11.7	1.2	30.2
-50°C	34.7	4.5	84.0	6.3	1.2	16.0	12.0	1.2	29.9
-60°C	32.0	1.7	81.3	7.3	1.2	18.7	11.7	2.3	29.5
-70°C	35.0	4.0	83.5	6.7	1.8	16.6	10.7	1.8	25.3
-80°C	32.0	2.6	80.5	7.7	0.9	19.5	12.7	2.8	32.5
LN	33.7	2.0	80.9	8.0	1.5	19.1	10.7	3.0	26.6

Continued.

Continued Appendix 6.4C. Effect of the temperature to which cells were cooled and the trehalose concentration on the proportion of viable, dead and viable permeable cells after cooling in FBS

	Live			Dead			Viable permeable		
Temp.	N	SEM	%	N	SEM	%	N	SEM	%
0.5 M T									
RT	31.0	2.6	96.1	1.3	0.9	3.9	0.0	0.0	0.0
0°C	37.3	4.1	94.8	2.0	0.0	5.2	0.0	0.0	0.0
-10°C	34.7	0.3	88.3	4.7	1.3	11.7	4.0	0.6	10.3
-20°C	31.0	3.6	78.6	8.3	0.3	21.4	10.7	2.0	27.0
-30°C	33.0	6.7	79.7	8.0	1.0	20.3	13.7	1.5	34.8
-40°C	32.0	2.1	77.4	9.3	1.5	22.6	8.3	0.7	20.3
-50°C	32.7	2.7	83.5	6.7	1.8	16.5	11.3	1.3	29.8
-60°C	33.0	5.2	81.9	7.0	1.0	18.1	14.7	2.3	39.3
-70°C	35.7	3.5	80.7	8.3	1.2	19.3	10.0	2.6	22.2
-80°C	36.0	5.3	86.1	6.0	1.5	13.9	12.0	2.1	29.7
LN	38.3	1.5	87.9	5.3	0.7	12.1	14.7	0.9	33.6
1.0 M T									
RT	22.3	3.2	91.5	2.0	0.6	8.5	0.0	0.0	0.0
0°C	22.7	4.2	94.4	1.3	0.9	5.6	0.0	0.0	0.0
-10°C	25.0	2.1	86.2	4.0	1.0	13.8	4.7	0.9	16.0
-20°C	26.0	1.5	85.7	4.3	0.9	14.3	12.7	2.3	41.5
-30°C	27.0	4.5	80.4	6.3	0.9	19.6	12.0	1.5	36.3
-40°C	25.0	3.2	80.5	6.0	1.2	19.5	12.7	2.6	43.2
-50°C	26.3	0.7	85.9	4.3	0.9	14.1	15.3	1.9	50.1
-60°C	24.0	4.6	78.9	6.0	1.2	21.1	13.7	2.3	45.2
-70°C	27.7	2.0	80.6	6.7	0.7	19.4	16.7	0.9	48.7
-80°C	26.7	1.9	84.1	5.0	1.2	15.9	13.3	0.9	42.2
LN	23.7	2.0	82.1	5.3	1.3	17.9	11.0	0.0	38.9

Appendix 6.4D (Fig. 6.23). Number of viable and dead cells after re-freezing (snap freezing) in Cytomix with or without trehalose

Trehalose	O M Trehalose				O .3 M Trehalose				O .5 M Trehalose				1.O M Trehalose			
	Live		Dead		Live		Dead		Live		Dead		Live		Dead	
Temp	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM
RT	0.0	0.0	26.7	3.7	0.0	0.0	27.3	1.9	0.0	0.0	26.3	2.6	0.0	0.0	24.0	5.3
0°C	0.0	0.0	28.7	3.8	0.0	0.0	24.0	0.6	0.0	0.0	23.0	1.2	0.0	0.0	28.7	3.8
-10°C	0.0	0.0	30.3	4.7	0.0	0.0	26.0	0.0	0.0	0.0	29.0	3.0	0.0	0.0	33.0	2.0
-20°C	0.0	0.0	23.0	3.6	2.0	0.6	26.0	2.9	1.0	0.6	25.7	2.9	1.3	0.9	24.7	2.0
-30°C	0.0	0.0	22.0	5.5	1.7	0.3	28.0	6.7	1.7	0.9	23.7	4.7	1.7	0.9	28.7	2.8
-40°C	0.0	0.0	28.7	4.7	2.3	1.2	24.3	4.4	2.0	0.0	26.7	6.4	1.7	0.3	32.3	3.8
-50°C	0.0	0.0	26.7	2.7	2.0	0.0	22.7	1.3	1.0	0.0	24.0	4.6	2.0	0.0	25.3	3.4
-60°C	0.0	0.0	24.0	5.1	4.3	0.7	28.3	2.3	2.7	0.3	28.7	2.2	1.0	0.6	23.7	4.9
-70°C	0.0	0.0	22.7	3.3	2.7	0.3	21.3	4.5	1.7	0.9	25.0	3.2	2.0	0.6	30.0	4.0
-80°C	0.0	0.0	24.7	0.7	3.0	0.6	23.0	5.6	1.7	0.3	25.3	5.2	1.3	0.7	22.0	3.1
LN	0.0	0.0	31.7	2.0	2.7	0.7	17.3	1.7	2.0	1.0	20.7	3.8	1.7	0.9	27.0	4.9

Appendix 6.4E (Fig. 6.24). Number of viable and dead cells after re-freezing (snap freezing) cells in FBS with or without trehalose

Trehalose	O M Trehalose				O .3 M Trehalose				O .5 M Trehalose				1.O M Trehalose			
	Live		Dead		Live		Dead		Live		Dead		Live		Dead	
Temp	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM	N	SEM
RT	6.0	1.0	22.3	3.2	3.3	0.9	24.0	4.6	3.3	0.9	24.0	4.6	1.3	0.3	28.7	4.5
0°C	5.0	1.0	26.0	3.2	4.7	0.9	28.3	3.5	4.7	0.9	28.3	3.5	2.3	0.3	34.3	5.5
-10°C	5.0	1.5	26.7	5.3	3.7	1.5	32.3	1.5	3.7	1.5	32.3	1.5	3.0	0.6	28.3	6.8
-20°C	4.0	0.6	20.7	2.6	5.0	0.6	29.3	1.8	5.0	0.6	29.3	1.8	2.0	0.0	23.3	5.0
-30°C	4.3	1.2	23.0	2.1	5.3	1.2	28.7	2.7	5.3	1.2	28.7	2.7	2.3	0.7	30.3	5.0
-40°C	5.0	1.0	23.7	5.0	5.7	1.2	30.0	3.1	5.7	1.2	30.0	3.1	2.7	0.7	31.0	6.1
-50°C	4.7	0.3	26.3	1.9	3.3	0.9	28.0	3.2	3.3	0.9	28.0	3.2	3.0	0.0	30.0	3.8
-60°C	3.7	1.2	24.7	5.0	4.0	1.2	24.7	5.8	4.0	1.2	24.7	5.8	3.3	0.9	32.3	4.3
-70°C	3.3	1.8	20.0	2.6	4.3	1.2	22.7	4.4	4.3	1.2	22.7	4.4	2.7	0.7	23.7	5.9
-80°C	3.3	0.3	22.7	2.7	4.3	0.7	26.0	2.9	4.3	0.7	26.0	2.9	2.7	0.3	34.0	4.0
LN	4.7	0.9	28.7	2.3	3.3	0.7	29.0	3.5	3.3	0.7	29.0	3.5	2.3	0.9	26.7	4.3

Appendix 6.4F (Fig 6.25). Number of cells plating from cells slow cooled in 10% DMSO in FBS then diluted in FBS containing 0, 0.3, 0.5 or 1 M trehalose and then re- frozen by snap freezing.

	0M Trehalose		0.3 M Trehalose		0.5 M Trehalose		1.0 M Trehalose	
Temp	N	SEM	N	SEM	N	SEM	N	SEM
RT	6.33	1.67	4.67	1.76	5.33	0.33	4.67	2.19
0°C	6.67	1.76	5.00	1.00	6.00	0.58	4.67	0.88
-10°C	6.00	0.00	5.33	1.45	6.67	1.45	4.00	1.73
-20°C	7.67	0.88	3.33	1.33	6.67	0.67	5.67	0.33
-30°C	6.67	1.86	5.67	1.45	6.67	0.33	5.00	1.53
-40°C	6.67	1.86	4.33	1.45	5.67	0.33	4.67	0.67
-50°C	6.67	1.20	5.33	1.45	5.33	2.03	5.00	0.00
-60°C	6.00	1.00	5.00	1.53	6.67	0.67	5.67	2.03
-70°C	6.00	1.15	5.67	2.03	5.67	1.20	4.67	1.76
-80°C	6.67	2.33	5.00	1.15	6.67	1.86	6.33	1.33
LN	5.67	0.88	4.00	0.00	6.67	1.33	4.33	0.88

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