ROLE OF AMINO ACIDS IN THE LYOPHILIZATION OF RBC

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CERTIFICATE

This is to certify that the thesis entitled "**ROLE OF AMINO ACIDS IN THE LYOPHILIZATION OF RBC"** submitted by **Ms. PARUL PATEL** in partial fulfillment of the requirements for the degree of **Bachelor of Technology in BIOTECHNOLOGY** embodies the bonafide work done by him in the final semester of his degree under the supervision of the undersigned. The thesis or any part of it has not been submitted earlier to any other University / Institute for the award of any Degree or Deploma.

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CONTENT

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LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Transfusion of RBCs saves millions of life worldwide each year. To cater the emergency need, preservation of blood is necessary. In addition, it is performed by two methods either by storing at Low temperature (at 4°C for 42 days) or by Lyophilization. Low temperature preservation usually means preservation at 4°C,-20°C,-80°C,-150°C or-196°C.Storing below 0°C involves ice related injury. So normally in clinical scenario, blood is stored at 4°C.Cells stabilizer ADSOL, CPDA need to added to prevent the storage lesion associated with low temperature storage. Also, transportation in the liquid form at low temperature is also a problem. The shortcomings of low temperature storage is overcome by Lyophilization of the blood which convert RBCs in to dry form lyophilization involves sublimation of ice at low temperature and low pressure. But it also involves dessication and low temperature related injury .HES and Trehalose has shown good efficacy in protecting the RBCs against those injury. The present study tried to find out the relative efficacy of HES and TREHALOSE. Different amino acid has shown good biostablizing property in the cryopreservation. The study also tried to find out the relative efficacy of different amino acids in the lyophilization of RBCs.

CHAPTER-1: MOTIVATION

In the preservation of the cells, the intrigity and functionality of the cells need to be maintained. Normally the native environment inside the cell residues provides the ideal environment for the cells. But to prevent the cellular damages due to external environmental variables, additional protective components are also needed. Conventionally RBCs are preserved in glycerol at cryotemperature with CPDA or ADSOL at -4°C .Over the years different Biostabilization protocols as well as biopreservation and storage methods have been researched and developed. But no existing preservation protocol gives enough viability to make lyophilized RBCs clinically usable. The Hemoglobin that is released due to the hemolysis is toxic to our body and that need to be removed before the transfusion. The main driving force for extensive research in this field is the enormous need of improved blood banking techniques, easier worldwide transportation of RBCs particularly to remote locales and site of immediate requirement.

CHAPTER-2: OBJECTIVE AND OVERVIEW OF THESIS

2.1- OBJECTIVE

The main aim of this work is how we can enhance the durability of lyophilize RBCs. Normally RBCs can't be stored more than 42 days at normal freeze and it is well known that RBCs are vulnerable to damage during storage of whole blood. The overall objective of the study is to preserve the RBCs in-vitro by applying freeze drier technique and how their viability can be improved.

2.2- OVERVIEW OF THESIS

In this study, various protective solutions is applied for the preservation of RBCs. The different protective solutions (15% HES and 15% Trehalose) in different concentrations of amino acid have been used for Lyophalization of Blood sample. Fresh blood is collected in EDTA solution and this whole blood was used for the Lyophilization of the RBCs. In the preliminary study, the collected blood sample was mixed with 15% HES and 15% Trehalose to check the relative efficacy .The study use the ADSOL as the protective reconstitution solution. 15% HES has showed better viability than the 15% Trehalose. In the second study, additional protective role of many amino acids was checked with HES.Microscopic study, viability by Drabkins method and serum potassium assay were done to check the preservation process damage.

CHAPTER-3: **LITERATURE REVIEW**

Porter WL et al,1978, in his experiment found that in the presence of phosphate buffer, oxygen uptake of lyophilize RBC was extremely slow while in presence of catalytic ion oxygen uptake was much improved [1]. Pellerin-Mendes C et al,1997, used disaccharide trehalose and the polymeric carbohydrate (dextran, 40 kDa) as a substitution of glycerol in the cryopreservation of RBC but the cell recovery in the presence of dextron was 35% of glycerol recovery[2] . Valeri CR et al,2000,had modified the viability, functionality, and %hemolysis of human RBCs stored at 4 degrees Celsius in MODIFIED additive solutions (AS-1, AS-3, or AS-5) with pyruvate, inosine ,phosphate and adenine solution .After the cryopreservation for 24 hours , the RBC recovery was approximately 85%[3]Jianping Yu. et al,2004 in his study "Freeze-drying of human Red Blood Cells in the Influence of carbohydrates and their concentrations" illustrated about the factors affecting the recovery of the cell in lyophilization[4]. Satpathy GR et al,2004, in his study found that loading of trehalose gives osmotic protection to the RBCs which help in maintaining the viability of dried RBC [5]. Kheirolomoom A. et al ,2009 illustrated that addition of phospholipids vehicle significantly decreases the hemolysis of freeze-dried RBCs after rehydration[6]. Hsu CC ET AL,2012 describes how to determine the optimum residual moisture in lyophilized protein pharmaceuticals.He concluded that each protein necessarily possess the moisture content which is required to protect the polar groups and also the excess of drying will lead to disclosure of these groups[7].

CHAPTER-4: RBC, HES, TREHALOSE, AMINO ACIDS AND LYOPHILIZAION

4.1-RBC STRUCTURE

RBCs are bioconcave blood cells, which possess hemoglobin (Hb) .Hb carries O2 from lungs to tissues and CO2 from tissues to the lungs. The main damage in the lyophilization process occurs in RBC membrane.RBC Membrane contain equimolar quanties of phospholipids ,unestrified cholesterol with a small amounts of glycolipids and free fatty acids,52% of RBC membrane is comprised of membrane proteinsMembrane protein provides the structural support to the membrane. Many injuries and some medical situation cause anemia and at that time clinically qualitative as well as immediate transfusion of RBC is required . Thus by increasing the concentration of RBC in a patient lacking oxygen carrying capacity,might be saved, actually this lacking of RBC is may be either because of their decreased production in bone marrow, or because of hemaglobiopathies and thalassemias which is also called defective hemoglobin and usually because of hemolytic anemia's. In this way today's world demands an urgent need of blood and its products but of the total donated blood only 30 % blood is used in developing and rest 70% is consumed in saving the lives of people. So preservation of RBCs is too much necessary which is performed either by storing at low temperature (at 4°C for 42 days approxmately) or by Lyophilization process.

4.2-PRINCIPLE OF RBC LYOPHILIZATION (source: A Guide to Freeze drying for the Laboratory – NBTC)

In Lyophilization there is a removal of most unbound water from the cells by the controlled freezing followed by the vacumed ice sublimation[8-9]. For the sublimation process, proper control over pressure and temperature is necessary. As latent heat of fusion is 80 calories /gm of ice and latent heat of vaporization is 540 calories/gm of water that is why latent heat of sublimation would be 680calories/gm of ice. Lyophilization has 3 stages

1. Freezing
2. Primary drying
3. Secondary drying

FREEZING- the sample needs to be frozen before lyophilization .The biological sample undergo freezing related injury during the cooling. The slow cooling leads to ice formation extracellular and fast culling leads to the ice formation intracellular. Extracellular ice formation affects the solute concentration, which is due to the separation of solutes from ice. It leads to osmosis and damaging of the cells. And the intracellular ice formation leads to the loss of higher cell viability because of ice related injury on cell's proteins and membranes. For lyophilization, slow cooling is preferred.

PRIMARY DRYING

Primary drying needs a very watchful control over two parameters.

1. Temperature
2. Pressure.

The difference between vapor pressure of the product and vapor pressure of the ice collector decides the rate of sublimation of ice from a frozen product. It is tremendously significant that the freeze-drying temperature of the product should form a balance between the temperature maintaining the frozen integrity of the product and the temperature maximizing the vapor pressure of the product. This balance is very necessary for optimum drying. The phase diagram has been shown in Figure 1 exemplifies this view. Most of the products are frozen and sound less than their eutectic or glass transition point (Point A), then the temperature is raised just to less than this critical temperature (Point B) and they are targeted to a subdued pressure which shows that the freeze drying process is going to begin. Figure 1 (typical phase diagram

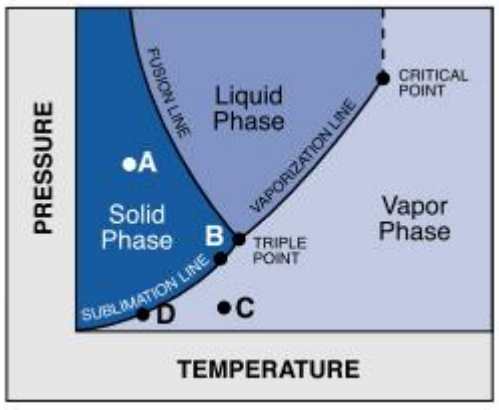

Fig: 1 A typical phase diagram (source: A Guide to Freeze drying for the Laboratory – NBTC**)**

Table 1: Relationship between Vapor Pressure/Temperature

Vapor Pressure (mBar)	Temperature (°C)
6.104	0
2.599	-10
1.034	-20
0.381	-30
0.129	-40
0.036	-50
0.011	-60
0.0025	-70
0.0005	-80

SECONDARY DRYING

When primary freeze-drying is completed, and all ice is sublimated in the product, the bound moisture is still present that may go up to 7-8%. Continued drying is vital at the warmer temperature for reducing the residual moisture content to the optimum level. This process is known as isothermal desorption because of the desorption of the bound water from the product. Secondary drying is mainly started with the product temperature more than the ambient one but well-suited with the product‘s sensitivity. Other conditions like pressure, collector temperature, should remain same because the vacuum should be the lowest for the desorptive process and the collector should be very cold. The Time required in secondary drying is approximately 1/3 to 1/2 of the time needed for primary drying.

4.3-HYDROXYETHYL STARCH

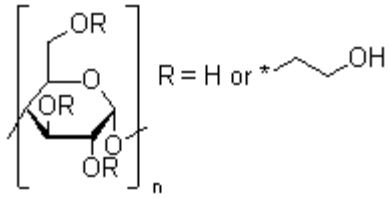

Fig-2: structure of hydroxyethyl starch

HES is made of glucose units derived from amylo-pectin and then it is modified when hydroxyethyl is substituted with –OH group in glucose molecule[29]. Hydroxyethyl starch helps in delaying the depredation of the compound. Thus, it is used as a plasma volume-expanding agent. It is used as a lyoprotectant for RBC preservation. The advantages are that it is a great substitution of plasma and there is no necessicity of its removal after thawing[30]. For preservation purpose mainly HES with high molecular weight is used. Investigators mainly HMW-HES and MMW-HES use as a protectant.

4.4-TREHALOSE-

It is made up of ά glucose subunits with 1,1 glycoside linkage between two units. It is a non reducing sugar and less soluble than the sucrose. It is normally found in plants, animals and bioorganism.In insects like bees and grasshopper; it is present, as a blood sugar when they need energy to fly it breaks in to glucose in the presence of catabolic enzyme trehalase.and becomes the source of energy for flying.

4.5-AMINO ACID

As a protectant, we used amino acids (150 Mm and 1ml equivalent weight each) alanine, arginine, proline, tryptophan, tyrosine, glutamine, glycine, cystine and lysine.

ALANINE helps in converting simple body glucose sugar in to energy and help in eliminating the excess toxicity from the liver. Also helpful in protecting cell of being damaged during aerobic functionality.

Argentine is very helpful for our body keeping joints liver muscles and skin healthy by regulating hormones sugar and many other things properly.

Now a days it is very helpful in treatment of AIDS and CANCER like diseases, which are directly linked to our immune system by increasing the number of T-CELLS.

TYROSINE helps in speeding up the metabolism and help in stimulating the nervous system.

GLUTAMINE helps in building and maintains the disorders of the body. Help in removing the toxic ammonia from the liver and maintain a healthy CNS. Also promote a healthy digestive tract by balancing acid/base level in our body.

It helps in promoting the cell division in our body. It also develops thombrocytes, lymphocytes and macrophages.

GLYCINE, not very essential but helps in maintaining the healthy nervous system. It is also used in formation of normal DNA & RNA strands. It also converts glucose in to energy needed for our body and regulate sugar level in our body.

LYSINE it has antiviral property. It is very essential for heron production and for their growth also.

It involves in production of antibodies.

CHAPTER -5: MATERIALS AND METHODS

This chapter describes the material used and the principles and protocols for the various experimental setups and design for the preservation of RBC in different amino acid protective solution; Later on, we check their viability in terms of percentage hemolysis and plasma potassium concentration.

5.1-CHEMICALS & REAGEANTS USED

1- Hydroxyethyl starch

2-Trehalose

3-Adsol solution

4-Amino acids

a- Tyrosine

b- Tryptophan

c- Lysine

d- Alanine

e- Arginine

f- Glutamine

g- Cystine

h- Proline

i- Glycine

j- Cysteine

1- Blood collection by the adult donor of laboratory.

5- Drabkin’s reagent (Crest Biosystems)

6-Potassium assay kit (Crest Biosystems)

All chemical used above are purchase from HiMedia, otherwise mention

5.2-APPARATUS & IMPORTANT WEARINGS

Glass wares like measuring cylinder, beakers, small petri plates, test tubes flasks etc and Plastic wares like syringe, falcon tubes, and eppendof tube

5.3-INSTRUMENT

- Spinix
- Spectrophotometer
- Multitask ELISA Plate reader
- Microscope
- Freeze-drier
- Ultra deep freezer
- Mini-centrifuge

5.4-BLOOD SAMPLE COLLECTION

The blood is collected from healthy human adult volunteers (5 ml) in EDTA solution and then makes 15 % w/v HES and Trehalose each. Then this blood sample is further formulated in different Lyoprotective solution (1:1). This formulated solution is incubating for 1-2 hr at -80°C.

Table: 3 Concentration of protective solution

Solute	Concentration
HES	15 % w/v
trehalose	15 % w/v
tryptophan	150mM
Cystine	150 mM
Lysine	150 mM
Alanine	150 mM
Arginine	150 mM
Glutamine	150 mM
Proline	150 mM
Glycine	150Mm

Fig3: freeze drier

5.5LYOPHILIZATION OF RED BLOOD CELLS

Procedure-

- Collect the whole blood in falcon tube containing EDTA.
- Lyoprotectant solution is prepared in ADSOL solution.
- Mix the lyoprotectant solution and isolated blood in 1:1 ratio (total volume 1ml).
- Freeze the sample at -80°C for 1 to 4 hrs.
- Lyophilize the freeze blood sample in vacuum freeze drier. During drying shelf temperature should be maintained at -70 °C to -80 °C and vacuum pressure should be at 80-90 mm

- The lyophilization process is done for 8 to 12 hrs.
- after lyophilization, the blood sample is reconstituted in to 1 ml ADSOL solution and RBC hemolysis is determined by Drabkin's method.
- The lyophilized blood sample is also observed under the light compound microscope to study the morphological changes.

5.6-CELL VIABILITY ASSAY:

5.6.1-Percentage Hemolysis using Drabkin's Reagent

Principle

Hemoglobin is converted in to methemoglobine by potassium ferricynde in the sample. Then a complex cynamethmoglobin is formed when methenoglobin further reacts with potassium cyanide. The complex is very stable and its formation intensity is directly proportional to concentration of plasma hemoglobin.

The actual happening in hemolysis is that the RBC gets breaked and starts releasing hemoglobin. Therefore, we can measure hemolysis by measuring the concentration of hemoglobin in the cell suspension, after centrifuging all blood sample containing different amino acid solutions by using mini-centrifuge. Then we measure the Absorbance at 540 nm of their supernatant.

Procedure

For hemoglobin assay 20µl of blood added to 5ml of Drabkin's solution. Incubated the sample at room temperature for 5 -10 mins and the absorbance were taken at 540 nm in a spectrophotometer.

$$\text{Relative hemolysis} = \frac{\text{OD}_{540\text{nm}} \text{ of sample}}{\text{OD}_{540\text{nm}} \text{ of whole blood}} \times 100$$

5.6.2-Determination of K^+ ions in supernatant

Like sodium ion potassium ion is also the main part of intra cellular and extra cellular fluids.

Principle

When potassium reacts with tetraphenyl boron in the presence of a special prepared buffer, it forms a colloidal suspension. And the produced turbidity is directly proportional to potassium concentration in the sample.

Tetra phenyl boron + K^+ → White turbidity

Procedure

- Reconstitute the lyophilize blood sample in ADSOL solution.
- Centrifuge the sample at 2500 rpm for 10 min.
- Add 100 µl of Tetra phenyl Boron reagent in 96 well plates.
- Then 10 µl of supernatant plasma solution is added in 96 well plate containing TB Reagent.
- Mix the sample at 1500 rpm for 5 min and wait for 2min.
- Take the Absorbance at 620 nm using ELISA plate reader.

$$Plasma\ K^+\ Conc\ (mmole/l) = \frac{Absorbance\ of\ test\ at\ 620nm}{Absorbance\ of\ std\ at\ 620nm} \times Conc\ of\ std$$

CHAPTER-**6:** **RESULTS and DISCUSSION**

In order to study the percentage hemolysis, taking place during the lyophilization, we formulate different protective solution of Amino acid in ADSOL solution containing 15% HES and Trehalose. The freshly isolated blood was suspended in varying amino acid lyophalization solution. Then above two solutions are mixed and incubate the solution at -80°C for 1 to 4 hr. After freezing the whole sample, the sample was transfered to Freeze-Drier. After sample is converted into dried form, reconstitute the sample in ADSOL solution and check their viability. The viability assay (percentage hemolysis) of RBC was determined by Drabkin's method. In each case, hemolysis was observed as function concentration of protective solution. And it was found that HES is better protective than the trehalose. For the next lyophilization study, HES was taken as a protective with amino acids additives. And the results were like that.

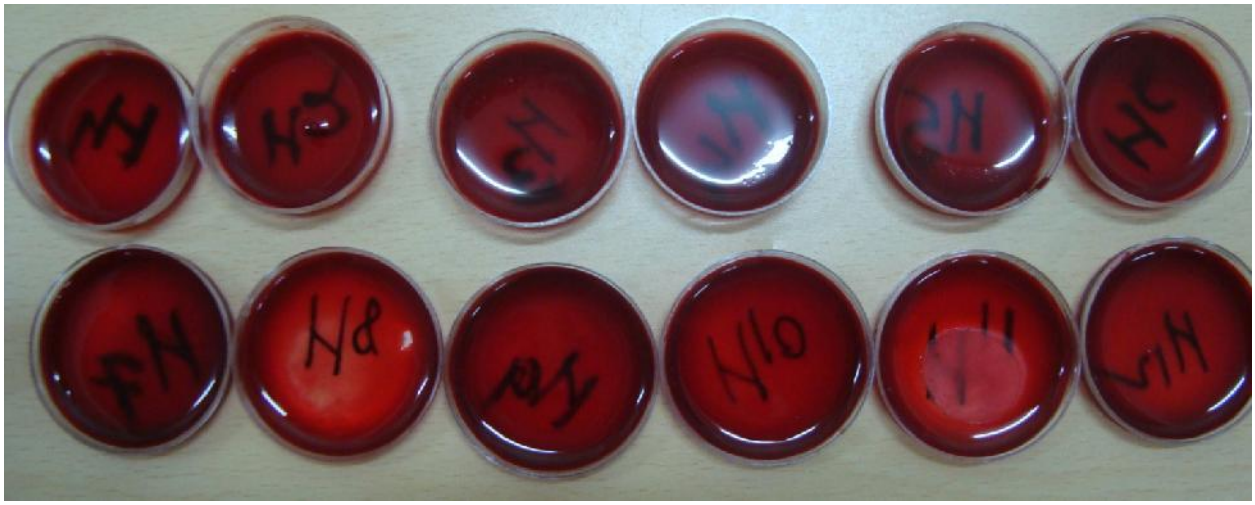

Fig4:blood sample containing Lyoprotective, before lyophilization

Fig-**5: Lyophilized RBCs.**

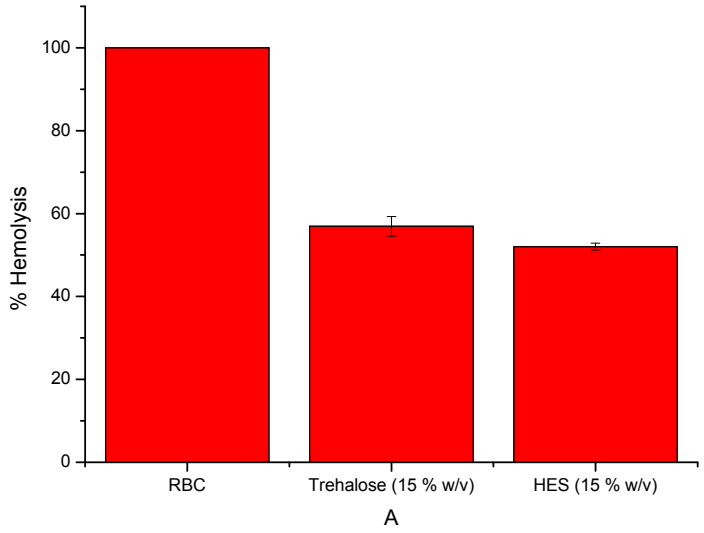

Fig-6: Comparative analysis of 15% HES and 15% Trehalose –It is clear from the graph that the percentage hemolysis of HES is less than the TREHALOSE. So for the the next lyophilization we will take HES as a protective with amino acids additives.

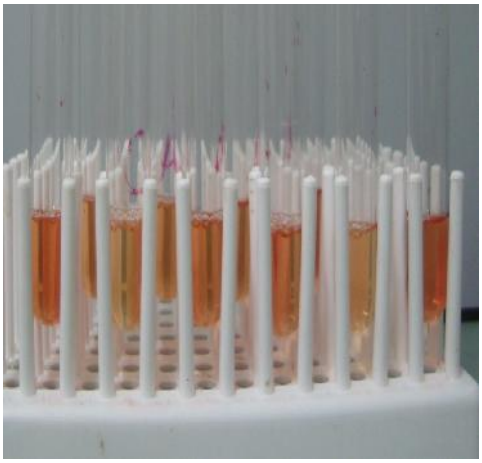

Fig 7: Different color intensity shown by Drabkin's reagent in the presence of Hb.

Different color intensity of Drabkin's reagent in the presence of plasma of lyophilize blood sample. The color intensity is directly proportional to plasma free Hb.

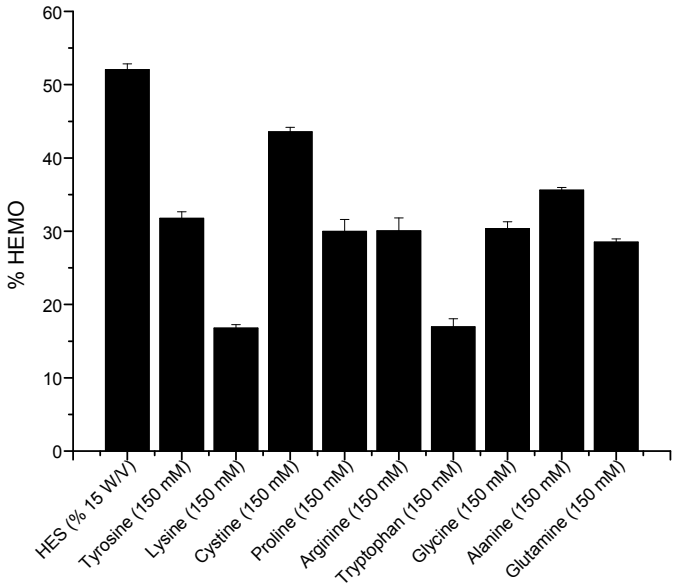

Fig-8: Percentage hemolysis in RBC sample after lyophilization in 15 % hydroxyl ethyl starch containing 150 mM of amino acid-.which shows clearly that lysine and tryptophan are very good in provoking cell damage in lyophilization. Except these two, glutamine, proline and aregenine are also leaving good impact inhibiting the cell damaging.

MICROSCOPIC IMAGES OF LYOPHILIZED RBCs-

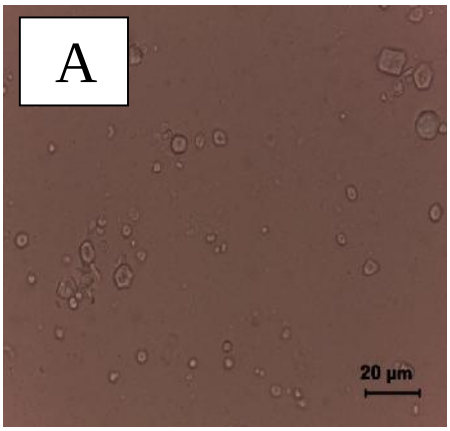

FIG: 9A- Microscopic image of lyophilize RBC control sample

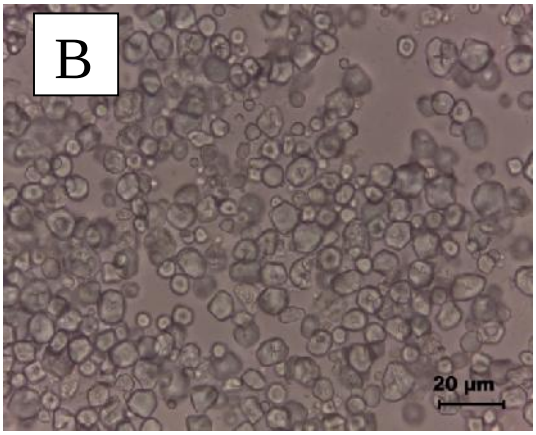

FIG: 9B-Microscopic image of lyophilize RBC containing LYSINE

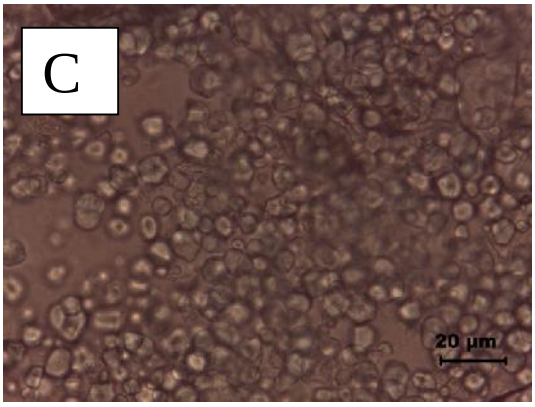

FIG: 9C- Microscopic image of lyophilize RBC containing PROLINE

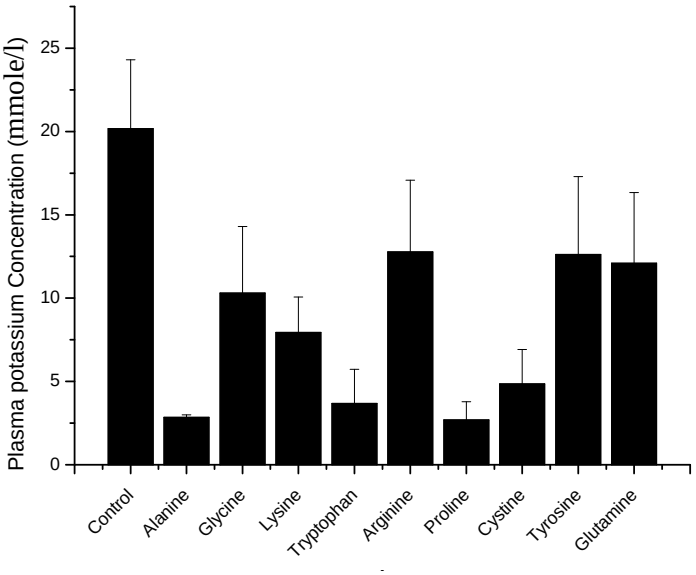

Fig-:10- Plasma potassium concentration of blood sample after lyophilization

From the potassium assay estimation, it can be concluded that Alanine and proline are doing better in RBC storage lesion, As well as glycine ,glutamine and lysine are also contributing enough in preventing the cell damage. For the extensive researches this estimation can be performed by taking different concentrations of amino acids.

Ronald h. nag et al, 1992 had given a technique for the calorimetric determination of potassium in the plasma and serum. The technique involves reflectance photometry with dry chemistry reagent. Though this technique involves large designated instrument but its accuracy in estimating the potassium concentration for near-patient testing is much better than the commercial methods .This new technique requires a dry chemistry reagent for K+ evaluation. But it does not need any instrumental maintenance.

Dorn A et al, 2005 had proposed a technique named as high –throughput fluorescence assay method to evaluate HERG potassium channel inhibition .Enamurous projects related to drug development did not get succeed because of the cardiac sides effects as QT prolongation and this effect is developed by myocardial HERG potassium channel. Somehow, if we provoke the molecular interaction with these channels we will be succeed to develop the system. Therefore, authors have involved a cell based fluorescence assay using a membrane sensitive fluorescence die. This system is very rapid in testing large numbers of compounds in a very short period of time [10].

CHAPTER-7: CONCLUSION

Tryptophan is showing very good results in both hemolysis as well as in potassium assay .Proline, lysine and glutamine are also playing very important role in preventing cell from damage during their lyophilization. And further studies are needed to check the RBC storage lessons. In the preliminary study, the relative efficacy of 15% HES and 15% trehalose was checked. And HES was showing better viability of RBCs.In the second study role of amino acids in the lyophilization was checked with HES.

And the ascending order of the percentage hemolysis of amino acids was-

Tryptophan<lysine<glutamine<cysteine<tyrosine<argentine<proline<alanine<HES

To check the functionalality of the cells another serum potassium assay was performed by taking these amino acids residues.

And the ascending order of efficiency the of amino acids-

Proline<alanine<tryptophan<cysteine<lysine<glycine<glutamine<argentine<tyrosine<CONTROL

.

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