RED BLOOD CELL STABILIZATION: EFFECT OF HYDROXYETHYL STARCH ON RBC VIABILITY, FUNCTIONALITY AND OXIDATIVE STATE DURING DIFFERENT FREEZE THAW CONDITIONS.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF

Master of Technology
Biotechnology

By
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Department of Biotechnology and Medical Engineering
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CERTIFICATE

This is to certify that the thesis entitled, "**Red Blood Cell stabilization: Effect of hydroxyethyl starch on RBC viability, functionality and oxidative state during different freeze thaw conditions.**" submitted by **Ms. Deepanwita Das** in partial fulfillment of the requirements for the award of Master of Technology in Biotechnology and Medical Engineering with specialization in "Biotechnology" at the National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any other Degree or Diploma.

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(DEEPANWITA DAS)

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Abstract

R. P. Goodrich and co-workers, 1989 have reported that red blood cells (RBCs) can be preserved in the dry state by addition of mixtures of hydroxyethyl starch (HES) and glucose (59,60). In this thesis work we tried to investigate the effect of HES alone on the viability, functionality and oxidative state of red blood cells after freeze thaw stress. Here we prepared eight formulations having varying concentrations of HES along with Adenine glucose mannitol sodium chloride (ADSOL) and Phosphate buffer saline (PBS) which were ADSOL, 5% HES in ADSOL, 10% HES in ADSOL, 15% HES in ADSOL, PBS, 5% HES in PBS, 10% HES in PBS, 10% HES in PBS and finally 15% HES in PBS as protective solutions during freeze thaw. Control sets used in this study did not contain HES. Red blood cell suspensions were prepared using the formulations at around 25% hematocrit and were frozen in liquid nitrogen for 10 mins. After thawing at different temperatures (4°C, 37°C and 60°C) the percentage hemolysis, percent methemoglobin oxidation, Thiobarbituric acid reactive species (TBARS) and catalase activity were determined using spectrophotometric assays. In all formulations percentage hemolysis observed was found to be more than 30% which has been reported in normal freeze thaw experiments on RBCs. In case of red blood cells in 15% HES in ADSOL which were thawed at 4°C showed moderate amount of hemolysis, lowest amount of methemoglobin, lipid peroxidation and highest Catalase activity. Therefore, it was found out to be the best formulation to preserve cells against freeze thaw stress among all the formulations used in this work. Thus, it can be concluded that freeze thaw experiments using HES alone in ADSOL or PBS showed a trend of protective effect in 15% concentration of HES but it is not sufficient alone for providing protection against different stresses during freeze thaw experiments due to significant amount of percent hemolysis. Further combination of disaccharide along with HES needs to be investigated and also the cooling rate during freeze thaw experiments needs to be controlled.

Keywords: Red blood cells, lyophilization, freeze thaw, hemolysis, lipid peroxidation, thiobarbituric acid reactive species, catalase, methemoglobin, freeze thaw, hydroxyethyl starch ADSOL, PBS.

ABBREVIATIONS

ADSOL	Adenine glucose mannitol sodium chloride
CPDA	Citrate phosphate dextrose adenine
fL	Femto litres
gm	Grams
Hb	Hemoglobin
HES	Hydroxyethyl starch
KH_2PO_4	Potassium dihydrogen phosphate
MDA	Malondialdehyde
min	Minutes
ml	Milliliters
mM	Millimolar
mOsm	Milliosmolal
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
OD	Optical Density
PBS	Phosphate buffer saline
RBC	Red blood cell
ROS	Reactive Oxygen Species
TBARS	Thiobarbituric acid reactive species
Tg	Glass transition Temperature

INTRODUCTION:

1.1 Motivation

Red blood cell (RBC), or erythrocyte, which comprises 99% of all blood cells, is a flexible biconcave disc 7.2 μm in diameter. It is specialized to carry oxygen from the lungs to the tissues of the body and to perform this it contains a pigment, hemoglobin. During its 120 days lifespan, it travels about 300 miles around the arteriovenous circulation, repeatedly passing through the capillary bed. As the mean diameter of a capillary is about 3 μm, the red cell has to retain a high degree of flexibility which requires energy. Energy is generated as adenosine triphosphate (ATP) by the anaerobic, glycolytic pathway.

Motivation for this study comes from the fact that Biopreservation of human RBCs by freeze drying has received lot of scientific attention since 20^{th} century and still remains an area with scope for extensive research. The main driving force for extensive research in this field is the enormous need of improved banking techniques, autologous RBC products for transfusion purposes and easier worldwide transportation of red blood cells particularly to remote locales and site of immediate requirement. Storage of red blood cells in a dry state (freeze dried) offers a possibility of storing the cells for long duration of time under conditions which are easier to maintain and easier to transport to site of immediate requirement. Can be readily shipped and easily transported to site of immediate requirement, Prevented sample shrinkage, minimized chemical changes, and maintained product solubility allows easy rehydration. The development of viable freeze dried RBCs will not only be a significant achievement in the field of Biostabilization, Cell preservation and Transfusion medicine but will also initiate modern blood banking by the means of autotransfusion. Autotransfusion is a process in which a person receives his/her own blood for a transfusion, instead of banked donor blood. This will further eliminate the potential risks faced by patients associated with hemotherapy, including immune and nonimmune–mediated transfusion related adverse reactions. Other circumstances in which it will be advantageous to store red blood cells for a longer period include extension of shelf-life, provision of transfusion material to

individuals with rare blood groups, stockpiling against disaster, insurance against irregular supply, and the avoidance of infectious disease, any attempt to prolong the shelf-life must provide a product that has all the aforementioned functions intact.

Because the interest and need for dry storage of pharmaceuticals and foods is not a new concept, success in these industries has served to demonstrate what may be possible for mammalian cells and to encourage ongoing research in red blood cell desiccation.

1.2 Objective

The aim of this work is to develop a relationship between the in-vitro damages on RBCs during various stresses and the biochemistry of the cell leading to such damage. The overall objective of the study is to investigate in-vitro effects of protective agents (excipients) on the survival of red blood cells after freezing and then thawing under different conditions and formulations. Specifically, there are two major objectives. First objective is to monitor the viability of the red blood cells after freeze thawing by analyzing the percentage hemolysis. Secondly, monitoring the oxidant and antioxidant biomarkers of RBCs using Hemoglobin oxidation assay, Lipid peroxidation assay and Catalase assay.

The specific aim of the first objective is to investigate the effect of different formulations and thawing conditions on the viability of red blood cells. Freshly isolated red blood cells were used in the freeze thaw experiments, which were suspended in different formulations using Hydroxyethyl starch, phosphate buffered saline and ADSOL solution. Fresh isolated red blood cells suspended in formulations without HES were used as control for this study.

The specific aims for the second objective are: (a) to compare and judge the oxidative state of hemoglobin, (b) to investigate and correlate the oxidative state (membrane peroxidation) of the red blood cell membranes with different formulations and thawing conditions, and (c) to evaluate the activity of antioxidative enzyme, Catalase, to evaluate the degree of oxidative stress implied on the red blood cells during the freeze thaw experiments.

1.3 Overview of thesis

In this study, the effect of Hydroxyethyl starch as excipients during different freeze thaw cycles on red blood cells viability and functionality will be investigated. Different formulations included in this study will contain combinations of varying percentage of Hydroxyethyl starch in either ADSOL or phosphate buffer saline as protective solutions. Fresh blood will be collected in CPDA bags and red blood cells would be isolated using simple centrifugation method. After the isolation of red blood cells as preliminary study, they will be stored in ADSOL and phosphate buffer saline for few days to check the viability and oxidative state of red blood cells during normal hypothermic preservation in these two solutions. Further, fresh red blood cells will again be isolated and suspended in the different formulations after which freeze thaw experiments will be performed. Complete freezing of the solutions will be done using liquid nitrogen for half an hour, after which they will be thawed under three different conditions at three different temperatures namely, 4°C, 37°C (room temperature) and 60°C. For 4°C refrigerator will be used whereas for 60°C water bath will be used to provide constant temperature condition. Following freeze thaw different parameters will be measured for investigating the viability and oxidative state of red blood cells.

Various assays used for investigating the status of red blood cells after freeze thaw include percentage hemolysis assay using Drabkin's Reagent, Hemoglobin oxidation assay, Lipid Peroxidation assay, Catalase assay.

In percentage hemolysis assay the amount of free and intact hemoglobin will be measured using the hemoglobin assay which utilizes drabkin's reagent for the quantification of hemoglobin spectrophotometrically. The amount of hemoglobin present in the supernatant is directly proportional to the percentage of hemolysis in the sample and it is therefore a viability marker. In hemoglobin oxidation the percentage of hemoglobin present as methemoglobin will be quantified spectrophotometrically. Methemoglobin is the oxidized form of hemoglobin and therefore its quantification is a marker of oxidative stress on red blood cells. In case of lipid peroxidation assay it measures the secondary product of peroxidation of red blood cell membranes which is malondialdehyde using Thiobarbituric acid reactive specie which is quantified

spectrophotometrically. As red blood cell membrane is important for the cell's integrity and functionality the lipid peroxidation assay is again an oxidative stress and functionality marker for red blood cells. Finally, catalase, is an antioxidant in red blood cells tells us about the oxidative state and capability of the cells to fight against oxidative stress. Catalase activity is measured using spectrophotometry to quantify the time needed for conversion of hydrogen peroxide and activity is given as Units/ml of enzyme.

LITERATURE REVIEW

2.1 Biopreservation and Biostabilization

Biopreservation is the process of maintaining the integrity and functionality of cells held outside the native environment for extended storage times. (1) Whereas, Biostabilization, is termed as the application of knowledge gained from the study of biological systems to stabilization of macromolecules, cells, tissues, and even intact plants and animals (Hightower et al 2000). Thus, both the terms go hand in hand when we talk about long time preservation and storage of cells outside their environment. This involves a wide range of application like storage of platelets, red blood cells, organs and shelf life extension of protein-based drugs and enzymes. Biostabilization and Biopreservation of susceptible biological organisms, and materials is of increasing importance. Over the years, different biostabilization protocols as well as biopreservation and storage methods have been developed for cells, bacteria, proteins, whole blood, and blood products. Advances in Biostabilization techniques may result in storage of platelets and red blood cells (2) by freeze drying. Further improvement of the existing methods to match the increasing demand of the medical technologies requires basic understanding of the interactions of the biological material with its environment.

2.2 Why Red Blood Cells

2.2.1 *Red blood cell*

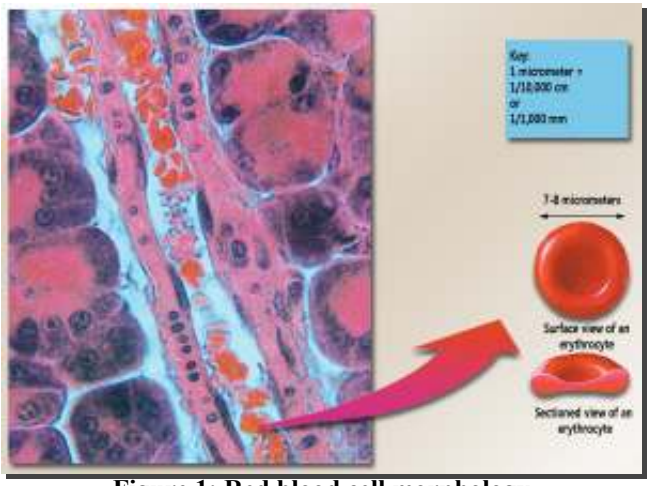

Figure 1: Red blood cell morphology

Derived from pluripotent stem cells in bone marrow through a maturation process called erythropoiesis, mature RBCs are biconcave disks approximately 7.2 lm in diameter, 1.5 to 2.5 lm thick, with a mean volume of 90 fL.(1) Along the developmental process, there is a reduction in cell volume, condensation of chromatin, loss of nucleoli, decrease in the nucleus, RNA, mitochondria, and an increase in hemoglobin synthesis, resulting in a mature RBC, which lacks a nucleus and organelles. The primary function of RBCs is to transport oxygen from the lungs to the body tissues, where the exchange for carbon dioxide is facilitated through synergistic effects of hemoglobin, carbonic anhydrase, and band3 protein, followed by carbon dioxide delivery to the lungs for release. Successful oxygen transport is dependent on efficacy of the 3 elements of RBC metabolism: the RBC membrane, hemoglobin, and cellular energetic (1). Continuous research of oxygen transport is crucial for development of improved RBC storage and biopreservation technologies.

2.2.2 *Red blood cell membrane*

Like other cell membranes, the RBC membrane is a fluid structure composed of a semipermeable lipid bilayer with an asymmetrically organized mosaic of proteins. Membrane lipids compromise approximately 40% of the RBC membrane mass, with equimolar quantities of unesterified cholesterol and phospholipids, and small amounts of free fatty acids and glycolipids. (1) Membrane proteins comprise approximately 52% of the RBC membrane mass and can be categorized into integral and peripheral proteins according to their location relative to the lipid bilayer. Integral membrane proteins, such as glycophorin and band 3 protein, transverse the membrane and contain extensions into or out of the RBC. The main function of integral membrane proteins is to carry RBC antigens and to act as receptors and transporters. In contrast, peripheral proteins are only found on the cytoplasmatic surface of RBC membrane forming the RBC cytoskeleton. The red cell cytoskeleton is organized in a two-dimensional hexagonal network and is predominantly composed of spectrin, ankyrin, protein 4.1, actin, and adducin.3 along with adaptor proteins like ankyrin, protein 4.2, protein 4.9, adducin, tropomyosin, myosin and tropomodulin. These proteins form a mesh-like network of microfilaments that strengthens the RBC membrane while maintaining RBC

shape and stability. 3 Unusual properties of the RBC membrane, such as high elasticity, rapid response to stresses, and the ability to undergo large membrane extensions without fragmentation, have been summarized by the term cellular deformability.

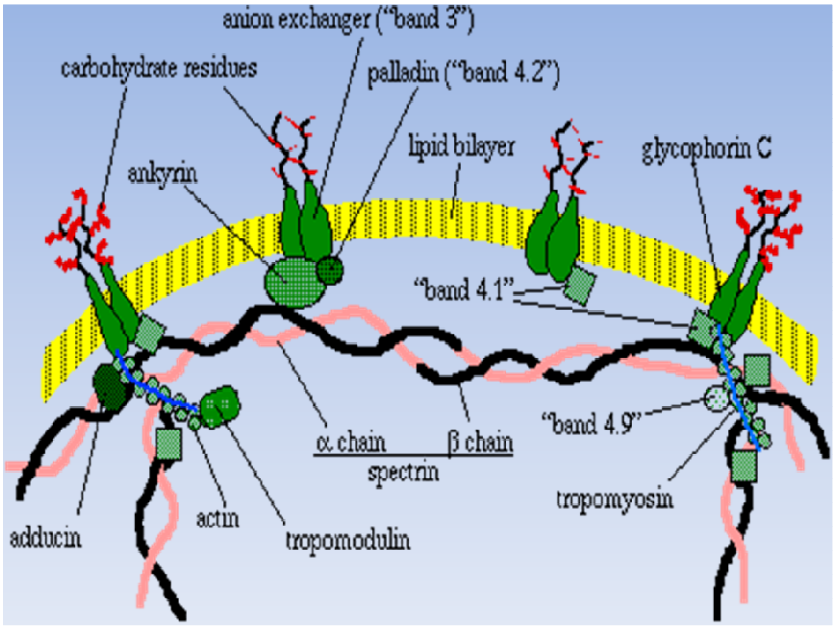

Figure 2: Red blood cell membrane

A wide variety of injuries and medical conditions resulting in symptomatic anemia require transfusion of red blood cells. (6) Red blood cell transfusions save lives by increasing RBC mass in patients that have low oxygen–carrying capacity due to increased RBC loss (traumatic/surgical hemorrhage), decreased bone marrow production (aplastic anemias), defective hemoglobin (hemaglobinopathies and thalassemias), and decreased RBC survival (hemolytic anemias). (1) Thus, there is an urgent need in majority of the world's population for safe blood and blood products. Of the estimated 80 million units of blood donated annually worldwide, only 38% are collected in the developing world where 82% of the world's population lives. (WHO Fact sheet 2004). Also, every year in the US, around 14 million units of blood are collected, and approximately 13.9 million units of RBCs are administered to 4.8 million patients (5).Therefore, Biopreservation and Biostabilization of red blood cells (RBCs) is needed to ensure a readily available, safe blood supply for transfusion medicine. Effective biopreservation procedures are required at various steps in the production of a RBC product including testing, inventory, quality control, and product distribution. The

biopreservation of RBCs for clinical use can be categorized based on the techniques used to achieve biologic stability and ensure a viable state after long-term storage. (1) Maintaining the quality and safety of RBCs delivered to the patient, as well as the overall clinical use of blood products, requires effective techniques for the preservation of RBC viability and function.

2.3 Biopreservation approaches

The American Association of Blood Bank Standards requires a product with 80% immediate survival (less than 20% hemolysis) and a 24h survival time of at least 70% of the transfused cells (7). Generally when donor blood is received at a processing center, RBCs are separated and stored in liquid dextrose based preservative media for 35–42 days as a unit of packed erythrocytes with a volume of approximately 250mL and a hematocrit value of 55– 80% at 4°C. Another method for long-term storage includes treating the cells with glycerol solution and freezing them at -80°C, followed by storage at this temperature for up to 10 years (7). Broadly there are three approaches for Biopreservation of red blood cells for clinical use categorized based on the techniques used to achieve biostability and ensure viability after long term storage. These approaches are hypothermic storage, cryopreservation, and lyophilization. (1)

Hypothermic preservation of RBCs is the earliest and most investigated approach of biopreservation based on the principle that biochemical events and molecular reactions can be suppressed by a reduction in temperature. In the context of biopreservation, hypothermic conditions are those in which the temperature is lower than the normal physiological temperature but higher than the freezing point of the storage solution (1) the currently licensed additive solutions for such storage are saline-adenine-glucose-mannitol (SAGM), ADSOL (AS-1), Nutricel (AS-3), and Optisol (AS-5) where the stogare duration is of 42 days (8,9,10). Although the quality of hypothermically stored RBCs has improved with the use of anticoagulant/ additive solutions, these storage solutions do not fully preserve RBC viability and function. Cellular metabolism is not completely suppressed at hypothermic temperatures and thus the preservation time remains short and also such preservation is highly susceptible to microbial

contamination (4). Hypothermic storage is also at time unavailable and quite inconvenient for transportation and distribution of the RBC supply.

Cryopreservation is the process of preserving the biologic structure and/or function of living systems by freezing to and storage at ultralow temperatures. As with hypothermic storage, cryopreservation uses the beneficial effect of decreased temperature to suppress molecular motion and arrest metabolic and biochemical reactions. Below -150°C, a state of "suspended animation" can be achieved as there are very few biologically significant reactions or changes to the physicochemical properties of the system. (11) In order to take advantage of the suspended animation state for cryopreservation of red blood cells for long duration, damage due to freezing and thawing must be minimized such damage is also referred to as "cryoinjury". Cryoprotectants used for cryopreservation can be non-permeating like sugars, sugar alcohols, polymers and starches such as Hydroxyethyl starch (HES) or permeating like dimethyl sulfoxide and glycerol. In contrast to hypothermic storage, RBC physiology, including hemoglobin structure, and membrane and cellular energetics, is unaffected by extended storage in the frozen state.(1) Although cryopreservation is the only current technology that maintains ex vivo biologic function and provides long-term product storage it requires ultra low temperature refrigerator or a liquid nitrogen container and transport of the red blood cells is difficult and may even be completely impossible in some environmental conditions (1). The above mentioned biopreservation methods are widely used in current clinical practice, but they cannot meet the current clinical demand for blood supplies worldwide. For these reasons, a search has begun for better methods for the preservation of red blood cells (3).

Compared to conventional Biopreservation methods, Lyophilization has many advantages, such as room temperature storage, lower weight, and greater convenience for transportation (4). Effective lyophilization prevents sample shrinkage, minimizes chemical changes, and maintains product solubility to allow easy rehydration. The adequate removal of residual moisture would accommodate easy storage and transport of a compact, lightweight product, stable at room temperature for extended periods.

Eliminating the need for expensive refrigeration devices would substantially reduce the current cost associated with the storage and transport of frozen blood making lyophilized RBCs ideal for remote storage and military applications (1). These advantages make the lyophilization of red blood cells more suitable for some particular environments, such as war or disasters, by making it possible to save lives, regardless of environmental conditions. Additionally, successful desiccation of RBCs and storage in the dry state would offer numerous practical advantages such as possibility for extending their shelf life for longer periods of time under conditions that are far easier to maintain (e.g., room temperature). (3) Despite claims to the contrary, (14,15) lyophilization of red cells to moisture contents that facilitate stable storage at room temperature for indefinite periods has not been demonstrated to date.

2.4 Freeze drying or Lyophilization

Lyophilization (freeze-drying) involves the removal of most unbound water from biologic materials through controlled freezing followed by the sublimation of ice under vacuum.(12,13) Freeze-drying RBCs has received a lot of attention over the years, and several approaches for lyophilization have been reported (14, 15, 16, 17), none of which have been particularly successful.

2.4.1 Overview of the Lyophilization Process

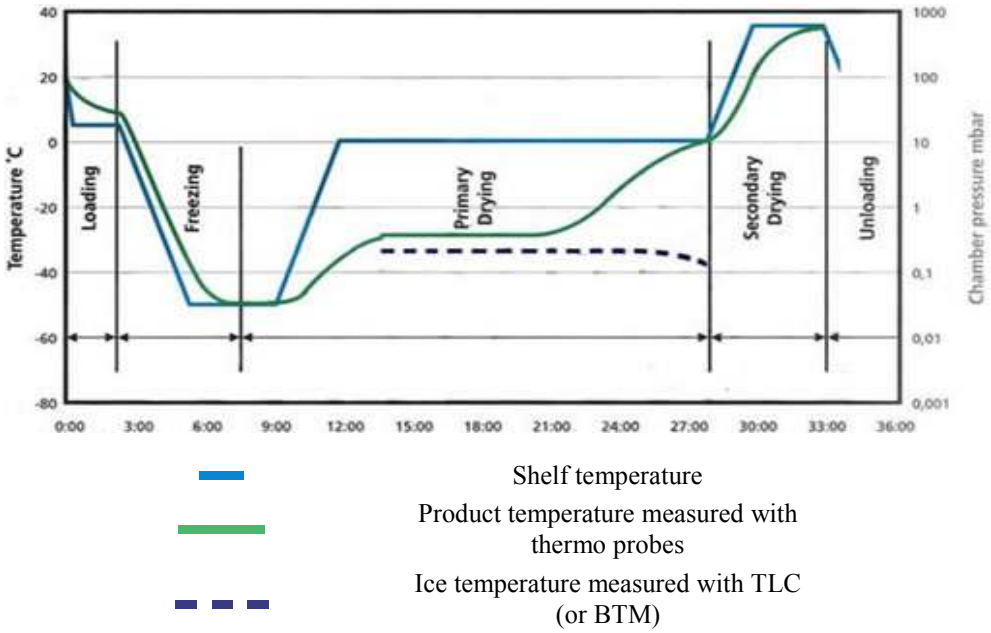

Figure 3: Overview of freeze drying cycle

Lyophilization and freeze drying refer to the same process. Lyophilization comes from the Greek and means to make easily dispersed or solubilized (Luo-Loosen, philos-loving). Freeze drying can be defined as the drying of a substance by freezing it and removing a proportion of any associated solvent by direct sublimation from the solid. Figure 3 shows the freeze drying cycle.

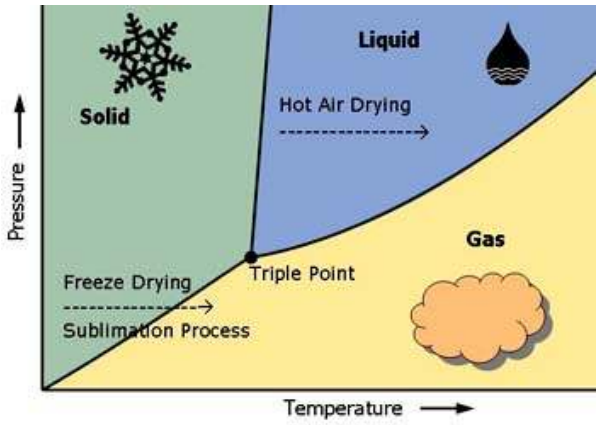

Figure 4: Principle of freeze drying mechanism

Figure 4 shows the principle of freeze-drying of controlling the water phase transitions under certain temperatures and pressures. Water can be transited through three phases. During lyophilization, the temperature and pressure are controlled to make sublimation

instead fusion or vaporization occur. Latent heat of vaporization causes ~540 calories/g water, heat of fusion (melting ice) causes ~ 80 calories/g ice, latent heat of sublimation (freeze drying) causes ~680 calories/g ice. Therefore, lyophilization, essentially sublimation is very energy costly. However, due to the many advantages of lyophilization, such as, prolonged shelf life, accurate and easy dosing, lyophilization is still attractive in pharmaceutical and biotechnological industries.

In principle, lyophilization is split into three separate stages: freezing, primary drying, and secondary drying (2). Freezing is to immobilize the product being freeze dried. The product structure, size and shape are fixed after freezing. Primary drying is to remove the free moisture that has been frozen. Secondary drying is to remove the bound moisture, which did not separate out as ice during freezing. Factors that may influence the recovery of cell viability during the freeze-drying process include the lyoprotectants (sugars, polymers), the operating condition during the drying process (primary and secondary drying), and the rehydration process. Freezing considered synonymous with drying because water is removed from a frozen specimen in the form of ice, leaving the specimen with a lower water content, (around 20%w/v) (22).

Cell damage and protein destabilizing can occur at any step of the whole process. Attempts to improve the recovery of viability and functionality have been focused on the entire process of lyophilization and rehydration. It was not clear at which stage(s) during the process the damage arose or the excipients were at work. Recent research shed some lights on the separate stresses and stabilization by using infrared spectroscopy or other complimentary tools. A broad view of the various or even controversial stabilization mechanisms will certainly help us to develop the rationale and strategies to stabilize and preserve red blood cells at their optimum.

2.4.2 Factors Responsible for Red blood cell damage during Lyophilization

2.4.2.1 During Freezing

A common approach in developing a freeze drying protocol for complex systems, such as red blood cells, is the gradual optimization of individual "subprocesses." One essential subprocess is the freezing step, after which the freeze drying procedure can easily be interrupted to obtain an interim result on the way to a freeze dried product (16). Freezing is a critical step in producing an acceptable lyophilized product. Freezing immobilizes the red blood cells. After freezing, the product structure, size and shape becomes fixed. During freezing, the shelf temperature and the complete product matrix is reduced and maintained to a temperature that is significantly below the glass transition temperature (eutectic temperature) of the product formulations, to ensure that the product is completely frozen. The formulation and freezing process dictate the ice crystal morphology, size distribution and porosity of the cake. Upon freezing, solutes and solvents are separated, the mobility of the water in the interstitial region reaches zero (25). Generally, freezing can be classified as supercooling, nucleation (ice growth), phase transition and frozen stages. Freezing is also a process that can be damaging to unprotected red blood cells. When a red blood cell formulation is frozen, the water in the extracellular medium freezes out of solution resulting in the concentration of extracellular solute in the unfrozen fraction. Figure 3 With additional cooling, more ice will form extracellularly, and the cell will become increasingly dehydrated. (1) Also the proteins and additives will separate into pockets surrounded by the ice crystals and as the ice nucleates and crystallizes, the protein hydration shell is disrupted. Additionally, the increased concentration of the extracellular solute and protein can lead to aggregation (26). Buffer salts in the formulation become concentrated and lowering the temperature causes decreased solubility of the salts, which eventually lead to their precipitation and separation into the pockets. Therefore, freezing subjects the red blood cell protein to increased concentrations of proteins and excipients from the formulation (27).

Freezing is a crystallization process performed at atmospheric pressure. Freezing step could be more important than drying step because it shapes the cake, determines the drying rate and final products of the cake. Most importantly, it will affect the stability

of various proteins including membrane proteins and thus the red blood cell viability and functionality if not protected properly.

2.4.2.1.1 Low Temperature

Understanding the damages that occurs during exposure of RBCs to low temperatures has been central to the development of protocols for the preservation of these cells for clinical and research purposes. In 1972, the 2-factor hypothesis of Mazur and colleagues (28) summarized the current understanding of the major forms of damage that result from low temperature, a hypothesis that is still valid today (Table 1).

Cooling Rate	Physical Response	Cryoinjury
Slow	Extracellular ice formation	RBC packing (mechanical damage)
	Water loss/volume reduction	Membrane permeability Ion leakage Influx of extracellular solute
	Dehydration/solute concentration	solute toxicity (biochemical damage)
Rapid	Supercooling	Intracellular ice formation (mechanical damage)

Table 1: Physical Changes and Associated Cryoinjury in Response to Cooling Rate

During freezing, low temperature also disturbs the various physico-chemical properties of the RBC proteins and the solvents, such as, the pKa of ionizable groups, the dielectric constant, surface tension, and viscosity of the solvents. The cold temperature also favors the water-solute hydrogen bindings. While the hydrophobic interactions become weaker, the interactions between the amino acid side chain and the polypeptide backbone become disrupted, the proteins reach a maximum exposure of polar groups of proteins, the protein becomes more unfolded (47). Therefore, as a RBC formulation is frozen, it is subjected to stresses that may be sufficient for permanently damaging the red blood cells.

2.4.2.1.2 Instant Concentration Change

As the ice nucleates and crystallizes out, water in the extracellular medium freezes out of solution, resulting in the concentration of extracellular solute in the unfrozen

fraction. Also the hydration shell of proteins is disrupted (29), the increased concentrations of protein tend to aggregate. The buffer salts are concentrated and crystallized; however, the proteins, cryoprotectants and lyoprotectants are concentrated but not crystallized.

There are two main changes of the salts: Lowering temperature causes the decreased solubility of the salts, leading to the precipitating of the salts. Further, precipitating of the salts will cause a significant pH change. Crystallization of disodium phosphate monohydrate during freezing shifts the pH 7 to pH 3.5. However, by selecting the buffer carefully, pH can be maintained at a comparatively more stable level. Buffer crystallization does not occur when the buffer is a minor component compared with the other solutes in the formulation. For example, the potassium phosphate buffer shows less change in pH than sodium phosphate buffer when temperature is decreased (27).

All physical properties related to concentration may change, such as ionic strength and relative composition of solutes, due to selective crystallization. Chemical reaction may accelerate in a partially frozen aqueous solution due to increased solute concentrations (45). The chemical reaction may reach several orders of higher magnitude relative to that in solution. Lastly, the oxygen concentration (ROS) was reported to be 1150 times higher in $-3^{\circ}C$ than in solution at $0^{\circ}C$, which significantly accelerates the oxidation stress on the red blood cells (30).

2.4.2.1.3. Rates of Freezing and Thawing

In accordance with Mazur's two-factor hypothesis (28), the cell damage after cryopreservation (i.e., freezing and thawing) is, among other things, strongly cooling rate dependent. Two opposite conclusions existed whether fast or slow freeze-thawing cause more damage to red blood cells. Some investigators thought that fast freezing caused less damage to proteins by preventing extensive crystal growth and substantially hindering the concentrating effect on salts and other additives. Therefore it can prevent possible further denaturation of the proteins. Red blood cell injury during slow cooling has been correlated with excessive cell shrinkage and toxicity due to the increasing concentrations of solutes (1). Slow freezing will cause protein concentration followed

by the removal of water, bringing the protein molecules into the actual physical contact. New disulfide bonds could form, causing denaturation and aggregation of the proteins. As injury during slow cooling is dependent on the changing solution composition and properties of the cryopreservation media, it is commonly referred to as "solution effects" injury. Pioneering work by James Lovelock in 1953 demonstrated that there was a critical temperature range where intra- and extracellular salt concentrations exceed 0.8 mol/L during freezing, causing irreversible damage to RBCs after prolonged exposure and thawing. (31) Support for the theory of Lovelock that damage to RBCs during freezing and thawing is the result of solution effects has been expounded upon by Mazur et al and Pegg and Diaper (51,52). In the late 1960s, Meryman provided evidence that RBCs can maintain osmotic equilibrium until a minimum cell volume is reached at which time water molecules are unavailable for exchange and the external osmotic pressure gradient results in an irreversible change in membrane permeability, ion leakage, and the influx of extracellular solute. (32) Other evidence suggests that water loss and volume reduction, rather than absolute electrolyte concentration, are responsible for RBC injury that results from slow cooling, perhaps through a mechanical resistance to volume change (53,54). Also, it has been proposed that cell damage is a result of physical forces exerted by interactions with ice crystals(55) and/or the tight packing of RBCs in unfrozen channels(1).

Other researcher got opposite conclusion: Fast freezing leads to a larger ice-liquid interface between the cellular components, proteins and the tiny ice crystals. These ice crystals may act as sites for protein denaturation and particulate formation. On the other hand, it is less possible to aggregate during a slow freezing, because the pure ice crystals are much larger and hence, the surface areas are less. Moreover, the slower cooling causes a lower solidification, the pure water is selectively frozen first, which is followed by the concentrate producing clusters of macromolecules of the proteins. Therefore, during thawing, the outer molecules will shield the inner ones from recrystallization damage (33). Such a protective effect can not be seen during a fast freezing, because the individual protein molecules are trapped in solidifying glass or ice crystals and no protective clusters are formed. Briefly, the mechanisms of cell damage

are solution effects at low cooling rates and intracellular freezing as well as devitrification and recrystallization at high cooling rates.

The rate of thawing influences the cell damage and protein inactivation also. During thawing, it is possible that the glassy state will re-crystallize and a larger ice crystal instead of a smaller ice crystal will grow. A rapid thawing is less detrimental to the protein. A slower warming will cause more recrystallization, hence, cause more damage to the proteins and the cell, due to interfacial tension, shearing forces upon the entrapped macromolecules, or, due to the extraction of the bound water into the growing ice crystals (33).

2.4.2.1.4. pH Changes and Other Factors during Freezing

Extreme pHs, as charges in proteins increase the electrostatic repulsion, cause protein unfolding and denaturation. pH changes during freezing will affect the rates of protein aggregation and chemical degradations. Freezing a buffered protein solution may selectively crystallize one buffering species, leading to pH changes. Disodium phosphate is easier to crystallize due to its lower solubility than that of the monosodium form, which causes dramatic pH changes during freezing. However, potassium phosphate buffer does not show significant pH changes during freezing. Potentially, storage stability of lyophilized proteins can also be affected by pH drop during freezing.

2.4.2.2 During Drying or Desiccation

During the lyophilization process, drying is divided into two phases: primary and secondary drying. The primary drying removes the free frozen water, and secondary drying removes the non-frozen bound water. At the end of primary drying, the left moisture content is about 10-15%, at the end of secondary drying, the water content should be around 3-5% or an optimal level that will provide better stability in the dry state. In protein formulations, typically less than 1% of the residual moisture content remains in the specimen (2). Understanding the physiological effects of desiccation or drying on mammalian cells is essential for the development of methods for inducing or

enhancing desiccation tolerance (2). Unlike plant seeds and certain bacteria, mammalian cells lack natural mechanisms that would allow them to cope with desiccation injuries. It is thought that the ability of cells to retain normal biological activity after desiccation and reconstitution without major changes to the cell proteome is unlikely. This is particularly the case for denucleated cells such as RBCs that have no capacity to up-regulate protein expression or compensate for desiccation-induced damage.

Desiccation stress has tremendous physiological effects on cells, including changes in osmotic pressure, cell volume, membrane properties, shrinkage of cell organelles, enzyme activity, down-regulation of metabolism, increases in intracellular salt concentrations and cell viscosity, and production of stress proteins (2) (Figure 4). In response to desiccation stress, the cell membrane undergoes tremendous changes that may be deleterious to the cells. Rupture of the plasma membrane commonly occurs during freeze-drying of mammalian cells. The extensive loss of water causes the nonaqueous cell components such as membranes to condense, leading to morphological changes from a lipid bilayer conformation to a deleterious hexagonal II phase, which is believed to be involved in the mediation of membrane fusion.(2) Cell shrinkage is the most common reaction to desiccation stress, and it has been proposed that cell damage may be a result of the cells dropping below a minimum critical volume (MCV). But till date the mechanism by which damage occurs when cells undergo excessive cell shrinkage has not been resolved. Another detrimental effect is associated with the loss of membrane surface area and the formation of microvesicles as has been observed with freeze-dried RBCs. (34) The mechanism of desiccation damage in mammalian cells is poorly understood and requires further investigation.

2.4.2.2.1. Removal of Water Molecules

Cells contain numerous proteins that are essential for normal function. These proteins are also subject to dehydration injury, which may result in denaturation and loss of biological activity. (35) Denaturation can occur when the monolayer covering of water on the protein surface (hydration shield) is removed, leading to disruption of the native

shape of the protein as well as protein aggregation.(29) A hydrated protein transfers protons to ionized carboxyl groups during dehydration. As many charges as possible in the proteins will be eliminated (36). The decreased charge density may favor protein-protein hydrophobic interaction to cause protein aggregation.

As mentioned, water molecules are very critical for maintaining an active site in proteins. Removal of the functional water molecules during dehydration inactivates the proteins, For example, lysozyme loses its activity upon removal of those water molecules locating in the active sites (48). Also, dehydration during lyophilization may cause uneven moisture distribution in different locations of the product cake, which potentially cause overdrying and denaturation in part of the product (45).

2.4.2.2.2. Drying Temperature

Both high and low temperature destabilizes a protein. Under high pressure, RNase A denatures below –22°C and above 40°C (16, 17). During freeze-drying, temperature changes dramatically; extra caution should be paid to prevent the proteins from destabilization and to prevent the cakes from collapse. During lyophilization or storage, the protein products should be kept at least 2-5°C lower than the collapse temperature or glass transition temperature (Tg). Excipients that raise Tg should be incorporated into the formulation to decrease molecular mobility.

2.4.3 Red Blood Cell damage during Freeze-drying

2.4.3.1. Hemolysis

Almost all studies reported before 1980s showed that no intact red blood cells where recovered when lyophilization was the preservation method. (24) Red blood cells have no nucleus and the protection of the integrity of the plasma membrane is a major challenge for lyophilization. Although many studies have been reported, researchers have all faced the challenging problem of hemolysis. Some studies showed a high concentration of free hemoglobin in the supernatant, this may have resulted from the damage to the cell membrane during lyophilization and/or during rehydration (15)

2.4.3.2. Oxidative damage

Oxidative stress has been defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage". Oxidative stress has been involved in aging as well as in the pathogenesis of several diseases (atherosclerosis, cancer, neurodegenerative diseases including Alzheimer's dementia, etc.). Very little is known about the extent of oxidative damage that occurs during freeze drying in red blood cells. The evaluation of oxidative damage in dry state is challenging, because most oxidative stress indicators (ROS) are extremely unstable in the dry state.

Mammalian red blood cell is particularly susceptible to oxidative damage (40), because;

1) It is an an oxygen carrier (it is exposed to high oxygen tension)
2) It has no capacity to repair its damaged components
3) The hemoglobin is susceptible to autooxidation
4) It`s membrane components are susceptible to lipid peroxidation

Oxidative stress induces generation of free radicals which can further react with all the cellular macromolecules leading to lipid peroxidation and protein oxidation. Lipid peroxidation can lead to membrane damage whereas protein oxidation can lead to cytoskeleton and cytosolic protein damage. Damage to proteins, particularly when they are enzymes, can lead to impairment of their function.

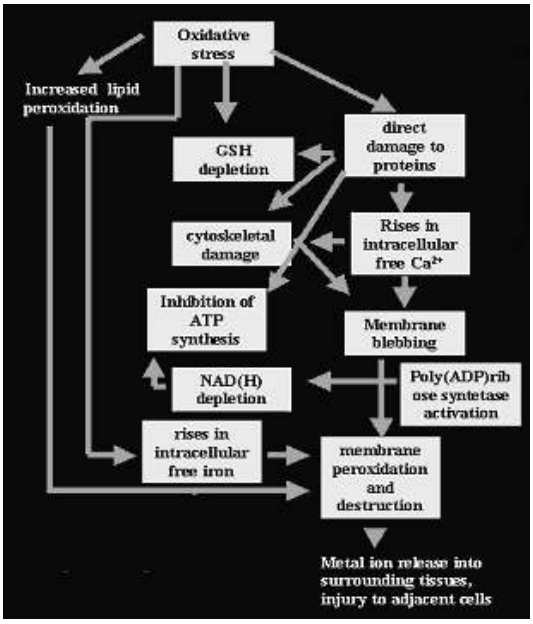

Figure 5: Oxidative damage in red blood cells

Oxidative injury to hemoglobin (Hb) is associated with formation of methemoglobin (MetHb) and degradation to a group of pigments collectively called reversible (rHCRs) and irreversible hemichromes (iHCRS) with concomitant production of superoxide radicals, which can further oxidize themselves, with an increase in accumulation of damaged, nonfunctional proteins (Heinz bodies and Hemin) and/or attack membrane proteins and lipids. The striking membrane damage is evidenced by an increased permeability to potassium, lipid peroxidation and crosslinking of membrane proteins, decreased deformability, and destabilization of protein interaction (37).

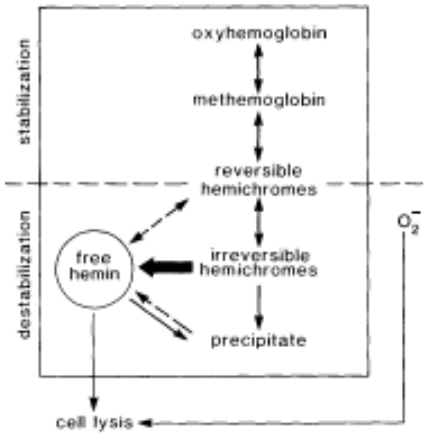

Figure 6: Effect of oxidation products of hemoglobin and Hemin on red blood cell membrane skeleton stability.

The possible Hb content and oxidative alterations occurring in RBC cytoskeletal components in the course of storage in citrate-phosphate-dextrose-adenine (CPDA) have been studied which showed the first evidence for a progressive oxidation of cytoskeletal proteins and accretion of denatured Hb was proportional to the age of storage and suggested a possible role for these modifications in the phenomenon of RBC storage lesion as an Hb- and cytoskeleton-associated pathology (38).

RBCs undergo major biochemical and mechanical changes during storage that are collectively referred to as "RBC storage lesion" and that could affect their after-transfusion performance. Reflecting the storage-induced cellular stress, the membrane of stored RBCs is characterized by various modifications in lipid and protein compartments, such as lipid peroxidation and phosphatidylserine externalization, decline of critical antigenic markers, protein aggregation, membrane-hemoglobin (Hb) association and oxidation. Several of these factors that alter dramatically during storage are potent regulators of membrane skeletal organization. Not surprising, events that are progressively observed in storage include the defective deformability, surface area loss, spheroechinocyte transformation and microvesiculation of red cells that precede the hemolysis of a subpopulation of them (38).

Oxidative damage has been shown to change a number of **RBC properties**.

1) Increased membrane rigidity and decreased RBC deformability can be induced by oxidative cross-linking of spectrin.

2) Oxidative damage can alter membrane permeability and lead to hemolysis.

3) Oxidative damage can also cause immune recognition of RBC.

4) Heinz body formation also takes place; Heinz bodies are refractile, irregularly shaped inclusions precipitated in erythrocytes by polymerization of oxidized hemoglobin.

5) Oxidants can increase membrane fragility by damaging α spectrin **or** protein 4.**1** with a consequent defective formation of the spectrin-4.1-actin complex.

Hence it has been identified that oxidative damage is a major determinant of RBC survival, the detail mechanism of these damages to RBC aging is largely ill-defined.

2.4.4. Stabilizing Mechanisms during Lyophilization

2.4.4.1 Thermodynamic Mechanism of Cryoprotection

Thermodynamically, Arakawa and Timasheff proposed a mechanism to explain the mechanism of stabilizing proteins in solutions, as we discussed (43). Carpenter and Crowe et. al. assumed that the basic mechanism to stabilize proteins in solution still hold because the hydration shell still exist during freezing(44), they extended this proposal to cryoprotection for several reasons: 1) Like in solution, the free energy change will favor the folded states more since the unfolded protein exposes more protein surface to the aqueous environment. 2) The thermodynamic stability is related to degradation. The degradation in the unfolded state is much faster than that in the native state. 3) In the early freezing stage, the freeze concentrate is relatively dilute solution. Carpenter et. al., found that the cryoprotectants that increased the stability of LDH in solution also improve the protein stability after freeze-thawing. However, this mechanism becomes questionable in the later stage of freezing. The preferential exclusion mechanism can not fully explain the cryoprotection provided from proteins and polymers.

2.4.4.2. Diffusion Restriction Mechanism and Other Mechanisms

At the beginning of freezing, initially the rate of chemical reaction may be come high due to the concentrated solutes; however, since freezing increases the solution viscosity rapidly, the rate of chemical reaction will drop gradually (45). Restricting diffusion of reacting molecules by higher viscosity can explain well why trehalose is more effective than sucrose, maltose, glucose or fructose in stabilizing liquid proteins.

2.4.4.3. Water Replacement Mechanism

Crowe *et al*. suggested that the accumulation of intracellular sugars provides defense against desiccation damage by maintaining the membrane lipids in a fluid state when water is absent serving as water substitute when the hydration shell of the proteins is removed. The mechanism was described as “the water replacement hypothesis,” by which soluble sugars form hydrogen bonds with the membrane lipids in exchange for the lost water (2). This action is believed to inhibit deleterious effects such as fusion and

membrane phase separation. FTIR has shown that hydrogen binding occurs between proteins and stabilizing carbohydrates (46).

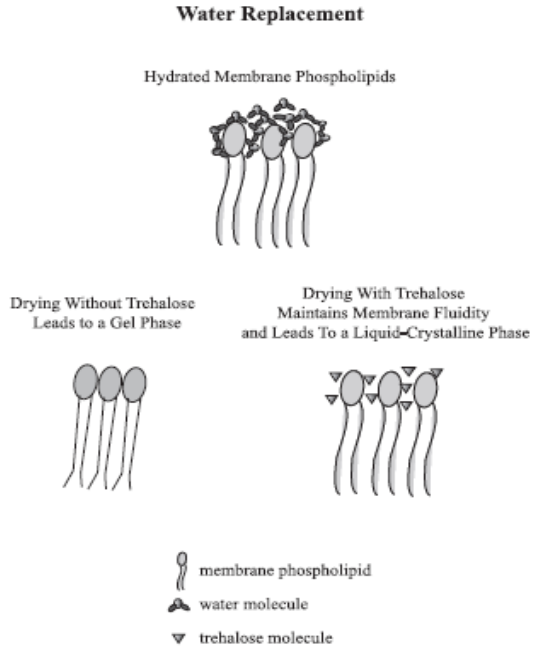

Figure 7: The postulated mechanisms of trehalose in protecting the cell against desiccation damage by "water replacement" mechanism.

2.4.4.4. Single Amorphous State Immobilization Mechanism

Because an amorphous mixture of proteins and stabilizers allows maximal H-bonding between stabilizers and proteins, crystallization of any amorphous protein stabilizers increases protein destabilization due to inefficient hydrogen bonding. Mannitol can be crystallized during lyophilization, which is not good for protein protection (47).

The amorphous excipients are good glass formers but inert. The proteins are sufficiently immobilized that translational motion and relaxational processes are hindered, hence aggregation, unfolding, and certain types of chemical degradation are prevented. Carpenter. et al., argued that single additive partitioning into the amorphous solid is not enough to protect a protein because the protein by itself could form into a glass. Thus, there is a conflict with the "water replacement mechanism" as stated above.

2.4.4.5. Hydration Protection Mechanism

To be stabilized, the proteins need a monolayer of water molecules to form a hydration shell. During heating or drying of lyophilization, the removal of both the hydration shell and the bound water cause inactivation of the proteins. Since a hydrated protein is easier to transfer protons to ionized carboxyl groups, most charges in the protein is removed. The decreased charge density may facilitate protein-protein hydrophobic interaction to cause protein aggregation (3).

Investigators believed that the bound water at the active sites help maintain the activities of the proteins. For example, dehydration of lysozyme caused activity loss due to removal of those water molecules residing functionally in the active site (48). Changing protein hydration with additives can stabilize proteins.

2.4.4.6. Vitrification Mechanism

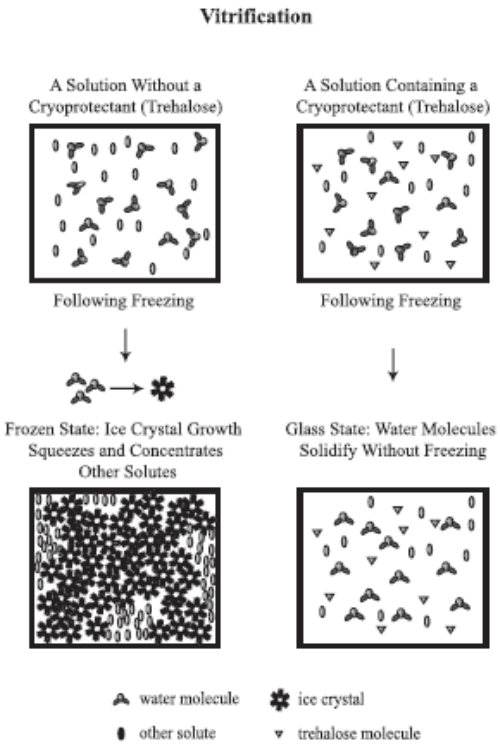

Figure 8: Vitrification mechanism of stabilization by trehalose

Sugars are also believed to protect against desiccation damage by the formation of an intracellular glass. As desiccation progresses, the cell solutes may either crystallize or form into an amorphous solid as a consequence of the increased viscosity. Amorphous solid is more like a liquid than crystalline materials in structure. This amorphous form

of solid “plastic” is termed a glass, and the formation of a glass is termed vitrification. Vitrification is believed to enhance desiccation tolerance by various mechanisms including the dilution of the biomaterials in the glass matrix, which limits their mobility and reduces the probability for chemical and/or physical interactions, and the prevention of cellular collapse by filling space that was once occupied by water(2). The glass formed in such processes can be classified into two types: Fragile and strong glass. Below the glass transition temperature, the viscosity of a fragile glass enhance more than a stronger glass at a given temperature drop (49). Both trehalose and sucrose can form a fragile glass. Excipients forming fragile glasses are better stabilizers during drying (50).

2.4.4.7. WLF (Williams-Landel Ferry) Kinetic Mechanism

Coupling with the “amorphous immobilization hypothesis”, WLF mechanism predicts that the stabilization of proteins during drying proportional to the difference between current temperature (T) and the glass transition temperature (Tg). Therefore, the degradation rate is a function of the variable, T-Tg (45).

2.4.4.8. Scavenger Mechanism and Other Mechanisms

Carbohydrates may protect proteins from oxidative damage in the dry state by acting as a scavenger of radicals that can oxidize them. However, carbohydrates do not protect proteins in solution, which indicates that the antioxidant action of carbohydrates may be limited to the dry state. Excipients can stabilize proteins by preserving a protein’s internal mobility. By forming multiple electrostatic interactions with proteins, polyelectrolytes can stabilize proteins. It has also been observed that the addition of aminophospholipid vesicles, in combination with amphitathic drugs and hypertonic conditions markedly reduced pressure induced red blood cell membrane damage. The membrane lesion leading to Hemolysis of RBCs can be mitigated by addition of liposomes added to the freeze-drying buffer (3).

2.4.5 Strategies to Stabilize Red Blood Cells during Lyophilization

As described above, formulation, freezing temperature, freezing rate, drying rate and rehydration process all affect the stability of red blood cells. During lyophilization, both freezing and drying stabilization should be considered.

2.4.5.1 Buffers

Used in formulation to prevent pH shift and stabilize the red blood cells during freezing, buffer selecting is very important. Generally, different proteins need different buffering agents for maximum stabilization in solid state. pH always changes when temperature shifts. Therefore, before the buffer is made at room temperature, the pH shift caused by temperature under the operating conditions should be considered. In addition, buffer concentration need to be concerned because it affects the ionic strength and Tg of the formulation, during lyophilization and storage.

2.4.5.2. Cryoprotectants

The chemicals, or CPAs, that are used for the cryopreservation of RBC can be classified into 2 major groups based on their mechanism of action and permeability across the plasma membrane. The first group, nonpermeating CPAs, includes sugars, sugar alcohols, polymers, and starches such as hydroxyethyl starch (HES), polyvinyl pyrrolidone (PVP), and polyethylene oxide. These CPAs are usually effective in millimolar concentrations and generally act by dehydrating the cell at high subfreezing temperatures, thereby reducing the incidence of intracellular ice formation and allowing rapid cooling before intracellular solute concentrations reach critical levels.(28,32) Extracellular CPAs may also act by stabilizing membranes and maintaining macromolecules in their native form.(46) Some extracellular solutes prevent RBC lysis in hypotonic environments by promoting RBC leakage of solutes in response to osmotic stress.(53) The second group of CPAs are those chemicals, like glycerol and dimethyl sulfoxide, that permeate into cells. These CPAs protect cells from injury caused by slow cooling by preventing excessive volume reduction and the lethal concentration of electrolytes,thereby reducing or abolishing the temperature at which a critical salt concentration is reached.(31, 53) Permeating CPAs act to depress the freezing point and

lower the chemical potential of a solution, reducing the amount of ice formed at any given temperature.(32) Glycerol is an attractive RBC CPA because it is relatively nontoxic at high concentrations and readily permeates the cell at 37°C. However, post-thaw removal of glycerol is necessary to prevent posttransfusion intravascular hemolysis. Both permeant and nonpermeant CPAs have been used successfully for the cryopreservation of RBCs. Glycerol probably reduces the freezing damage by keeping the salt concentration in the unfrozen phase at a lower concentration(1). It also leaves a greater volume of fluid in the unfrozen phase and modifies the shape of the ice crystal. Therefore, it reduces the mechanical stress of freezing (31). The changes in the nucleation and freezing characteristics may explain protective effects of sugars, polyhydric alcohols and polymers as well.

Sugars provide various cryoprotection. Glucose and maltose at 100mM offer "complete protection". Trehalose, sucrose and galactose provide partial protection, while lactose and proline are ineffective. Trehalose loading is required to achieve the stabilization of hemoglobin (6).

Some cryoprotectants may induce unwanted effects on the structure and function of biomolecules specifically. The toxicity of cryoprotectants limits the concentration of additives that can be used. Sometimes, they play a role in cryoinjury. The cryoprotectant, similar to the presence of ice, may result in dehydration and several toxic effects.

2.4.5.3 Lyoprotectants

Polyhydric alcohols and sugars increased the transition temperature of some proteins in aqueous solution; this stabilizing action was ascribed to a decreased hydrogen bond rupture potency. The positive surface free energy perturbations by sugars play a predominant role in their preferential interaction with proteins and membranes of red blood cells. The exclusion volume of sugars and the chemical nature of the protein surface are also two factors. That's why during drying, sugars especially disaccharides play a predominant stabilizing function. Disaccharides are essential to satisfy the

hydrogen bonding requirement to replace the lost water during sublimation for stabilization by “water replacement” mechanism (2).

However, among the sugars, non-reducing sugars should be selected. Non-reducing sugars will not cause the Maillard reaction during storage. Trehalose, as a nonreducing disaccharide, plays a role in protecting the cytoactivity when the cells are freezing, drying or lyophilization. It has been a biomembrane protectant applied to lyophilization of human blood cells (platelets and erythrocytes), and from which astonishing results have been obtained. Having powerful hydration, distinctive vitrification transform and crystal transform and unique resistance of high temperature and humidification, trehalose is thought of a preferred protectant in the study of cell preservation. In recent years, people concerned trehalose on its protective mechanism, experimental means of transit trehalose to mammal cells and the mechanism of loading in red blood cells. Browning reaction can cause significant destabilization of lyophilized proteins during storage. Even sucrose is a non-reducing sugar, it can be easily hydrolyzed into two reducing sugars either in liquid or solid states: D-glucose and D-fructose, especially at low pH's during storage.

2.4.5.4 Process Optimization

Freezing temperature and freezing rate should be controlled to get homogeneous ice crystals, larger crystals are preferred because less surface areas of ice crystals will protect the protein from shearing, aggregation and interface tension. The freezing period should be long enough to ensure a complete frozen matrix, short enough to prevent cryoinjury. The chamber should be degassed to minimize oxidation. The buffer factor should be minimized. Therefore, when the buffer salts become concentrated, the pH will not shift too significantly.

2.4.6. Formulations to Stabilize Proteins

2.4.6.1. Surfactants

Except sodium dodecyl sulfate (SDS), most surfactants are used for protein stabilization, including polymers, polyols, nonionic and anionic surfactants. The mechanisms include: binding to the proteins and reducing the proteins' available hydrophobic surface areas, therefore decreasing the proteins' self-association and any

deleterious interactions with non-specific hydrophobic surfaces; preventing surface-induced deactivation of proteins; inhibiting aggregation and precipitation (24).

Nonionic species such as Tween and pluronic can prevent proteins from adsorption onto the surfaces, to inhibit aggregation and precipitation , hence denatuartion(24).

However, some reports showed that surfactants are effective protein stabilizers during lyophilization but not good stabilizers for long-term storage. A study showed that Tween 80 could inhibit aggregation of FIX during freeze-thawing, but could not protect lyophilized proteins during storage. Studies also showed that Tween 20 and Tween 80 at various concentrations inactivated proteins stored at 40°C, 60°C (24). It has been stated clearly that the use of any surfactant in any formulation must be carefully considered and restricted to the lowest levels possible because of possible toxicity and hypersensitivity reactions.

2.4.6.2 Sugars/Polyols

Polyols includes a class of excipients like sugars (e.g., mannitol, sucrose, and sorbitol), and other polyhydric alcohols (e.g., glycerol and propylene glycol). They are both cryoprotectants and lyoprotectants. Polyols can protect proteins from both physical and chemical degradation pathways. Concentrations of sugars /polyols will determine the degree of stabilization. Lower concentrations of sugars/polyols may or may not have any great effect during lyophilization. At 5 to 100 mM, both trehalose and glucose could not stabilize proteins to a desired degree (44), Arakawa recommended a concentration of 0.3M as the minimum to achieve significant stabilization during freeze-thawing (43).

Other sugars/polyols which can stabilize proteins during freeze-thawing include lactose, glycerol, xylitol, sobitol, mannitol at 0.5-1M (44), as well as of sucrose, maltose, glucose or inositol. Higher concentrations of sugars/polyols also are for lyoprotection. Till now, dissacharides (such as trehalose, sucrose, maltose and lactose) are the best stabilizers, equal to or better than monosaccharides (such as glucose, galactose) in stabilizing proteins without the millard reaction (19).

Among the most commonly used disaccharides (sucrose and trehalose), trehalose is preferable for biomolecules due to its higher glass transition temperature. The advantages of trehalose can summarized as: 1) More flexible formation of hydrogen bonds with proteins due to the absence of internal hydrogen bonds; 2) less hygroscopicity; 3) Low chemical reactivity; 4) Prevention of water to plasticize the amorphous phase partly by forming trehalose-protein-water microcrystals (6). However, sucrose can be as effective as trehalose, depending on both the protein and sugar concentration. In addition, they found that the most effective lyophilization solutions contained membrane stabilizing agents capable of permeating the membrane, such as carbohydrates and polyols, as well as high-molecular-weight polymers (1).

2.4.6.3 Polymers/Proteins

The mechanisms of polymers/proteins stabilization are varied, such as: Surface activity, preferential exclusion, steric hindrance of protein-protein/polymer interactions, and limited protein structural movement due to increased viscosity.

The most extensively used polymers in RBC and platelet cryopreservation and lyopreservation are hydroxyethyl starch (HES), dextran, polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), and albumin (24). With the exception of PEG, all of these colloids have been used as plasma replacement fluids. Because of this, it has been generally assumed that they are ideal for use in the development of frozen or freeze-dried blood products that can be transfused without cryoprotectants removal.

PVP and PEG have been explored both as cryoprotective and lyoprotectant agents for RBC freezing procedures. PVP has been recognized as a polymer that can provide significant advantages in the preparation of lyophilized RBCs(64). PEG on the other hand is a key component in the development of various blood substitutes. PEG-modified hemoglobin and PEG modified liposome-encapsulated hemoglobin have been shown to have few side effects and prolonged persistence in the circulation compared with their unmodified counterparts (24).

The inclusion of a polymer with a high glass transition temperature is necessary to offset the low glass transition temperature of the low MW cryoprotectants. Dextran was reported to stabilize proteins by increasing Tg and inhibiting crystallization of small

stabilizing excipients, such as sucrose. However, Dextrans at very high concentrations destabilizes proteins due to its inability to form enough hydrogen bonding with the proteins (24). PEG 3350, dextran, PVP and BSA can dramatically inhibit the pH drop during freezing and inhibit the crystallization of small molecules due to increased polymer-induced viscosity (24). Similarly as the glass transition temperature of a compound is a direct function of its MW, HMW-HES and MMW-HES are preferred over other polymers in lyophilization media striving for high glass transition temperatures (24).

2.4.7 Hydroxyethyl Starch as a Lyoprotectant

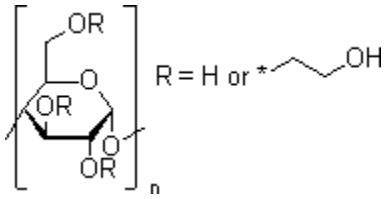

Figure 9: Structure of hydroxyethyl starch.

Sowermimo and Goodrich, 1992-1993, first reported that red blood cells (RBCs) can be preserved in the dry state by addition of mixtures of hydroxyethyl starch (HES) and glucose (15, 14). Spieles *et al.*attempted to freeze- dry red blood cells in the presence of HES and reported that the cells were completely destabilized during the freeze-drying and rehydration. Franks attacked the Goodrich *et al.* results, calling them a ‘‘confidence trick.’’ Curiously, both Spieles *et al.* and Franks ignored the report of Goodrich *et al.* that HES is in itself insufficient for the preservation; a monosaccharide in practice, glucose) is required as well (60). Hydroxyethyl starch (HES) is a modified natural polysaccharide obtained from maize and potatoes, and, by its chemical structure, is similar to glycogen (23). HESs are polymers of glucose units derived from amylopectin and modified by substituting Hydroxyethyl for hydroxyl groups on glucose molecules. The presence of hydroxyethyl groups contributes to highly increased solubility and resistance to hydrolysis of the compounds by plasma amylase, delaying its degradation and elimination from the circulation compared with underivatized starch; thus, it can function as a plasma volume-expanding agent (24).

Beside of solute concentration and weight averaged mean MW (the arithmetic mean of MW of all HES molecules), the pharmacokinetics of HES is, in contrast to other colloids, influenced also by other physical-chemical characteristics: MS, the molar ratio of the total number of hydroxyl ethyl groups to the total number of glucose units; DS, the degree of substitution defined as the ratio of substituted glucose units to the total number of glucose molecules; and, the C2/C6-ratio, which is the ratio of the number of substituted hydroxyl ethyl groups in glucose molecule in C2 position to the number of hydroxyl ethyl groups in C6 position. Higher molecular weight and more extensive degree of substitution result in slower elimination. The C2/C6 ratio is a factor which modifies HES resistance for degradation by alpha-amylase, and possible responsible for its side-effects (*e.g.* accumulation, tissue accumulation, bleeding complications). The water binding capacity of HES ranges between 20 and 30 ml/g, and the expanded volume initially is higher than the volume infused. However, following the infusion of HES, larger molecules rapidly undergo amylase-dependent breakdown, and molecules smaller that 50-60 kDa are eliminated by glomerular filtration. Anaphylaxis is not frequent, and the reported incidence is of less than 0.1%.

The advantages of Hydroxyethyl starch as a lyoprotectants for RBC Biopreservation are that it is a well established plasma substitute and therefore does not need to be removed after thawing (Knorpp et al., Science 157, 1312, 1967). Moreover, in the case of hypovolemia, it serves as a plasma substitute. After numerous in vitro (Cryobiology 27, 667, 1990) and animal experiments (Cryobiology 28, 546, 1991), autologous studies were carried out on 7 healthy volunteers (Cryobiology 30, 657, 1993; CryoLetters 16, 283-288, 1995).

HES is the most commonly used high–molecular- weight cryoprotectant used in freezing and freeze-drying protocols. All types of HES including HMW-HES, MMW-HES, and LMWHES have been used in the development of RBC freezing and freeze-drying procedures. For investigators developing lyophilized red blood cells, HMW-HES or MMW-HES might appear to be the right choice for an extracellular protectant. Lyophilization media often are developed according to the glass transition theory. They

are usually mixtures of low MW carbohydrates and high MW polymers. The inclusion of a polymer with a high glass transition temperature is necessary to offset the low glass transition temperature of the low MW cryoprotectants. The latter assures successful cake formation during freeze drying and higher product stability. Because the glass transition temperature of a compound is a direct function of its MW, HMW-HES and MMW-HES are preferred over LMW-HES in lyophilization media striving for high glass transition temperatures.

2.4.8 Research Work done so far

Because sugar has extensive protective effects on the freezing or lyophilization of biological cells, people began to use these sugars to preserve mammalian cells by freezing or lyophilization (4). Based on this theory, human platelets have been successfully lyophilized after being loaded with the disaccharide trehalose; when rehydrated these cells are capable of responding to physiological agonists (65). Recently, a method was developed for freeze-drying human platelets; the rehydrated cells were capable of responding to physiological agonists and were stable during storage in the dry state, resulting in more than 90% recovery after 2 years (18).

Meryman 1960 (20) and MacKenzie 1971 (21) did early work on lyophilization of RBCs focusing on the use of extracellular agents such as dextran, PVP, PEG to protect cells during freezing, drying and subsequent rehydration. The successful recovery of RBCs and encouraging posttransfusion survival in rats suggested that clinical use of lyophilized RBC was feasible. All other studies before 1980s showed that no intact RBCs were recovered when lyophilization was the preserving method. Goodrich et al. 1990s reported successful rehydration of RBCs that had been stored at ambient temperatures for 7 days at a low moisture content (1-2%) with acceptable maintenance of RBC metabolic, cellular, and rheological properties (14). Goodrich Jr et. al. 1992 reported the recovery of metabolic functions in lyophilized RBCs, but the water content was about 25-30%. (14) Rindler et al. 1999 adopted HES and maltose as protective agents, and investigated the effect of shelf temperature on the survival rates of lyophilized RBCs, and also provided various strategies to improve cell survival after lyophilization (16, 17). He concluded that the highest recovery rate of RBCs was

obtained at a cooling rate of 220 K/min and at cooling temperature of -35°C. Weinstein et al.investigated the "behavior" of rehydrated erythrocytes after freeze drying by re-infusing them into the original donors, and discovered that the cells survive normally in the circulation with no adverse clinical affects of re-infusion except some slight decrease in deformability of the cells(66). Jiang et al. carried out some research on the effect of the process (freezing and drying condition, residual water content) on recovery of protein activity after freeze drying (67). Zimmermann et al.investigated the influence of different parameters of lyophilization (*e.g.*, the protective effect of CPAs, freezing velocity, and thermal treatment) (68). Furthermore, Crowe's group reported that trehalose loaded platelets were successfully freeze dried, with an excellent recovery. Rehydration from the vapor phase led to a platelet survival rate of 85% (65). Also, Trehalose loaded RBCs lyophilized in the presence of liposomes demonstrated high survival and low levels of methemoglobin during 10 weeks storage at 4 °C in the dry state. (3) Satpathy and Torok et al. reported that introduction of trehalose into RBCs significantly increased cell survival (45% hemolysis) during freeze drying. Han et. al. reported HSA (25% wt/vol) had a positive effect on cell survival (70% Hb recovery) during lyophilization and also that extracellular sugar had no positive effect on RBC survival. (4)

MATERIALS AND METHODS

3.1. General

This chapter describes materials used and principle and protocols for the various experimental setup for experiments of Red blood cells in different formulation and freeze thawing conditions and later on the viability and biochemical investigation of the cells in presence of the excipient Hydroxyethyl Starch.

3.2. Chemicals and Reagent

All chemicals used for buffers and reagent were of analytical reagent grade. Hydroxyethyl starch and Thiobarbituric acid were purchased from Sigma Aldrich all other chemicals were obtained from Merck Specialities Kolkata and Himedia, Mumbai, India (Annexure–II). Liquid nitrogen was obtained from Cryogenics Laboratory, Department of Mechanical Engineering of our institute. Bovine Serum Albumin (BSA) was purchased from Himedia, Mumbai, Drabkin's reagent and Hemoglobin standard reagent was purchased from Crest Biosystems. All reagents were prepared in double distilled water. Blood was donated by adult volunteers of our department and was collected in Blood bank of Community Welfare Society Hospital, Rourkela, Orissa.

3.3. Glassware, Plasticware and Apparatus

All glass wares (Conical flasks, Measuring cylinders, Beakers, Petri plates and Test tubes etc.) are purchased from M/s Bhattacharya & Co. Ltd (Kolkata, India) under the name Borosil and all plastic wares (sterile pipettes, centrifuge tubes, Parafilm) were purchased from Tarson. The 0.22µm filter unit with Durapore® PVDF membrane was purchased from Millipore. The instruments and apparatus used throughout the experiments are listed in Annexure-I.

3.4. Buffers and protective (freezing) formulations

Phosphate buffered saline (PBS) (300mOsmol, pH 7.2) was used as an isotonic buffer for the formulations. ADSOL (462mOsm, pH 7.2) was used as a preservative solution in the formulations. Freezing solution or formulation used for the freeze thaw cycles contained 100mOsmol ADSOL and 6.6mM K-phosphate (pH 7.2). All buffers were prepared using double distilled water and were filter through 0.22µm Millipore filter unit. The protective solution or formulation also contained Hydroxyethyl Starch along with ADSOL or PBS. The isotonic PBS (pH 7.2) and ADSOL were used as base solution. Eight freezing buffers were tested which included ADSOL, 5% HES and ADSOL, 10% HES and ADSOL, 15% HES and ADSOL, PBS, 5% HES and PBS, 10% HES and PBS and finally 15% HES and PBS. Unless indicated otherwise by the terminology or the context, all concentration percentages are expressed as weight/volume (0.01 g/mL).

Table 2: Composition of buffers and protective solutions used.

PBS (300mOsm, pH7.2)	ADSOL (462mOsm, pH7.2)	ADSOL (100mOsm, pH7.2)
154mM NaCl	111mM glucose	24mM glucose
1.06mM KH_2PO_4	2mM adenine	0.43mM adenine
5.6mM Na_2HPO_4	154mM NaCl	33mM NaCl
	41mM mannitol	8.9mM mannitol

3.5. Blood Sample collection

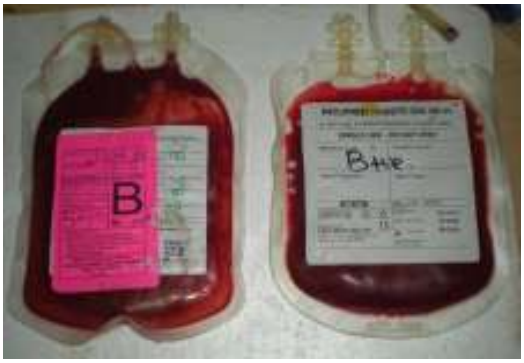

Figure 10: Blood collected in CPDA bags from CWS Hospital.

Whole human blood was drawn in citrate-phosphate-dextrose bags from healthy, adult volunteers after obtaining informed consent according to approved institutional protocols. The blood was collected from adult volunteers from our Department in the blood bank of Community Welfare Society Hospital, Rourkela, Orissa and processed immediately.

3.6. Red blood cell isolation

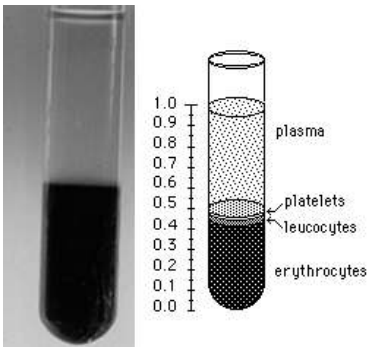

Figure 11: Red blood cell separation after centrifugation.

The cells were washed three times in PBS (pH 7.2) by centrifugation at 1 kg for 10 min at 4°C to remove the buffy coat and plasma. After each spin, RBCs were collected from the bottom portion of the packed red cells, and the buffy coat along with the upper layer of RBCs was left behind. Erythrocytes were separated from platelet rich plasma by centrifugation at 329g for 14 min; afterwards the cells were washed three times in 300 mOsm, pH 7.2, cold PBS by centrifugation at 515g for 10 min to remove the buffy coat and remaining plasma. A small portion of the top layer was removed at each washing and erythrocytes were collected from the bottom portion of packed red cells. The washed erythrocytes were stored in 462 mOsm ADSOL, pH 7.2 at 4°C with a hematocrit (Hct) of approximately 60-65% and used within 2 days.

3.7. Preliminary experiment: Hypothermic storage in ADSOL and PBS solutions

The freshly isolated red blood cells were stored in 300 mOsm, pH 7.2 Phosphate buffer saline (PBS) and 462 mOsm, pH 7.2 ADSOL for 2 weeks to investigate the changes in viability, functionality and biochemistry of red blood cells in these solutions during hypothermic preservation at 4°C.

3.8. Freeze Thaw Experiments

Red blood cells were prepared for freezing in eight different formulations (Table 3) with a hematocrit of 25% approximately. 4ml of each cell suspension prepared with the eight different formulations were placed in vials of 5 mL capacity. They were dipped in liquid nitrogen container to attain a temperatures lesser than -100°C. The temperature decrease

was monitored using temperature probes for the duration the samples are dipped in liquid nitrogen. After that one third of the RBC samples in all the eight different formulations were thawed in a water bath at 60°C for 30 minutes. Another one third of the RBC samples of all the eight different formulations were thawed at room temperature for 30 minutes. Finally, the remaining RBC samples in different formulations were allowed to thaw at 4°C for 30 minutes.

Table 3: Formulations and conditions for freeze thaw experiments.

Preservation solution	Amount of Hydroxyethyl starch				Thawing condition		
ADSOL (462 mOsm, pH 7.2)	0% (Control)	5%	10%	15%	4°C	37° C	60°C
PBS (300 mOsm, pH 7.2)	0% (Control)	5%	10%	15%	4°C	37° C	60°C

3.9. Cell viability Assay: Percentage Hemolysis using Drabkin's Reagent

PRINCIPLE

Potassium ferricyanide converts the hemoglobin in the sample to methemoglobin. The methemoglobin further reacts with potassium cyanide to form a stable cyanmethemoglobin complex. Intensity of the complex formed is directly proportional to the amount of hemoglobin present in sample.

During hemolysis the red blood cells break open and release hemoglobin in the surrounding fluid. Therefore to measure the hemolysis in red blood samples, the amount of hemoglobin in cell suspension (total Hb) and in the supernatant (free Hb) after pelleting the cells by centrifugation (1960g for 1 minute) can be measured to quantify the percentage of hemolysis in the sample using the formula:

$$\% \text{ Hemolysis} = 100 \text{ X } (OD_{540} \text{ of the free Hb}) / (OD_{540} \text{ of the total Hb}).$$

PROTOCOL

For hemoglobin assay 20 ml of blood added to 4ml of Drabkin's solution. Incubated the sample at room temperature for 5 -10 mins and readings were taken at 540 nm in a spectrophotometer. Haemoglobin values calculated from a standard curve prepared using HiCN standard solution. Measurements of total Hb (g/l) were also used in the calculation of Thiobarbituric acid reactive substances (TBARS) in the lipid peroxidation assay (39).

Percentage hemolysis of the samples was determined by converting hemoglobin to cyanmethemoglobin using the Drabkin's reagent (Crest Biosystems) and measuring the absorbance of cyanmethemoglobin at 540nm. The percent hemolysis was calculated using the following formula mentioned earlier (6).

3.10. Oxidative stress parameters

3.10.1. Hemoglobin Oxidation Assay

PRINCIPLE

Hb has a characteristic absorption spectrum. (1) choleglobin is indicated by an increase in absorbance at 700 nm, (2) met-Hb gives a shoulder at 630 nm, (3) ferryl Hb is distinguished from met-Hb by its lack of a shoulder at 630 nm, (4) oxy-Hb at 577 and 542 nm bands, (5) hemichrome gives a shallower trough at 560 nm (58). Thus, any change in the characteristic absorption spectrum of Hb reflects the changes in the spin state of iron hem. Absorbance of this spin state band gives a clear report about hem-hem interaction and consequently its affinity to O2 and its delivery to tissue. Therefore, studying Hb oxidation by spectral analysis is advisable to record spectral change over the range of 200–700 nm and attempt to recognize features which are characteristic of different products**.**

Figure 12: Hemolysate preparation for HB oxidation assay, A- Hemolyzed RBC in ice cold water; B- intact RBC sample.

PROTOCOL

The percentage of oxidized hemoglobin (methemoglobin) in RBC suspensions was determined spectrophotometrically using the millimolar extinction coefficients of the

different Hb types (oxyhemoglobin, methemoglobin, hemichromes) at pH 7.4. Hemolysate was prepared using ice cold distilled water so as to adjust the concentration of Hemoglobin to 4×10^{-5} M. Briefly, RBC lysates were scanned from 500 to 700 nm while recording the absorbance values (OD) at 560, 577, 630 and 700 nm. The concentrations (mmol/l) of the different Hb types were then calculated as follows:

$$[\text{OxyHb}] = 29.8(OD_{577}-OD_{700})-9.8(OD_{630}-OD_{700}) -22.2(OD_{560}-OD_{700})$$

$$[\text{MetHb}] = 7(OD_{577}-OD_{700}) + 76.8(OD_{630}-OD_{700}) - 13.8(OD560-OD_{700})$$

$$[\text{Hemichromes}] = \_33.2(OD_{577}-OD_{700})-36(OD_{630}-OD_{700})+ 58.2(OD_{560}-OD_{700})$$

3.10.2. MDA (Lipid peroxidation) Assay

PRINCIPLE

Lipid peroxidation of biological membranes is one of the most studied indicators of oxidative stress. The process initiates when reactive oxygen species (ROS), remove a hydrogen atom from methylene carbons of fatty acid side chains of Polyunsaturated fatty acids (PUFAs) resulting in lipid radical which reacts with molecular oxygen to yield peroxyl radicals. Peroxyl radicals propagate the oxidative process by removing hydrogen atoms from adjacent fatty acids, and consequentially create various lipid hydroperoxides which further decompose to secondary lipid peroxidation products such as malondialdehydes (MDAs). MDAs have been widely used as lipid peroxidation indicators. MDAs have been shown to have adverse effects on cell integrity as shown by membrane damage and Hemolysis in red blood cells (RBCs).

This assay was introduced by Kohn and Liversedge and modified by Tamir Kanias (39). The detection of MDAs in biological specimens is based on the principle of the reaction between MDAs with thiobarbituric acid (TBA), which requires a low pH and heat. The resulting chromophore is named thiobarbituric acid reactive substances (TBARS), and can be quantified spectrophotometrically at 532 nm.

PROTOCOL

MDA was extracted from RBC samples by mixing with Trichloro Acetic acid in a ratio of 2:1. Then they were vortexed well and incubated for 10 min at 20°C after which the samples were centrifuged at16100g for 10min at 18°C to extract the MDA extracts. After

centrifugation supernatants were collected and mixed with Thiobarbituric acid in 4:1 ratio and then incubated in boiling water for 15min in screw capped boiling tubes. Immediately after boiling, samples were cooled on ice for 10 min. TBARS thus formed were quantified spectrophotometrically at 453nm and 532nm and values determined using a MDA standard curve.

3.11. Antioxidant status: Catalase Assay

PRINCIPLE

Catalase (CAT, H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6) is an enzyme that decompose hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (H_2O). This enzyme is found in peroxisomes and endoplasmic reticulum in hepatic and muscle cells and cytosol and cell membrane in erythrocytes. It was found that catalase and glutathione peroxidase were equally active in detoxification of H_2O_2 in human erythrocytes. As an antioxidant defense mechanism, Catalase enzyme can protect the cells from oxidative damage and thus, can be used as antioxidant marker. If the activity of this enzyme is decreased in the erythrocytes, then they will be susceptible to oxidative damage (41). In this assay Catalase enzyme is assayed by following the disappearance of hydrogen peroxide spectrophotometrically at 240 nm as described by Beers & Sizer (42).

PROTOCOL

Preparation of enzyme extract: Hemolysate preparation

The hemolysate was prepared by pipetting out 1 ml of washed red blood suspension in ice cold distilled water which was further diluted by phosphate buffer [pH 7.0].

Determination of enzyme activity

Catalase activity was measured spectrophotometrically at 25^0C by recording the change in absorbance at 240 nm (42) due to the conversion of H_2O_2 to the H_2O_2 and O_2. In the solution containing 0.1ml of hemolysate (enzyme extract), 2.9ml of substrate solution containing 0.036% H_2O_2 prepared in 50mM PBS, pH 7.0 was added. Enzyme activity was calculated using the following formula:

$$\text{Units/ml enzyme} = [(3.45)\ (df)] / [(min)\ (0.1)]$$

Where, 3.45 corresponds to the decomposition of 3.45 micromoles of hydrogen peroxide in a 3.0 ml reaction mixture producing a decrease in the A_{240} of 0.05 absorbance units, df is the dilution factor, min is the time in minutes required for the decrease in the A_{240} of 0.05 absorbance units and 0.1 = Volume (in milliliter) of enzyme used.

RESULTS AND DISCUSSION

4.1. Preliminary experiments: Hypothermic storage in ADSOL and PBS solutions

In order to study the various changes taking place during hypothermic (4°C) storage in the two different solutions, namely 300mOsm pH 7.2 PBS and 462mOsm, pH 7.2 ADSOL, freshly isolated red blood cells were suspended in the two solutions to a hematocrit of around 50%. The time of suspension in the two solutions was taken as zero hours and viability assay (percentage hemolysis) and oxidative assays (lipid peroxidation, hemoglobin oxidation and catalase activity) were performed successfully after 12 hours interval for four days and final readings were calculated after two weeks as shown in Table 4.

Table 4: Results of Hemolysis, Methemoglobin, TBARS and Catalase assay during preservation in ADSOL and PBS for 2 weeks.

Time (hrs)	Protective solution	Hemolysis (%)	Met Hb (% Hb)	TBARS (nmol/gm Hb)	Catalase (Units/ml)
0	ADSOL	0	1.6	2.01	790
	PBS	0	0.33	1.38	718.75
12	ADSOL	0	1.7	2.9	784.1
	PBS	0	3.6	9.7	690
24	ADSOL	0	1.8	3.4	750
	PBS	0	3.8	11.5	638.9
36	ADSOL	0	2.1	5.2	690
	PBS	0	4.5	13.6	575
48	ADSOL	0	2.6	6.2	668.5
	PBS	0	4.8	14.3	420.7
60	ADSOL	0	3.4	6.96	663.9
	PBS	0	5.3	16.0	383.3
72	ADSOL	0	3.8	8.2	644.8
	PBS	8.5	6.1	18.1	261.4
84	ADSOL	4.9	4.8	13.4	582.7
	PBS	32.5	10.4	20.1	172.5
96	ADSOL	13.9	4.9	13.7	579.2
	PBS	95.45	13.3	23.1	152.7
336	ADSOL	23.8	8.2	14.1	560.7
	PBS	97.32	15.7	25.6	145.0

4.1.1 Cell viability assay: Percentage Hemolysis

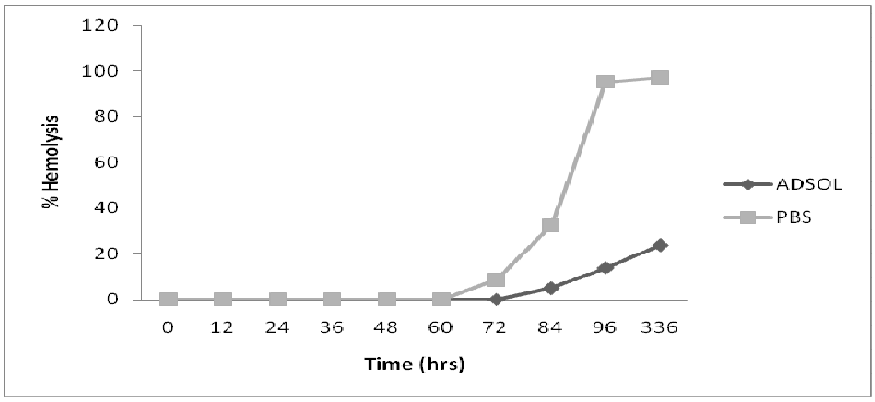

Figure 13: Percentage hemolysis in RBC samples during hypothermic storage in ADSOL and PBS.

Percentage hemolysis during hypothermic storage of red blood cells in ADSOL and PBS was measured using Drabkin's reagent. Hemolysis was measured as a function of time for two weeks as shown in Figure 13. A significant difference in the percentage hemolysis was observed for both the solutions and it was found out that after 72 hours of storage the cells in PBS hemolyzed rapidly compared to the cells in ADSOL solution. After 336 hours (2 weeks) the percentage hemolysis of cells in PBS was measured to be 97.32% whereas in case of cells in ADSOL it was only 23.8%.

4.1.2 Hemoglobin Oxidation Assay

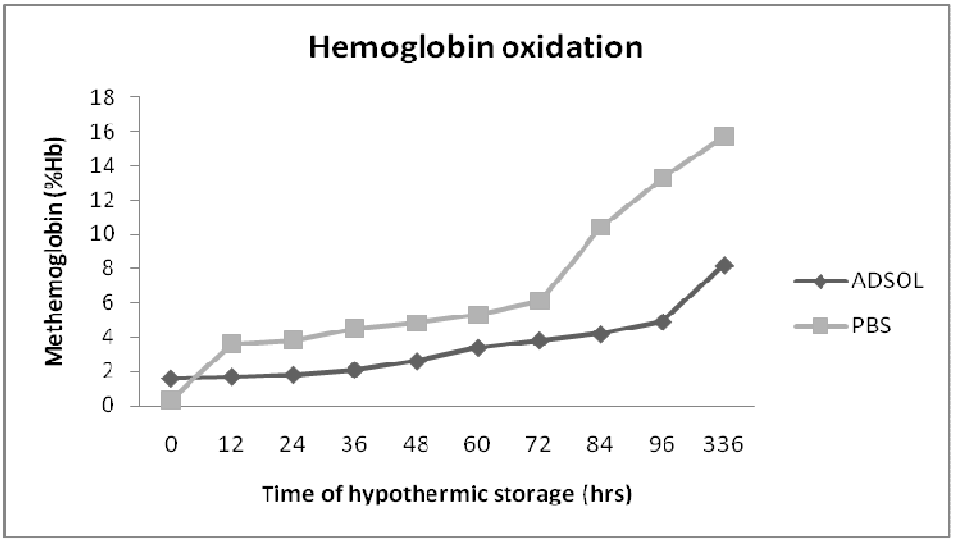

Figure 14: Hemoglobin oxidation in RBC samples during hypothermic storage in ADSOL and PBS.

Hemoglobin oxidation was investigated for hypothermic storage of RBCs in ADSOL and PBS by quantifying percentage of hemoglobin present as methemoglobin in the hemolysates spectrophotometrically. Figure 14 shows methemoglobin formation as a function of time in the RBCs stored at 4°C. As seen in case of hemolysis, methemoglobin formation also varied significantly for RBCs stored in ADSOL and PBS. It was observed that from initial hours, the methemoglobin percentage in RBCs stored in PBS increased abruptly compared to that of cells in ADSOL and it further increased after 72 hours of storage and finally after 336 hours the percent of methemoglobin in case of PBS had reached 15.7% compared to 8.2% in cells preserved in ADSOL.

4.1.3 Lipid peroxidation Assay

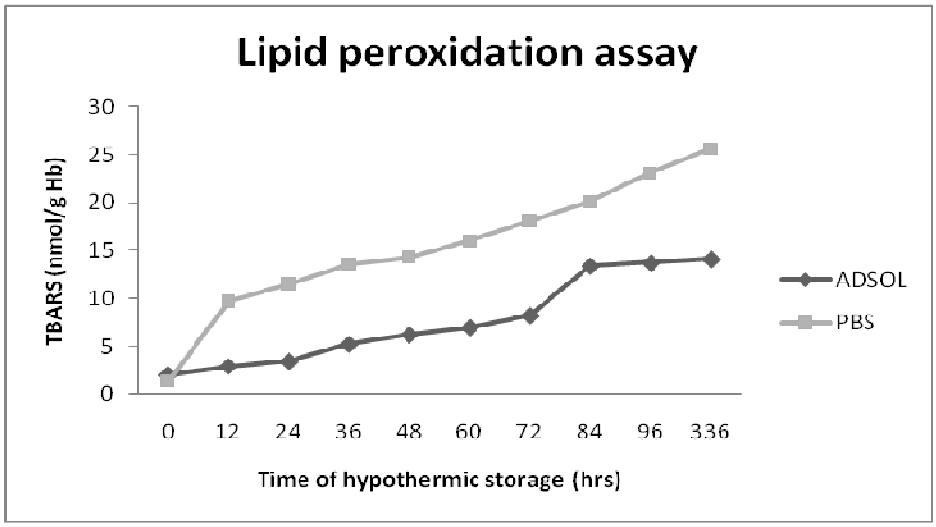

Figure 15: Lipid peroxidation in RBC samples during hypothermic storage in ADSOL and PBS.

Lipid peroxidation was quantified in the RBC samples preserved in ADSOL and PBS at 4°C by measuring the TBARS spectrophotometrically and by comparing with MDA standard graph. Figure 15 shows the increase in TBARS in both the samples, the increase in case of cells preserved in PBS was seen to be increasing almost linearly compared to that of cells in ADSOL and after 336 hours it was found to be 25.6 nmol/ gm of hemoglobin. Whereas in case of cells preserved in ADSOL there was an abrupt increase in TBARS after 72 hours which became constant in the later hours and the value was found to be 14.1 nmol/gm of hemoglobin.

4.1.4 Catalase Assay

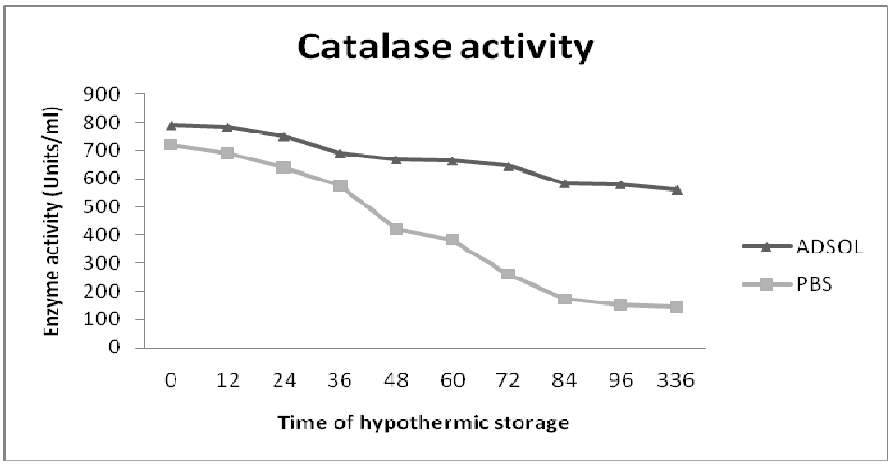

Figure 16: Catalase activity in RBC samples during hypothermic storage in ADSOL and PBS.

The catalase activity of red blood cells preserved in ADSOL and PBS at 4°C was quantified using catalase assay using hydrogen peroxide as substrate (H_2O_2). In case of cells preserved in PBS significant degradation of catalase activity (80%) was observed, it decreased from 718.75 units/ml enzyme to 145 units/ml enzyme wheras in case of cells preserved in ADSOL the catalase activity degraded but to a very lesser extent compared to cells in PBS, the activity in case of cells in ADSOL decreased from 790 units/ml enzyme to 560 units/ml enzyme which was around 30% decrease.

4.2. Freeze thaw experiments

Successful freezing was performed for red blood cells in eight different formulations using liquid nitrogen and later thawing was done at different temperature conditions namely 4°C, 37°C and 60°C. After the freeze thaw experiments different viability and functionality assays were performed successfully as described in the following sections.

4.2.1 Cell viability assay: Percentage Hemolysis

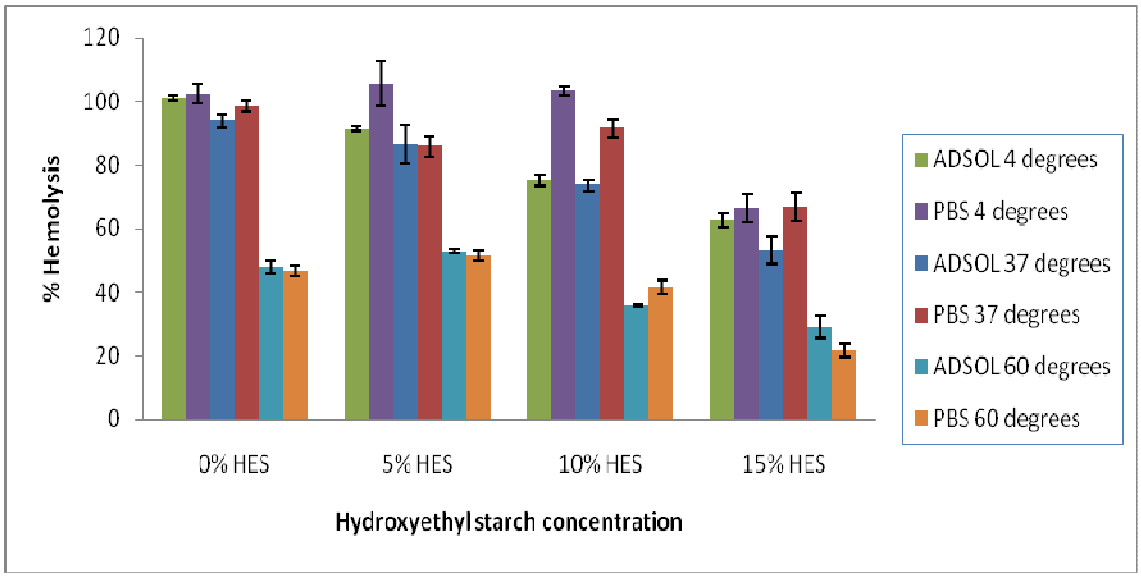

Figure 17: Percentage hemolysis in RBC sample stored in ADSOL and PBS with 0, 5, 10, 15% HES after freeze thawing at different conditions.

Percentage hemolysis for all the eight formulations after undergoing different thawing conditions was measured using Drabkin's reagent. Percentage hemolysis for all the samples was found to be more than 30% (Figure 17) which is quite normal as the same extent of hemolysis has also been reported during normal freeze thaw of red blood cells. This discrepancy is attributed to different damaging mechanisms involved with the respective sample processing subsequent to freezing or to increased devitrification and recrystallization at supraoptimal cooling rates for freezing. In four of the test samples the percentage hemolysis was found to be 100%. Percentage hemolysis for all the formulations after 60°C thawing was found to be the least and highest was found in case of 4°C thaw. Also, the least amount of hemolysis was seen in case of formulation having 15% HES.

4.2.2 Hemoglobin Oxidation Assay

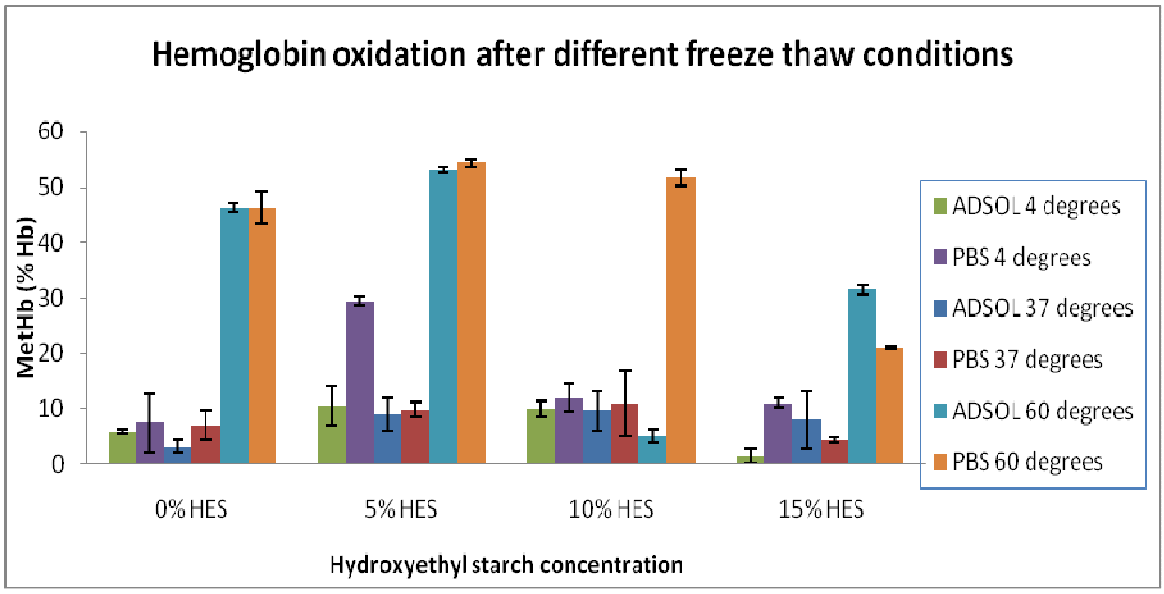

Figure 18: Hemoglobin oxidation in RBC sample stored in ADSOL and PBS with 0, 5, 10, 15% HES after freeze thawing at different conditions.

Hemoglobin oxidation was measured by spectrophotometrically quantifying the amount of methemoglobin formation after freezing and different thawing conditions as shown in Figure 18. Significant amount of increased methemoglobin was observed in case of 60°C thawing condition for all of the formulations used. Least amount of methemoglobin was observed in case of cells in 15% HES in ADSOL which was thawed at 4°C, which was 1.55% hemoglobin whereas the maximum amount of hemoglobin oxidation was found to be in 5% HES in PBS which was thawed at 60°C which was 54.29% hemoglobin.

4.2.3 Lipid peroxidation Assay

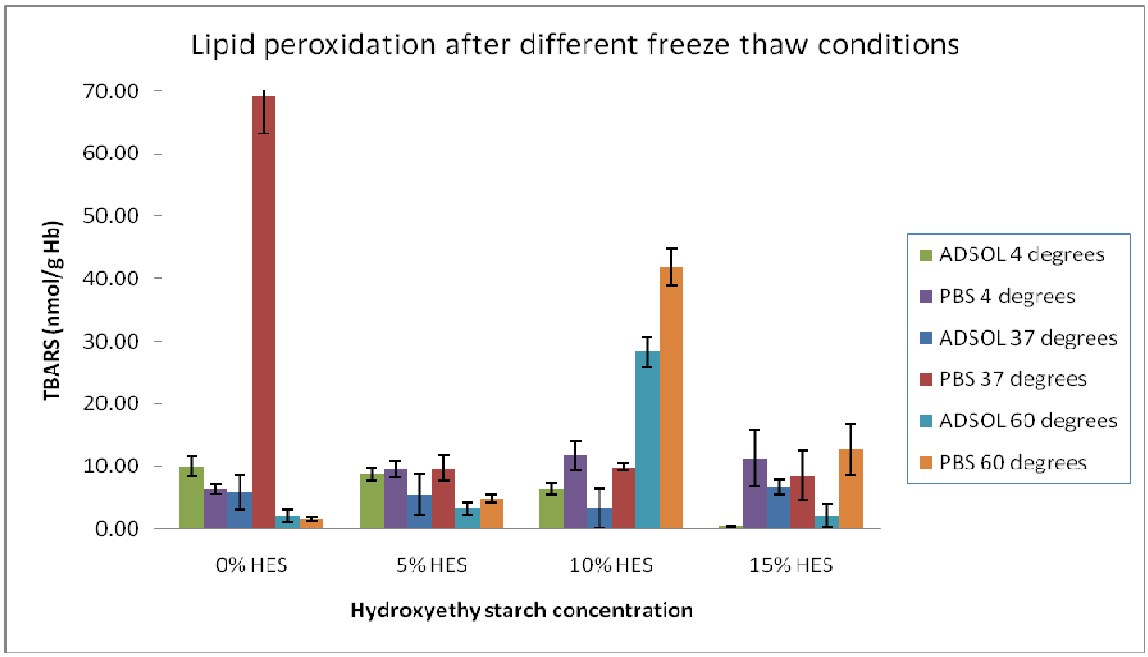

Figure 19: Lipid peroxidation in RBC sample stored in ADSOL and PBS with 0, 5, 10, 15% HES after freeze thawing at different conditions.

Red blood cell membrane lipid peroxidation was measured by quantifying TBARS spectrophotometrically. All values of TBARS were more or less around 10 nmol/ g of hemoglobin except three samples as seen in Figure 19. Cells in PBS thawed at room temperature (37°C) showed exceptionally high value of TBARS whereas 10% HES in ADSOL and PBS thawed at 60°C showed high values of TBARS compared to all other samples. It was found that cells in 15% HES in ADSOL had lowest TBARS, therefore showed lowest lipid peroxidation and again confirmed the protective effect of 15% HES under freeze thaw stress.

4.2.4 Catalase Assay

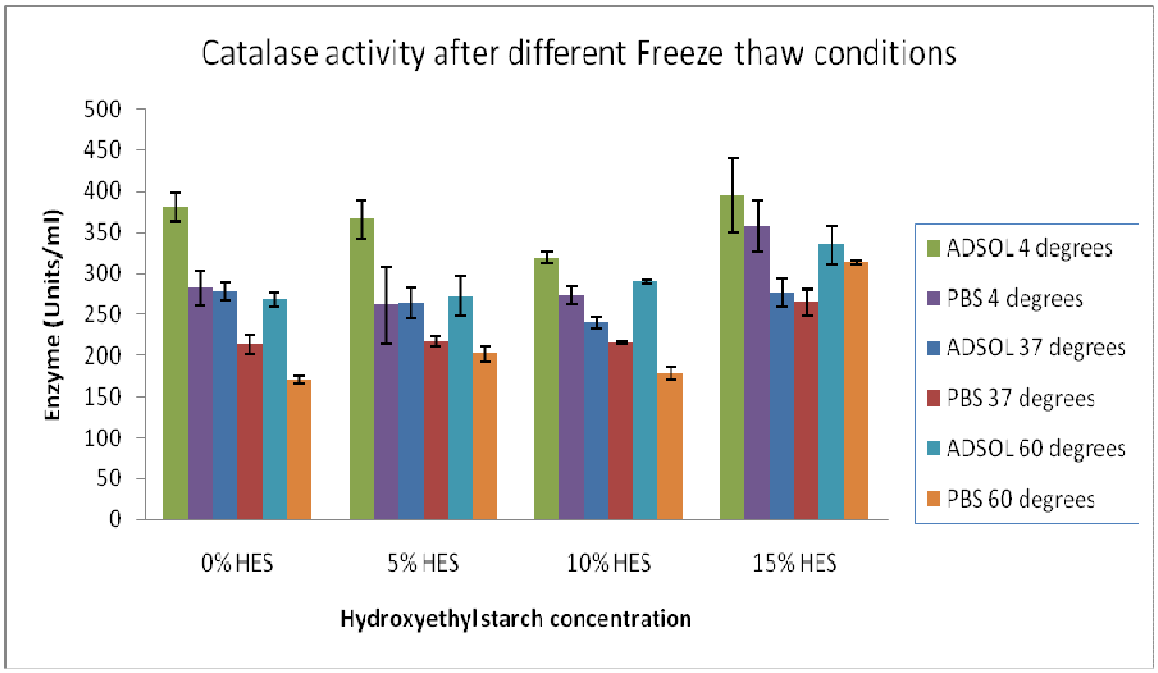

Figure 20: Catalase activity in RBC sample stored in ADSOL and PBS with 0, 5, 10, 15% HES after freeze thawing at different conditions.

Catalase activity was measured as a function of time taken to convert a specific amount of the substrate (H_2O_2) by quantifying the absorbance decrease. The catalase activity of all the formulation was found to be decreased compared to activity of fresh RBCs stored in ADSOL. The catalase activity was observed to be higher for formulations having 15% HES compared to all other formulations, out of which cells in ADSOL which were thawed at 4°C showed the highest activity.

4.3 Discussion

The preliminary experiments performed in this thesis work showed a significant difference in the viability, functionality and biochemistry of cells hypothermically stored in ADSOL and PBS solutions. Red blood cells stored in ADSOL showed better results compared to cells stored in PBS solutions, this confirmed the protective effect of ADSOL during hypothermic storage of red blood cells as reported by documented by Leonart(61).

Earlier, Sowermimo and Goodrich, 1992-1993, first reported that red blood cells (RBCs) can be preserved in the dry state by addition of mixtures of hydroxyethyl starch (HES) and glucose (14, 15). Therefore, the fact that HES acts as an excipients to protect red blood cells during long term storage against stress cannot be ignored. In this present work we were able to relate to the study done by Goodrich which reported that there is definitely a protective effect of higher concentration of HES (15% in this work) but it was not found to be significant enough to prevent hemolysis because more than 30% hemolysis occurred in all conditions, which is the same percentage of hemolysis which occurs during normal freeze thaw of red blood cells (31).

Thawing at 4°C and 37°C temperatures led to more than 50% hemolysis irrespective of presence or absence of HES. But the percentage hemolysis in case of 60°C thawing was less compared to other thawing conditions. This data obtained was somewhat similar to data obtained by Han et. al. in 2005 (4) who reported lower concentration of free hemoglobin at higher temperatures than 4°C using red blood cells rehydrated in 6% HES. The above mentioned data obtained can be the result of two reasons. Firstly, as discussed earlier the rate of thawing influences cell damage and slower thawing rates (4°C) cause more damage compared to faster thawing condition (60°C) as reported by Fishbein (33) due to extraction of the bound water into growing ice crystals. Secondly, at 60°C there can be aggregation of hemoglobin which forms clumps inside the damaged red blood cells and does not allow the hemoglobin to reach the solution and thus the free hemoglobin is not available for correct percentage hemolysis measurements. Overall, amount of hemolysis was less in the 15% HES formulations compared to formulations

having no or less amount of HES independent of the thawing condition used which confirmed the protective effect of HES.

Hemoglobin oxidation, which was quantified as the percentage of hemoglobin present as methemoglobin, was found to be less than or equal to 10% in all the formulations except formulations subjected to 60°C thawing condition. Again the protective effect of 15% HES was visible as the cells in 15% HES in ADSOL thawed at 4°C showed the lowest amount of methemoglobin.

In case of lipid peroxidation assay tremendous high value was observed in case of cells in PBS without HES thawed at 37°C, this value could have generated due to an error or some other degradation mechanism due to absence of hydroxyethyl starch and protective solution ADSOL. In all other samples highest peroxidation was observed in case of cells in 10% HES thawed at 60°C. Overall the values of TBARS obtained were similar to values reported by Kanias et. al. (39) in case of fresh RBCs stored in PBS for 72 hours at 4°C. For lipid peroxidation assay also the protective effect of 15% HES was observed similar to that observed in case of methemoglobin.

In case of catalase assay not much difference was observed independent of percentage of HES present in the formulations. However, a slight trend was observed in the catalase activity depending on the thawing conditions. Comparing all three thawing conditions, cells which underwent 4°C thaw showed the maximum catalase activity compared to other two conditions.

Therefore, after performing al the assays red blood cells in 15% HES in ADSOL which were thawed at 4°C showed moderate amount of hemolysis, lowest amount of methemoglobin, lipid peroxidation and highest Catalase activity. Therefore, this formulation was found out to be the best formulation to preserve cells against freeze thaw stress among all the formulations used in this work. Conversely, hemolysis in all the formulations was observed to be significant enough to say that HES alone did not protect the cells against cell lysis. This damage can be explained on the basis of different reports

till date on freeze thawing. Firstly, as reported by Lovelock in 1953 (31) it may be the indirect effect of freezing mediated by the increase in concentration of the solutes which surrounded the cells during freezing also called the “solution effect”. Secondly, as per Mazur (51, 63) it is due to the reduction of the quantity of unfrozen water rather than the corresponding increase in salt concentration that accompanies freezing which is due to intracellular ice formation. Thirdly, hemolysis could also be due to the physical forces exerted by interactions with ice crystals as reported by Ishiguro et al (55). This can also be a result of cell damage due to intracellular freezing and devitrification at rapid cooling rates as already documented by Rindler (16). Such damaging effects of HES can also be related to reports of Goodrich (62) that HES in itself is insufficient for preservation of red blood cells, and it needs a monosaccharide or disaccharide in combination for its protective effect as a polymer.

Introduction of monosaccharide or disaccharide in combination with HES has been proposed due to the well known protective mechanism against freeze thaw stress called the “water replacement” mechanism (2). As discussed above we need to remove the available water for ice formation and substitute it with small molecules such as monosaccharides or disaccharides which are capable of mimicking the bound water present in and around RBCs. This would help in overcoming the damaging effects on red blood cell due to intracellular ice formation after freeze thaw and hence increase the viability of cells under such a condition.

CONCLUSION AND FUTURE STUDIES

5. Conclusion

In conclusion, in this work it has been demonstrated that freeze thaw experiments using HES alone in ADSOL or PBS showed a trend of protective effect in 15% concentration of HES compared to cells with no or lesser concentration of HES. But HES is not sufficient alone for preventing hemolysis and provide protection against different stresses during freeze thaw experiments. This favors the study reported by Spieles *et al*. on red blood cell freeze-drying and rehydration (60).

We suggest the combination of disaccharide along with HES and a plasticizer needs to be investigated and also the cooling rate during freeze thaw experiments needs to be controlled to prevent hemolysis and provide an apt formulation for protection of red blood cells.

6. Future studies

We intend to investigate the activity and effect of HES, HSA and trehalose together as excipients or lyoprotectants after the same freeze thaw experiments on red blood cells to study the protective effect of HES along with loaded and unloaded trehalose. We plan to observe other parameters like ATP detioration, membrane protein degradation, agglomeration or glutathionylation and activity of other powerful free radical scavengers during the freeze thawing experiments.

We also intend to study the effect of the same excipients after freeze drying also to compare the involvement of vacuum along with the freeze thaw stress. Further we also plan to experiment different formulations for rehydration after the freeze drying of red blood cells.

REFERENCES

1. Kirby L. Scott, Jelena Lecak, and Jason P. Acker. Biopreservation of Red Blood Cells: Past, Present, and Future. Transfusion Medicine Reviews, Vol 19, No 2 (April), 2005: pp 127-142
2. Kanias T, Acker JP: Mammalian cell desiccation: facing the challenges. Cell Preserv Technol 2006;4: 253–277.
3. Satpathy G.R. et. al. Phospholipid vesicles increase the survival of freeze-dried human red blood cells. Cryobiology 51 (2005) 290-305.
4. Han Y et al. Improved preservation of human red blood cells by lyophilization. Cryobiology 2005; 51:152.
5. Whitaker BI, Henry R (2005) 2005 Nationwide Blood Collection and Utilization Survey Report (National Blood Data Resource Center, US Department of Health and Human Services, Washington, DC).
6. Satpathy G.R. et al. Loading red blood cells with trehalose: a step towards biostabilization. Cryobiology 49 (2004) 123-136.
7. American Association of Blood Banking Technical Manual, 50th ed., Bethesda, MD, 2003.
8. Hess JR, Greenwalt TJ: Storage of red blood cells: New approaches. Transfus Med Rev 16:283 - 295, 2002
9. Harmening DM: Modern blood banking and transfusion practices. Philadelphia, PA, F.A. Davis Company, 1999, pp 9 – 11
10. Hogman CF: Preparation and preservation of red cells. Vox Sang 74:177 - 187, 1998
11. Mazur P: Basic problems in cryobiology, in Timmerhaus KD (ed): Advances in cryogenic engineering, vol 9. New York, Plenum Press, 1964, pp 28-37
12. MacKenzie AP: The physico-chemical basis for the freeze-drying process. Dev Biol Stand 36:51 - 67, 1977
13. Meryman HT: Freeze-drying, in Meryman HT (ed): Cryobiology. New York, Academic Press, 1966, 609- 663
14. Goodrich RP, Sowermimo CS, et al. Preservation of metabolic activity in lyophilized human erythrocytes. Proc Natl Acad Sci USA 1992;89:967–971.

15. Sowermimo CS, Goodrich RP, et al. Refrigerated storage of lyophilized and rehydrated, lyophilized human red cells. Transfusion 1993;33:322–329.
16. Rindler V, Heschel I, Rau G. Freeze-drying of red blood cells: how useful are freeze/thaw experiments for optimization of the cooling rate? Cryobiology 1999;39:228–235.
17. Rindler V, Luneberger S, Schwindke P, et al. Freeze drying of red blood cells at ultra-low temperatures. Cryobiology 1999;38:2–15.
18. F.W. Wolkers, N.J. Walker, Y. Tamari, F. Tablin, J.H. Crowe, Towards a clinical application of freeze-dried human platelets, Cell Preservation Technol. 1 (2003) 175–188.
19. Crowe JH, Tablin F, Wolkers WF, Gousset K, Tsvetkova NM, Ricker J: Stabilization of membranes in human platelets freeze-dried with trehalose. Chem Phys Lipids 2003;122:41–52.
20. Meryman HT. Drying of living mammalian cells. Ann NY Acad Sci 1960; 85:729–739.
21. MacKenzie AP, Rapatz GL. Freeze drying preservation of human erythrocytes. Crybiology 1971; 8:384.
22. Franks F. Freeze-drying of bioproducts: putting principles into practice. Eur J Pharmaceut Biopharmaceut 1998;45:221–229.
23. Dieterich HJ. Recent developments in European colloid solutions. J Trauma 2003; 5 Suppl 54:S26-S30
24. Irina Bakaltcheva and Thomas Reid. Effects of Blood Product Storage Protectants on Blood Coagulation. Transfusion Medicine Reviews, Vol 17, No 4 (October), 2003: pp 263-271
25. Jennings, T. A., Duan H., 1995. Calorimetric Monitoring of Lyophilization. J. Pareter.Sci. & Tech. 49, 272-282.
26. Eckhardt, B. M., Oeswein, J. Q., Bewley, T. A., 1991. Effects of freezing on aggregation of human growth hormone. Pharm. Res., 8, 1360-1364.
27. Van den Berg, L., Rose, D., 1959. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions: the reciprocal system KH_2PO_4-Na_2HPO_4-H_2O, Arch, Biochem. Biophys. 81, 319-329.

28. Mazur P, Leibo SP, Chu EHY: A two-factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. Exp Cell Res 71:345 - 355, 1972
29. Wang W. Lyophilization and development of solid protein pharmaceuticals. Int J Pharmaceut 2000;203: 1–60.
30. Wisniewski, R., 1998. Developing large-scale cryopreservation systems for biopharmaceutical products. Biopharm. 11, 50-60.
31. Lovelock JE: The haemolysis of human red blood cells by freezing and thawing. Biochim Biophys Acta 10:414 - 426, 1953
32. Meryman HT: Modified model for the mechanism of freezing injury in erythrocytes. Nature 218:333- 336, 1968
33. Fishbein, W. N., Winkert, J. W., 1977. Parameters of biological freezing damage in simple solutions: Catalase. Cryobiology.14, 389-398.
34. Török Z et al. Preservation of trehalose-loaded red blood cells by lyophilization. Cell Preserv Technol 2005;3:96–111.
35. Allison SD et al. Effects of drying methods and additives on structure and function of actin: mechanisms of dehydration-induced damage and its inhibition. Arch Biochem Biophys 1998;358:171.
36. Rupley, J. A., Careri, G., 1991. Protein hydration and function. Adv. Protein Chem. 41, 37-173.
37. Petr Jarolim, Meir Lahav, Shih-Chun Liu, and Jiri Palek. Effect of Hemoglobin Oxidation Products on the Stability of Red Cell Membrane Skeletons and the Associations of Skeletal Proteins: Correlation With a Release of Hemin. Blood, Vol76, No 10 (November 15). 1990: pp 2125-2131
38. Anastasios G. Kriebardis, Marianna H. Antonelou, Konstantinos E. Stamoulis, Effrosini Economou-Petersen, Lukas H. Margaritis, Issidora S. Papassideri .Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells. J. Cell. Mol. Med. Vol 11, No 1, 2007 pp. 148-155
39. Kanias et. al. Determination of lipid peroxidation in dessicated Red Blood Cells. Cpt 5 (2007) 165-174.
40. Kurata M, Suzuki M, Agar NS. Antioxidant systems and erythrocyte life-span in mammals. Comp Biochem Physiol 1993;106B:477-87.

41. Cetin Ozurk, Engin M. Gözükara. Erythrocyte Catalase Activities in Chronic Leukemias. Journal of Turgut Özal Medical Center 4(1):1997
42. Beers, R. F. & Sizer, 1. W. (1952). A spectophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Cbem 195, 133-1 40.
43. Arakawa, T., and Timasheff, S. N., 1985. The stabilization of proteins by osmolytes. Biophys. J. 47, 411-414.
44. Carpenter, J. F., Crowe, J. H., and Arakawa, T., 1990. Comparison of solute-induced protein stabilization in aqueous solution and in frozen and dried state. J. Diary Sci. 73, 3627-3636.
45. Pikal, M. J., 1999. Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of thermodynamic stabilization and glassy state relaxation dynamics. In: Rey, L., May, J. C., (Eds.), Freeze-drying/Lyophilization of pharmaceutical and Biological products, vol.96. Marcel Dekker, New York,161-198
46. Carpenter, J. F., and Crowe, J. H., 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochem 28, 3916-3922.
47. Franks, F., Hatley, R. H. M., Friedman, H. L., 1988. The thermodynamics of protein stability: Cold destabilization as a general phenomenon. Biophys. Chem., 31, 307-315.
48. Nagendra, H. G., Sukumar, N., Vijayan, M., 1998. Role of water in plasticity, stability, and action of proteins: the crystal sctructures of lysozyme at very low levels of hydration. Proteins: Struct. Funct. Gen. 32, 229-240.
49. Angell, C. A., 1995. Formation of glasses from liquid and biopolymers. Science. 267, 1924-1935.
50. Hatley, R. H., 1997. Glass fragility and the stability of pharmaceutical preparartion-excipient selection. Pharm. Dev. Technol 2, 257-264.
51. Mazur P, Rall WF, Rigopoulos N: Relative contributions of the fraction of unfrozen water and of salt concentration to the survival of slowly frozen human erythrocytes. Biophys J 36:653 - 675, 1981.
52. Pegg DE, Diaper MP: The bunfrozen fractionQ hypothesis of freezing injury to human erythrocytes: A critical examination of the evidence. Cryobiology 26:30 - 43, 1989
53. Meryman HT: Osmotic stress as a mechanism of freezing injury. Cryobiology 8:489 - 500, 1971 56.

54. Meryman HT: Freezing injury and its prevention in living cells. Annu Rev Biophys 3:341 - 363, 1974
55. Ishiguro H, Rubinsky B: Mechanical interactions between ice crystals and red blood cells during directional solidification. Cryobiology 31:483 - 500, 1994
56. Transfusion Medicine Technical manual. 2nd Edition, sponsored by W.H.O., published by D.G.H.S. Ministry of Health and Family Welfare, Govt. of India, 2003; 309-310.
57. Balasubramaniam P, Malathi A. Comparative study of hemoglobin estimated by Drabkin's & Sahli's methods. J Postgrad Med 1992; 38:8-9.
58. M.A. Ibrahim, M.I. El-Gohary, N.A. Saleh, M.Y. Elashry. Spectroscopic study on oxidative reactions of normal and pathogenic hemoglobin molecules. Romanian J. Biophys., Vol. 18, No. 1, P. 39–47, Bucharest, 2008.
59. John H. Crowe. et. al. Stabilization of Dry Membranes by Mixtures of Hydroxyethyl Starch and Glucose: The Role of Vitrification. CRYOBIOLOGY 35, 20–30 (1997)
60. Spieles, G., Heschel, I., and Rau, G. An attempt to recover viable human red blood cells after freeze-drying. Cryo-Lett. 17, 43–52 (1996).
61. Leonart, M.S.S., et. al. Enzymes and membrane proteins of ADSOL-preserved red blood cells. Sao Paulo Med J/Rev Paul Med 2000; 118(2):41-5.
62. Goodrich, R. P., Williams, C. M., Franco, R. S., and Weiner, M. (1989). ‘‘Lyophilization of Red Blood Cells.’’ U.S. Patent 4,874,690.
63. Mazur P, Rall WF. Physical – chemical basis of the protection of slowly frozen human erythrocytes by glycerol. Biophys J 0006-3495/78/0701-101. 36:653 - 675, (1978).

ANNEXURE - I

LIST OF EQUIPMENTS USED

Instruments	Make	Function
Analytical Balance	Afcoset ER-200A	Weight Measurement
pH meter	Systronics	Measurement of pH
Ultra Low Temperature freezer	Remi-RQFP 265	Preservation of reagents
Ultra pure water system	Millipore	Preparation of the buffers etc.
Spectrophotometer(UV/Vis)	Systronics 2203 Double beam	Different assays (Hemoglobin, MDA)
Refrigerator	Whirlpool	Preservation of Blood samples and RBCs
Ultra Centrifuge	Remi-C24BL	Isolation of RBCs
Water bath	LAUDA Ecoline-staredition RE-104	Heat treatment
Vortex Mixer	Genie	Proper mixing of reagents
Magnetic stirrer	Spint	Mixing

ANNEXURE – II

LIST OF CHEMICALS AND REAGENTS USED

S. No	CHEMICAL/REAGENT	SUPPLIER
1	Malondialdehyde	MERCK Specialities Pvt.Ltd.
2	Hydrogen Peroxide	MERCK Specialities Pvt.Ltd.
3	Sodium chloride	MERCK Specialities Pvt.Ltd.
4	Sodium dihydrogen phosphate	MERCK Specialities Pvt.Ltd.
5	Trichloro acetic acid	MERCK Specialities Pvt.Ltd.
6	Disodium hydrogen phosphate	MERCK Specialities Pvt.Ltd.
7	Potassium chloride	MERCK Specialities Pvt.Ltd.
8	Ethanol	MERCK Specialities Pvt.Ltd.
9	Thiobarbituric acid	SIGMA Aldrich
10	Hydroxyethyl starch	SIGMA Aldrich
11	Adenine	Himedia
12	Mannitol	Himedia
13	Glucose	Himedia
14	Sodium hydroxide	Himedia
15	Sodium hypochorite solution	Himedia
16	Drabkin’s Reagent	Crest Biosystems
17	Hemoglobin standard (60mg/dl)	Coral clinical systems