

# Techniques for cell preservation



**ASSIGN  
BUSTER**

## Summary

Many industries such as the food, pharmaceutical and horticultural industries require an extensive use of various types of cells. Hence there is a requirement to preserve cells so that they can be used either directly or for further research at the appropriate time. In order to preserve these cells, expertise from a variety of disciplines including but not limited to engineering, biology, biotechnology, cryobiology etc is required to design protocols that enable the development of precise and reliable preservation methods.

There are many methods that are used today to preserve cells such as cryopreservation, hypothermic preservation, vitrification, freeze drying, the later two stem from cryopreservation as they involve freezing in some way. Cell preservation techniques are cell specific so techniques used to preserve plant cells may not be useful in preserving mammalian cells or microorganisms. Presently bacteria can be preserved in a dry state, however, mammalian cells are desiccation sensitive and cannot be stored in a dry state without the use of biotechnology (Kanas and Acker, 2006). Using cryopreservation puts cells in an animated stage where metabolism is stopped as they are stored between -800C and 1960C. At these temperatures cells remain viable for a very long time but this can only be made possible by controlling variables in the correct way. If the cooling rate is too slow and the cell will dehydrate in an attempt to form equilibrium, the extracellular liquid becomes a brine solution thus causing cell denaturation as a pH shift occurs. If the cooling rate is too fast then intracellular ice formation damages the membrane of the cell. Hypothermic preservation on the other hand, uses <https://assignbuster.com/techniques-for-cell-preservation/>

temperatures above 00C and below 370C. This technique is mostly used for storage of tissue but can be used to preserve cells for a short period and is especially useful during transportation of cells as this technique doesn't require expensive refrigeration equipment. Vitrification is important to cryobiology as the natural state of liquid inside living things is disordered (Wowk, 2010). This provides a minimally disturbing way of preserving cells as during cooling, the liquid maintain properties of a liquid in terms of disorder of molecules while changing its physical properties to form glass (Wowk, 2010). Freeze- Drying/ lyophilisation is another preservation technique normally used for heat sensitive material (Ciu et al., 2006). This process involves 3 key stages- freezing primary drying and secondary drying (Ciu et al., 2006).

Storage of preserved cells is extremely important to ensure that quality is maintained before usage of cells and that scientific discoveries are not unreliable due to compromised integrity of samples (Mills 2009).

## **Contents (Jump to)**

Introduction

General Preservation Techniques

- Hypothermic Preservation

Cryopreservation

Thermodynamic Aspects of Cryopreservation

Freeze Drying

## Heat and Mass Transfer

### Vitrification

#### Thermodynamic Aspects of Vitrification

#### Storage

- Preservation of Mammalian Cells
- Preservation of Microorganisms
- Preservation of Plant Cells

#### Conclusion

## **Introduction**

Cell preservation is sustaining or maintaining healthy cell cultures by nurturing them or keeping them in an environment where they remain unchanged, this is needed for any procedure involving the use of cells. However this is difficult to achieve as cell cultures can easily become contaminated which renders cell cultures useless and it has been established that at high or low temperatures above about 60°C and below -200°C proteins that make up cell start to denature leading to cell death and therefore suitable cell preservation techniques must be applied to prevent loss of cells.

Following the natural process of the cell life, they can reach their death phase quickly and therefore in order for cells to be used and investigated with accurate (as reasonably possible) results and so as to not compromise the integrity of samples (Mills, 2009) they need to be stored properly by following certain protocols. These protocols are important as cell membranes <https://assignbuster.com/techniques-for-cell-preservation/>

are very sensitive and are easily damaged by freezing, drying and rehydration (Wolfe and Bryant, 1999). Rupturing of this membrane is a clear sign of cell death (Wolfe and Bryant, 1999). Discoveries were made in the 1940's to 1960's using cryopreservation techniques/ protocols were then continually developed and have been used for many years after that, even today. There are two primary types of cell preservation: for low temperatures- cryopreservation and at high temperatures- hypothermic preservation. There has been branches from these two main methods of cell preservation such as freeze drying, vitrification etc which basically use ideas from the main protocols and improve on them to result in a greater number of viable cells after preservation. Hypothermic preservation is usually for a couple of hours in a clinical setting with the anticipation of transplantation whereas cryopreservation is for long term storage, up to years.

## **General Cell Preservation Techniques**

Cell preservation techniques are specific to different types of cells as cells can vary widely from type to age. There are two main methods of low temperature preservation. These are cryopreservation- freezing preservation at temperatures below freezing, vitrification where fluid is in a glassy form and secondly, hypothermic preservation where temperatures are above freezing. Protocols for cryogenic preservation are different when ice forms as ice formation changes the biophysical environment that the cell experiences.

## **Hypothermic preservation**

Hypothermic preservation is not used mainly for cell preservation but mainly used for tissue and organ preservation but for transportation where the time period is short hypothermic preservation is a suitable for cell preservation

<https://assignbuster.com/techniques-for-cell-preservation/>

(Rubinsky, 2003). Cells intracellular and extracellular content are very different and specific and separated by a barrier known as a lipid bilayer (Toole and Toole, 1995). Positive effects include decrease in metabolic rate and oxygen demand, the negative effect is protein denaturation due to the fact that when the temperature is lowered (Rubinsky, 2003). Any temperature below the lipid phase transition temperature (6-180C) causes a disruption to these membranes, the lipid membrane bilayer of the cell transitions to a gel phase and the ionic leakage occurs as loss of efficiency of the active transport system of the cell (Rubinsky, 2003). As well as ionic imbalance, acidosis can occur which damages the cytoskeleton of the cell, free radical production, edema and protein denaturation (Rubinsky, 2003). When hypothermia is used to preserve single cells for a couple of hours, the cell can normally be restored to their original condition (Rubinsky, 2003). To combat these negative effects preserving solutions are designed such as nonelectrolytes (sucrose, raffinose, saccharoids), citrate and magnesium chelates to prevent intracellular edema, buffers, mannitol are used to deal with acidosis, free radical production (Rubinsky, 2003).

## **Cryopreservation**

James Arnott (1979- 1883) made modern cryobiology possible by using cold temperatures to kill cancerous tumours which paved the way for using low temperatures for preservation of biological material (Dias et al., 2010).

Cryopreservation has been used for many years and has been effective in preserving cells as it reduces problems associated loss of viable cells (Ryan, nd). Organisms have the ability to stay in an almost unaltered state when frozen below a certain temperature for considerable periods of time and this

is the basis of cryopreservation. The concept of freezing cells to preserve them was made possible because of the discovery that glycerol can be used to prevent damage from freezing by Polge, Smith and Parkes (Ryan, n. d.). Cryopreservation is usually done at temperatures of below -1300C or even lower, to stop a cell's biological time. Although this is the aim cells have been stored for years at -700C to -900C where cellular damage did occur as the biological time is only slowed down dramatically but not stopped.

To allow cells to stay in this almost animated state where there is little biological effect, the rate of the cooling process must be slow and gradual to prevent complications from arising when the cell cultures are thawed for usage (Ryan, n. d.). Cryopreservation can be described as having two stages: before and after freezing (Rubinsky, 2003). In the first stage, prior to freezing, cells experience hypothermia but as this is for a short while, cells aren't damaged or any damage done is minor (Rubinsky, 2003). As the temperature is lowered there is almost a stage wise process in which cell goes through (Ryan, nd). Cooling from room temperature to 00C, as metabolic rate is a strong function of temperature, the cell's metabolic rate slows and this disrupts the pumping of ions in the cell and active transport which are processes that requires energy (Ryan nd). Ice formation of a physiological saline, which most biological materials are, freezes at -0. 560C so as the temperature drops from 00C to -200C and ice crystals form in the extracellular environment, the solute concentration of the extracellular environment increases and therefore the cell loses water by osmosis in an attempt to establish equilibrium of solute and water concentration thus causing the cell to shrink and dehydrate (Ryan, nd). The rate at which water

leaves cells is dependent on the ratio of surface area to volume of the cell, membrane kinetics and proportion of free water (Gook and Edgar, 2007).

Ice formation usually occurs first in extracellular space as ice formation is a function of volume of solution (Rubinsky, 2003). Extracellular ice then forms in the direction of the temperature gradient and as ice forms energy is removed from the system (Rubinsky, 2003). When a solution starts freezing, solutes are rejected because ice can incorporate a small amount of solute and impurities (Rubinsky, 2003). This causes an increase of solutes between the interface of the ice and water and this build-up leads to the ice to form in a finger like form known as dendrites (Rubinsky, 2003). During the course of cryopreservation, the cells usually remain unfrozen between these dendrites along with the rejected solutes (Rubinsky, 2003). The solution between the dendrites become hypertonic as the temperature is reduced and this is a major cause of cell damage during cryopreservation (Rubinsky, 2003).

Damage to cells is related to chemical reactions which are related to time, temperature and concentration (Rubinsky, 2003).

Cooling rates have an optimum value for greatest survival (Woods et al., 2004). Even though some cells can survive with intracellular ice formation, majority require sufficiently low cooling rates (Woods et al., 2004). Mass transfer across the cell membrane is related to the effect of cooling rates and because mass transfer is a function of time and cell dehydration and chemical damage a function of temperature, so it would stand to reason that freezing cell rapidly to cryogenic temperatures eliminates damage (Rubinsky, 2003). A plot of cooling rates against survival rates has an inverse U shape (Woods et al., 2004). If the cooling rate increases beyond the



optimal cooling rate then survival of cells start to decrease (Woods et al., 2004). If the cooling rate is quick, then intracellular ice is formed before the cell has time to dehydrate completely which would have otherwise resulted in the disruption of the cell's organelles that is, the cell dies (Ryan, n. d.). To eliminate this problem the solution is not to drastically reduce the cooling rate as if the cooling rate is slow damage can occur as complete dehydration takes place and the physical strain caused to the cell can cause damage (Ryan, n. d.). Any unfrozen extracellular substance is basically a brine solution due to the solute can cause pH shifts which will induce protein denaturation resulting in the death of the cell (Ryan, n. d.). A cooling rate that is not too slow or fast will preserve the life of the cell as dehydration effects will be minimal and intracellular ice will not have formed. This is the case for most bacteria and prokaryotic cells but eukaryotic cells have a greater likelihood of dying and therefore a cryogenic agent is introduced (Ryan, n. d.).

The advantage of freezing cell cultures is that they are easily maintained as little input is required after the freezing period which is more desirable than maintaining a growing cell culture as it can easily get damaged or contaminated (Ryan, n. d.).

### **Thermodynamic aspects of cryopreservation**

Cryobiology is a change in temperature from physiological and temperature is a very important thermodynamic property (Elliot, 2009). Biological processes in the cells are made dormant due to the low temperature and the recovery of these cells depend on whether ice nucleation or osmotic transport across the cell damages it (Elliot, 2009). This can be understood

<https://assignbuster.com/techniques-for-cell-preservation/>

using thermodynamics developed for non biological applications (Elliot, 2009). Thermodynamic principles are also used to develop protocols for cryopreservation for example, isochoric cryopreservation of low temperature preservation (Rubinsky, 2004). Theoretical studies by Rubinsky et al (2004) show that pressure can act as an effective cryoprotectant when used with the usual cryoprotectant (Rubinsky, 2004). There is a large difference in the increase in concentration of solution during cryopreservation between isochoric cryopreservation and isobaric preservation (Rubinsky, 2004). The figure below shows that isochoric cryopreservation reduces the concentration of solutes (Rubinsky, 2004).

### **Freeze Drying**

The need for frozen storage can be avoided by allowing the ice in the frozen substance to sublime leaving a drying porous solid residue (Smith, 1970). This can easily be restored to its original form by the addition of sterile water (Smith, 1970). Freeze drying can be applied where freezing would normally prove lethal (Smith, 1970). The freeze drying process is made up of three main stages- freezing, primary drying where the sublimation of ice occurs and secondary drying where water is removed from the solute phase by desorption (Rambhatla and Pikal, 2003). To develop this process drying time needs to be minimised whilst maintaining the quality of the product (Rambhatla and Pikal, 2003). Principles of mass transfer are essential when considering primary drying in freeze drying processes.

### **Heat and Mass Transfer**

Scale up problems can occur as there will be differences in drying time due to the differences in supercooling between laboratory and manufacturing,

variability in heat transfer rates, differences in efficiencies of refrigeration systems (Rambhatla and Pikal, 2003). Carman considered sublimation of ice in a vacuum with the partial pressure of air zero and partial pressure of water  $p_c$  exerted at the surface of a condenser at temperature  $T_c$  considered as acting through the interspace (Smith, 1970). So

(1)

Where  $W_n$  is the net rate of vapour transfer in g/sec,  $T_i$  is the temperature at the interface in Kelvin. Carman then studied vapour diffusing through stagnant air which resulted in the equation

(2)

However Schrage objected to these equations as when there is mass transfer, equilibrium velocity distribution among vapour particles does not exist and therefore introduced multiplying factors for  $p_i/p_s$  (Smith, 1970). Practicable freeze drying rates are normally less than 1% that of pure ice and therefore  $W_n/W_0$  may be ignored (Smith, 1970). Stagnant air is the worst possible condition and it is assumed there is no mass transfer of ice fragments (Smith, 1970). Carman then plotted  $W_n/W_0$  against  $P$ ,  $p_c = 0$  and  $X = 1\text{cm}$  with the result showing that making  $P$  much smaller than  $p_s$  is pointless (Smith, 1970). If dry material is left in front of ice, when sublimation begins, the ice interface recedes (Smith, 1970). This becomes a heat and vapour barrier and reduces the rate of sublimation which is a function of temperature (Smith, 1970). As a result of dry temperature having a restricting effect when sublimation begins, the vapour pressure  $p_i$  at the interface will begin to approach  $p_s$  (Smith, 1970). Maintaining the

temperature just below that at which ice melt ensures that the rate of drying is at its maximum and most practical (Smith, 1970).

## **Vitrification**

Using the method of vitrification accomplishes the cells reaching cryogenic temperatures without formation of intracellular ice by the use of cryogenic agents. The ice free nature in the vitreous state is based on the exponential increase of the viscosity of the solution within the glass transition range of temperatures, the molecular translational movement is restricted therefore inhibiting ice growth (Baust, 2007). When the liquid is cooled sufficiently it becomes viscous in a glass like state or a non moving liquid, molecular diffusion stops and ice formation is insignificant. With the lack of ice formation, mechanical stress to the cell's structure is reduced. There are three main approaches to vitrification: ultra-rapid cooling, pre-freeze the addition of cryoprotective agents, freeze the cryoprotective agent during slow cooling.

Cryogenic agents do not need to permeate the actual cell as intracellular ice is prevented from forming by a slow cooling rate. There are various chemicals that provide cryoprotection include and some of these include ethylene glycol, methyl acetamide, methyl alcohol and polyvinyl pyrrolidone with the most widely used being dimethylsulfoxide (DMSO) and glycerol (Ryan, n. d). However, vitrification while being very desirable many of these agents have toxic effects to the cell culture as the concentration is high which make the use of these cryogenic agents difficult. High cooling rates needed for vitrification is difficult to achieve (10 to 10000C/min which depends on the concentration of the cryoprotectant used).

<https://assignbuster.com/techniques-for-cell-preservation/>

From reviews of studies on cell preservation using vitrification by Baicu et al, the studies showed that cell suspensions have little benefit where using this process is concerned.

### **Thermodynamic aspects of vitrification**

Under normal conditions, substances can exist as solid liquid or gas. When liquids are cooled, crystallisation into a solid occurs (Wowk, 2010). Crystal nucleation followed by crystal growth turns liquid into an ordered molecular arrangement of a solid crystal (Wowk, 2010). Some types of liquid remain uncrystallized in these conditions depending on their properties and retain the physical properties of a liquid (Wowk, 2010). As the temperature is lowered even further, the disordered pattern of a liquid remains but the physical properties become like a solid resulting in a solid liquid called glass (Wowk, 2010). Solutes in the unfrozen solution around the cell become concentrated during cooling thus increasing solute concentration inside the cell as a result of osmosis (Wowk, 2010). When the solute concentration becomes too high it cannot freeze, cooling further below the glass transition temperature leads to vitrification of cells and the liquid (Wowk, 2010). Cryoprotectants protect the cells by preventing salt concentration becoming too high. Figure 2 shows cryoprotectant concentration as a function of temperature followed by cells between ice crystals.

Figure 3. Path followed on a glycerol-water phase diagram by solution between the ice crystals during slow freezing, beginning with addition of cryoprotectant.  $T_m$  is the equilibrium melting temperature and  $T_g$  is the glass transition temperature. After recovery from initial supercooling, solutes concentrate during freezing so that the melting point of remaining unfrozen

solution equals the solution temperature. Vitrification occurs between ice crystals when the temperature drops below  $T'g$ , the intersection of the  $Tg$  and  $Tm$  lines. (In practice the path may deviate slightly to the left of the  $Tm$  line at very low temperatures because the equilibrium melting line can only be followed exactly if the cooling rate is infinitesimal. (Wowk, 2010)

During the process, in the cooling stage, a liquid or solid will want to occupy space in accordance with the equation

(3)

where  $T$  is temperature,  $V$  is volume at constant pressure  $\alpha$  and is the thermal expansion coefficient, representative values being  $85 \times 10^{-6}$  per  $^{\circ}C$  for liquid and  $40 \times 10^{-6}$  per  $^{\circ}C$  for glass (Wowk, 2010). Solutions of cryoprotectants, on cooling, undergo larger volume of contraction than the container it is in (Wowk, 2010). However a solid can be prevented from contraction if it is adhered to the wall of the container (Wowk, 2010). Volume change is proportional to temperature change. Stress usually develops in the vitrified system because of uneven temperature distribution during cooling (Wowk, 2010). Stress that this causes can be minimised by using non-adherent hydrophobic material for the container (Wowk, 2010).

### **Storage**

Storage is very important when preserving cells as mishaps will lead to loss of healthy cells. After the cryogenic agent is added to the cells it needs to be kept in a container that can withstand the low temperatures. This is a necessity in order to prevent damage to the cells which can be caused by leakage of the solution from the containers if the material of the container

becomes brittle during storage. The seal of these containers is also very important especially when storage uses liquid nitrogen as if there is a leakage, pressure can build up when being returned to a higher temperature which will lead to an explosion. This can be potentially very hazardous if the cell culture is of pathogens or toxic substances. Two types of vessels suitable to cryogenic storage are heat-sealable glass ampules and plastic screw cap vials. Glass ampules are however not widely used as there may be an invisible pinhole leak.

The technique used to recover the cell is very important in order to make the preservation techniques worthwhile. The major problems that arise from recovery of cells after cryogenic preservation are if the thawing rate is not fast enough or if the cryoprotectant isn't removed properly or in a way that prevents cell damage. Cryoprotective agents used to protect the cell during preservation need to be removed as quickly as possible during cell recovery by changing the medium between 6-8 hours of thawing. However this technique varies with the type of cryoprotectant for example, if glycerol is used, osmotic damage can be caused to the cell destroying it so equal volumes of warm medium has to added stepwise, every 10 minutes so osmotic equilibrium is reached before further progress in the recovery.

## **Preservation of Mammalian Cells**

Preservation of cells has a large impact on many industries and improving preservation techniques for different types of cells is an ongoing project. Using cell preservation techniques for mammalian cells means that farmers in the cattle breeding industry select valuable semen from bulls which can then be stored for years until required and during epidemics such as the foot

and mouth disease in that was in 2001, there will be a reserve of this sperm that could be used until the epidemic passed. Preservation of human cells such as red blood cells means there can be a continuous supply needed for tests and blood transfusions, preservation of oocytes and human sperm means individuals can retain the ability to have their own biological children who otherwise may not have been able to due to health reasons.

During the course of experiments, fowl sperm was frozen in 1.4M glycerol, which serves as a cryoprotectant and after thawing most of the cells were still living, showing that glycerol reduces the build up of ice (Smith, 1970). Mammalian spermatozoa was studied and it became apparent that with some species for example, with rabbit spermatozoa glycerol needs to be added slowly and the cooling rate needs to be as slow as about 10C/min and on thawing, the glycerol does not need to be removed slowly in order to gain maximum cell survival (Smith, 1970). However, in 1949, Polge, Smith and Parkes reported that storing fowl spermatozoa in 1.4 - 2.8 M glycerol in a buffered salt solution, cooled rapidly and with slow removal of glycerol from the cells after thawing, results in a good number of living cells (Smith, 1970). This process was done at -790C and therefore can only be carried out for short periods of time. For longer period of time, the temperature needs to be much lower at around -1900C (Smith, 1970). Different techniques are used for spermatozoa preservation, a technique that was widely used in the 70's was that where the semen is diluted with citrate-egg-yolk diluent, slowly cooled to 50C (Smith, 1970). Glycerol is then added and the mixture left at room temperature for 5 hours to allow the glycerol to penetrate the cells, cooled at 10C/min to -150C then 50C/min to -790C and stored in solid carbon



dioxide at -790C or liquid nitrogen at -1960C (Smith, 1970). Preservation of sperm has changed since the 70's and currently preservation of human spermatozoa in liquid nitrogen is the most successful preservation method (Dkhil, 2009). There are many other expensive and complicated protocols to preserve human spermatozoa but to overcome this, a simple method where human spermatozoa is preserved is by separating the sperm using a swim up technique (Younglai, 2001). After the swim up technique is applied, sperm is then storing in 800 mOsmol KSOM medium at a holding temperature of -200C (Dkhil, 2009).

In history the next cell to be successfully preserved after spermatozoa was the red blood cell or erythrocyte (Smith, 1970). In 1949 the method for preserving erythrocyte was rapid freezing without any cryoprotective agent and this was successful however only when vitrification was employed (Smith, 1970). The erythrocytes were placed between two thin sheets of glass and directly immersed in liquid nitrogen at -1960C (Smith, 1970). This technique however is not applicable to majority of human cells as they have nuclei because of their lower water permeability (Smith, 1970). The high water permeability of the erythrocyte is enough to prevent damage to the cell from high salt concentration as intracellular ice does not form (Smith, 1970). The process of preserving red blood cells is simple if sterilization is not required (Smith, 1970). A simple technique was described by Meryman and Kafig where blood is sprayed from a syringe with a fine needle onto the surface of liquid nitrogen where droplets freeze separately and can easily become thawed by sprinkling into warm saline (Smith, 1970). This preservation process is made more effective by adding sucrose of 1.2 M

concentration immediately after being placed in the liquid nitrogen, this give a survival rate of greater than 95% (Smith, 1970). Currently the most practiced preservation method for red blood cells is storage in liquid for up to six weeks (Kanas and Acker, 2010). There is an increasing need for red blood cells for transfusion medicine so alternative preservation methods are being researched (Kanas and Acker, 2010). Hypothermic preservation of red blood cells is where the temperature is above freezing but below the temperature of normal physiological temperatures (Scott et al., 2005). Current solutions of adenine is used so part of the ATP production pathway and the membrane's structure is maintained, glucose is also added to maintain metabolism of the cell, inorganic phosphate etc (Scott et al., 2005). As well as all the contents of the nutrient medium, certain parameters also need to be controlled such as the temperature (Scott et al., 2005). The temperature needs to be between 10C to 60C to prevent degradation by reducing the cell's metabolism by up to 40 times and this allows hypothermic storage for up to 42 days (Scott et al., 2005). Cryopreservation is employed to preserve red blood cells but to reduce or prevent cryoinjury, chemicals are added as cryoprotectants (Scott et al., 2005). These chemicals fall into two groups, nonpermeating CPA's or chemicals which protect red blood cells by dehydrating the cell at high subfreezing temperature therefore preventing solute concentration reaching critical levels and the second group are CPA's that permeate the cells and prevent lethal concentrations of electrolytes and so the temperature where a critical salt level is reached no longer exists (Scott et al., 2005). Permanent CPA's act to depress the freezing point and lower the chemical potential of a solution, reducing the amount of ice formed at a given temperature (Scott et al., 2005, pp-138).

Freeze drying or lyophilisation of red blood cells has researched a lot over the years with many techniques being unsuccessful (Kheirloom et al., 2005). Trehalose from one of these studies proved to be useful as it improved the viability of cells after freeze drying (Kheirloom et al., 2005). Membrane lesion leading to hemolysis can be alleviated by adding liposomes to the freeze drying buffer (Kheirloom et al., 2005). Using trehalose and liposome in the lyophilisation process shows a high survival rate of red blood cells in storage for 10 weeks at 40C (Kheirloom et al., 2005).

Bone marrow is a tissue in bones which can be used to form new cells. These cells circulate in the blood and therefore be used in the formation of red blood cells. Barnes and Loutit in Smith (1970) first showed that bone marrow cells could survive at -790C if cooled slowly and using 2 M glycerol (Smith, 1970). The essential steps used are: glycerol is added to the cell suspension of concentration below 4M and given time to penetrate the cells (Smith, 1970). The time the glycerol takes to penetrate the cells depends on the temperature but using previous results it takes an hour for equilibrium to be achieved, where the concentration of glycerol is equal in and out of the cell. It is noted that at room temperature the toxic effect of glycerol are greater than at a lower temperature but using a lower temperature means the time need to for the cell suspension to reach equilibrium is extensive (Smith, 1970). Cooling is then performed slowly as cooling from room temperature to -790C, damages the cells so their normal function cannot be carried out (Smith, 1970). After cooling bone marrow cells, storage must at least be -600C (Smith, 1970). Thawing is carried out quickly as if done slowly in a

stepwise manner, there is great cell loss. The most readily available method that isn't mechanically intrusive is to use a hot water bath at 40°C then when the ice has melted place the containers with the cell suspensions in melting ice to keep their temperature below 40°C (Smith, 1970).

Experimental work has shown that in animals fresh bone marrow is no different to bone marrow that has been preserved using glycerol (Smith, 1970). However with human bone marrow after a brief period of storage at -70°C, 75% of cells survived which decreased by 65% over a storage period of 3 years- this is problem is overcome at -196°C where there is no cell loss (Smith, 1970).

Preservation of bone marrow using glycerol provides a less toxic effect to cells than dimethyl sulphoxide (DMS