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STUDY OF INCONGRUENT UPSHOT OF ERYTHROCYTE & ITS RELEVANCES

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ABSTRACT

Erythrocytes are the Red Blood Cells, color is due to the heme group of hemoglobin. The size of erythrocytes varies widely among vertebrate species. The development of RBC takes place in bone marrow. Life span is approximately 120 days, Erythropoiesis is the development of erythrocytes. The membrane perturbation, electro-encapsulation, hypo osmotic lysis, lipid fusion endocytosis, these are the ways for the drug loading in erythrocytes. Drug content, osmotic fragility, osmotic shock, turbulence shock and in-vitro drug release are the evaluation parameter for it. These methods are effective for insertion of low molecular weight drug but it has very less % entrapment efficiency. These drug loaded erythrocytes having large number of biological and biomedical applications. The concept of employing erythrocytes as drug or bioactive carrier could surely find the cancer's cure. Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug carrier concept that one can reached to the better drug delivery to get maximum effective concentration i.e more quantity of bioavailable drug in the blood to get desire biological activity.

KEYWORDS: Oxyhemoglobin, Electroencapsulation, Hypoosmotic.

INTRODUCTION

The color of erythrocytes is due to the heme group of hemoglobin. The blood plasma alone is straw-colored, but the red blood cells change color depending on the state of the hemoglobin: when combined with oxygen the resulting oxyhemoglobin is scarlet, and when oxygen has

been released the resulting deoxyhemoglobin is darker, appearing bluish through the vessel wall and skin.

The size of erythrocytes varies widely among vertebrate species; erythrocyte width is on average about 25% larger than capillary diameter and it has been hypothesized that this improves the oxygen transfer from erythrocytes to tissues. The normal shape is a biconcave disc. The shape is maintained by a contractile protein. When suspended in media of varying toxicity the volumes of the cells change owning to the passage of water through the membrane. The cells act as osmometer. In hypertonic media they shrink and in hypotonic media they swell. On swelling the cells change from a biconcave disc to a sphere (spherocyte).

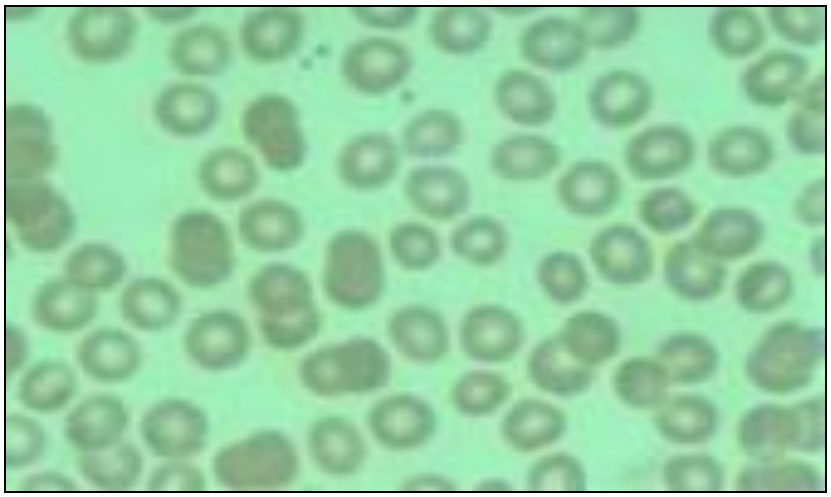

Fig.1 Microscopic erythrocytes

Characteristics of individual erythrocytes

- Diameter = 6 to 8 microns
- Thickness = 1.5-1.8 microns (a biconcave disk)
- Production rate = 2.5 million/sec. Or 200 billion/day
- Life span = approximately 120 days

Erythropoiesis is the development of erythrocytes.

Sites of development

Erythropoietin tissue originates in the yolk sac then moves to the liver and spleen during fetal life. Eventually erythropoiesis settles in the medullary cavity of the skeleton. By about 18 years of age the axial skeleton and proximal ends of the long bones is the site of erythrocyte production.

Erythrocyte maturation times

- Rubriblasts usually divide within 12 hours of stimulation to make 2 daughter cells (prorubricytes)
- Prorubricytes require about 20 hours developing.
- The rubricyte stage lasts about 30 hours.
- A metarubricytes last about 48 hours.
- Reticulocytes are released from bone marrow after 2 to 3 days and circulate for an additional 1 or 2 days before maturing into an erythrocyte.
- A mature erythrocyte lives about 120 days.

Composition of bone marrow

- Rubriblasts = 1% or less
- Prorubricytes = 1 to 4%
- Rubricytes = 10 to 20%
- Metarubricytes = 5 to 10%

Human red blood cells

Red blood cells are the most common type of blood cell and the vertebrate body's principal means of delivering oxygen to the body tissues via the blood. The cells are filled with hemoglobin, a bimolecular that can bind to oxygen. They take up oxygen in the lungs or gills and release it while squeezing through the body's capillaries. The blood's red color is due to the color of hemoglobin. In humans, red blood cells develop in the bone marrow, take the form of flexible biconcave disks, lack a cell nucleus, sub cellular organelles and the ability to synthesize protein, and live for about 120 day.

Red blood cells are also known as RBCs, red blood corpuscles (an archaic term), haematids or erythrocytes (from greek erythros for "red" and kytos for "hollow", with cyte translated as "cell" in modern usage). The capitalized term red blood cells is the proper name in the us for erythrocytes in storage solution used in transfusion medicine.

Drug loaded erythrocytes

Novel drug delivery systems are one of the widely used delivery systems. In the present scenario, amongst them, "drug loaded erythrocytes" is one of the growing and potential systems for delivery of drugs and enzymes. Erythrocytes are biocompatible, biodegradable,

possess long circulation half-life and can be loaded with variety of biologically active substances.

Carrier erythrocytes are prepared by collecting blood sample from the organism of interest and separating erythrocytes from plasma.[2] By using various physical and chemical methods the cells are broken and the drug is entrapped into the erythrocytes, finally they are resealed and the resultant carriers are then called "resealed erythrocytes". Surface modification with glutaraldehyde, antibodies, carbohydrates like sialic acid and biotinylation of loaded erythrocytes (biotinylated erythrocytes) is possible to improve their target specificity and to increase their circulation half-life. Upon reinjection the drug loaded erythrocytes serve as slow circulation depots, targets the drug to the reticulo endothelial system (res), prevents degradation of loaded drug from inactivation by endogenous chemicals, attain steady state concentration of drug and decrease the side-effects of loaded drug. Nowadays, nanoerythrosomes based drug delivery systems have excellent potential for clinical application.

Method of drug loading

The potential use of erythrocytes depends on their ability to encapsulate exogenous enzymes or other substances into erythrocytes.[3] Mostly hypotonic lysis of cell in a solution containing the drug/enzyme to be entrapped followed by restoration of tonicity to reseal them serve as a loading procedure. But other techniques like electrical breakdown, endocytosis chemical perturbation of membrane and lipid erythrocyte fusion have also been utilized.

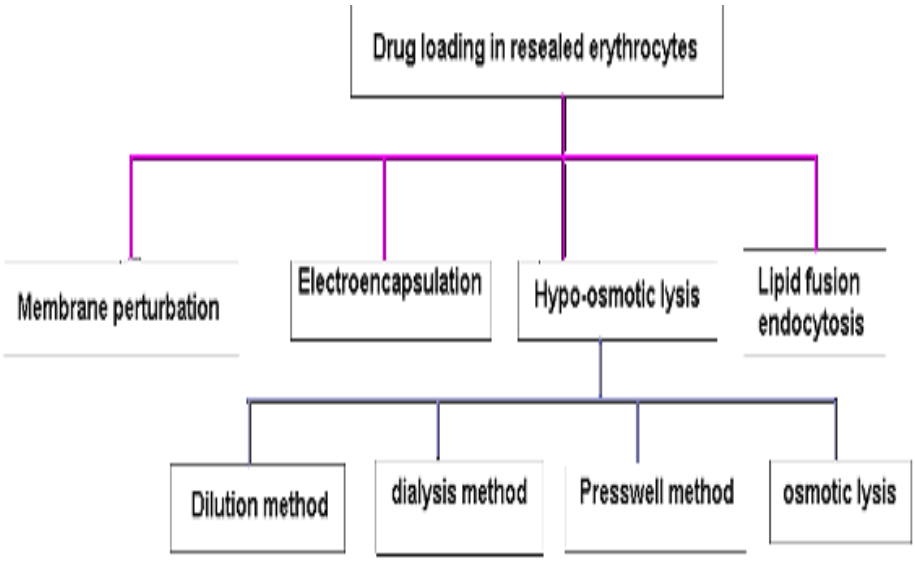

Fig.2 Various methods of preparation of resealed erythrocytes

Table 1. Comparison of various hypo osmotic lysis methods

Methods	% loading	Advantages	Disadvantages
Dilution method	1-8%	Fastest and simplest especially for low molecular weight drugs	Entrapment efficiency is very less (1-8%).
Dialysis	30-45 %	Better *In vivo* survival of erythrocyte	Timeconsuming heterogeneous size distribution
Press well dilution	20-70%	Good retention of cytoplasm *In vivo*	-------
Isotonic osmotic lysis	-----	Better *In vivo* surveillance	Impermeable only to large molecule, time consuming

By chemical perturbation of membrane (drug mediated loading)

This method is based on the observation that the permeability of the erythrocytic membrane is increased, when it is exposed to some chemical agents. Haemolysis technique in isotonic solution developed by lin and co-worker, 1975 is based on the use of an anesthetic, halothane, which changes the permeability and selectivity of the membrane. The In vivo survival of loaded erythrocyte by these techniques however was found to be poor.

Hypotonic haemolysis and isotonic resealing method

This method is based upon hypotonic lysis of cell in a solution containing the drug/enzyme to be entrapped followed by restoration of tonicity to reseal them. Three type of ghost can be distinguished type I, which reseal immediately after haemolysis; type II which reseal after reversal of haemolysis by addition of alkali ion and type III which remain leaky under different experimental condition. Erythrocytes have exceptional capability for reversible shape change with / without accompanying volume changes and for reversible deformation under distention (stress).

The swollen erythrocyte have a little capacity to resist volume greater than 50-70% of the initial volume .The principle of using erythrocyte as drug carriers reside in the fact that these ruptured membranes can be released by raising the salt concentration to its original (isotonic) level. Erythrocytes are resealed on addition of sufficient 1.54 m kcl, which restore is tonicity.

Electro-insertion or electro-encapsulation

The erythrocytes membrane is opened by dielectric breakdown and subsequently the pores are released by incubation at 370C. The method is based on creating electrically induced permeability changes at higher membrane potential difference.[8] The components can be

entrapped when an electric pulse of greater than a threshold voltage of 20micro second is given. The electromechanical compression of the membrane after breakdown leads to formation of pores. The extent of pore formation depends upon the electric field strength, pulse duration and ionic strength of the suspending medium.

The colloidal osmotic pressure of hemoglobin is about 30 mlOsm. The technique can also be used to insert protein into erythrocyte membrane. Erythrocytes are prepared by electro encapsulation method & can give a sustained release of entrapped drug. However the method is time consuming and cost factor is the major disadvantage as compared against presswell and dilution method.

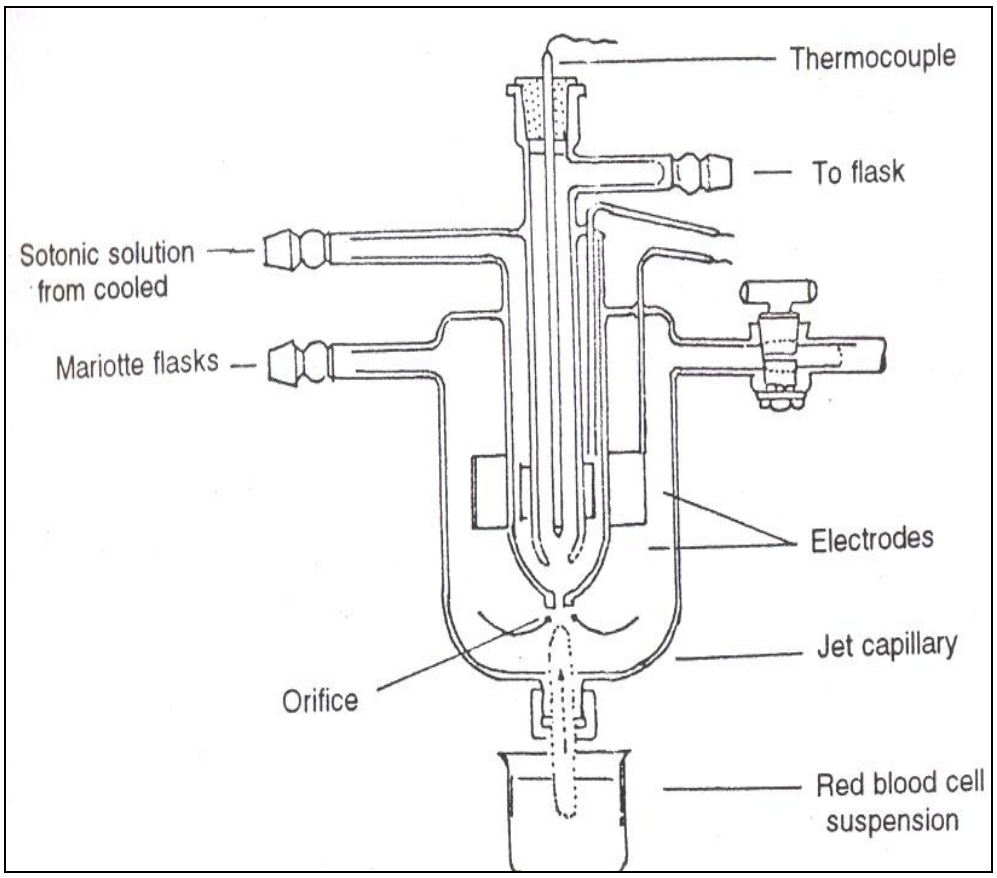

Fig.3 Schematic representation of the system used to produce resealed erythrocytes by electric breakdown method.

Loading by electric cell fusion

In this method, the molecules are first loaded in to erythrocyte ghosts. These ghosts are then caused to adhere to target cells. Electric pulses are applied to induce fusion of ghost with target cell with subsequent release of the encapsulated molecule. An antibody against a

specific surface protein of the target cell can be chemically cross-linked to drug loaded ghosts.[9]

Hypo-osmotic lysis method

In this process the intracellular and extra cellular solutes of erythrocytes are exchanged by osmotic lysis and resealing. The drug present will be encapsulated with in the erythrocyte membrane by this process.

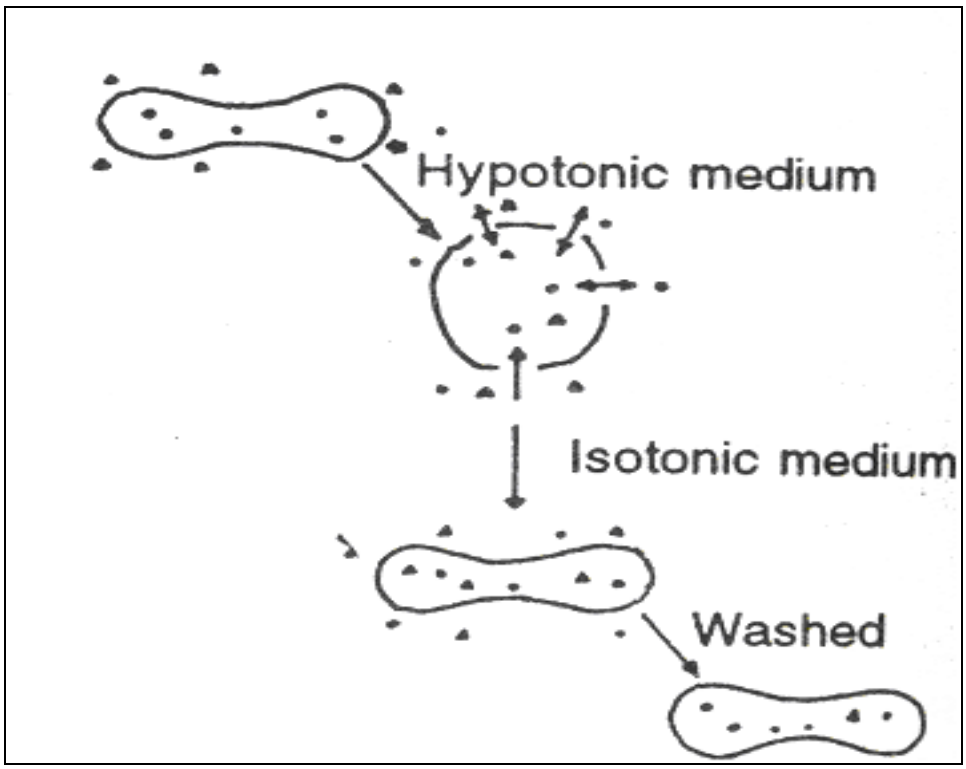

Fig. 4 Schematic representation of hyposmotic lysis procedure

Dilutional haemolysis

Erythrocyte could be treated with 2-20 volumes of material to be loaded in a hypotonic buffer at 00C or 5 mins. The method is rapid and simplest especially for low molecular weight drug.[4]

Dialysis

The major limitation of dilution procedure is, low entrapment efficiency and can be overcome by carrying out lysis and resealing within a dialysis tube.[7] The common principle that the semipermiable dialysis membrane maximizes the intra cellular: extra cellular volume ratio for macro molecules during lysis and resealing but also allows for free flow of small ion, responsible for lysis and resealing of erythrocytes.

Haemolysis and resealing is done in three steps

1. Washed erythrocytes are mixed with phosphate buffered saline containing drug/protein to be entrapped, this drug is then placed in a dialysis bag.
2. This bag is inflated with an air bubble and sealed such that it occupies not more than 75% of the internal volume.
3. The sealed bag is then placed in a bottle containing at least 200 ml of lysis buffer and placed on a mechanical rotator for 2hr at 40C.
4. The lysis bag is transferred to a bottle containing at least 200 ml of resealing buffer at room, temperature for 30 minutes.

Preswell dilutional haemolysis

The technique is based on initial controlled swelling of erythrocyte without lysis by placing them in slightly hypotonic solution followed by centrifugation at low 'g' to take them up to point of lysis.[5] The addition of small volume of drug solution to attain drug loaded released erythrocyte. The hypotonic buffer medium also contains the material to be loaded and the cells are allowed to remain lysed for 10 min.at 00C. This is followed by restoration of tonicity and resealing of membrane.

Isotonic osmotic lysis

Haemolysis in isotonic solution can be achieved both by chemical and physical means. Erythrocytes are incubated in solution of a substance with high transerrythrocytic membrane permeability the solute will diffuse in to the cell to inwardly directed chemical potential gradient.[6] This will be followed by water uptake until osmotic equilibrium is restored.

Various methods are based on this mechanism including

1. Conventional (classical) haemolysis in isotonic urea solution
2. Polyethylene induced haemolysis
3. Ammonium chloride induced haemolysis

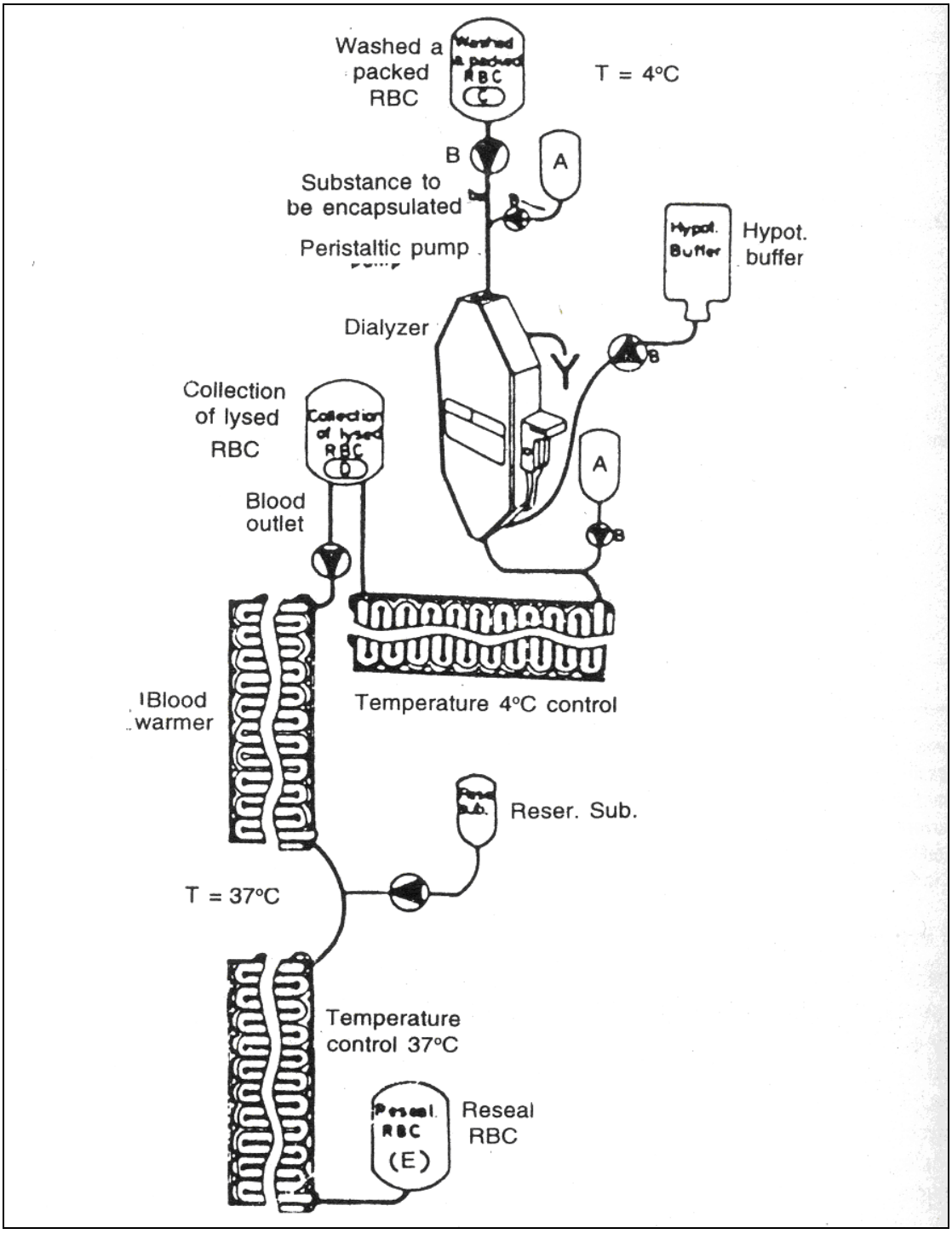

Fig.5 Continuous flow lysis

Loading by lipid fusion

Lipid vesicles containing drug can be directly fused with human erythrocyte leading to exchange of lipid entrapped drug. Nicolau and Gresonele, 1979 used this technique for loading of inositol hexaphosphate into resealed erythrocyte for the increased oxygen carrying capacity. The method, however gives very low encapsulation efficiency (1%).

Loading by "red cell loader"

Magnani and worker developed (1998) a novel method for a non diffusible drugs into human erythrocytes. The equipment designed for this method was termed as: red cell loader". It requires a less than 50 ml of blood. The method is based on two sequential and controlled hypotonic dilutions of washed RBCs followed by concentration with a haemofilter.

Entrapment by endocytosis

The vesicle membrane separate the endocytosed substance from the cytoplasm, which may shelter drug prone to inactivation in or protect the erythrocytes from drug the resulting erythrocyte contain vacuoles and have In vivo survival characteristic from released cell.[10] The drug substance is trapped in endocytic vacuoles several classes of drug as reported to produce this phenomenon. Drug like primaquine and related 8-aminoquinoline, vinblastine, hydrocortisone, propranolol, tetracaine and vitamin A.

Entrapment by endocytosis can be carried out in three steps

1 One volume of washed packed erythrocytes is added with nine volumes of buffer containing ATP< MgCl2 and CaCl2.

2 Resealing of erythrocyte membrane by the addition of NaCl to 154 mm followed by incubation for 2 min at 370C.

3 Entrapment of the material by allowing endocytosis following incubation of washed resealed cell with buffer containing the material to be entrapped for 30 min at 370C.

In vitro characterization

Released erythrocyte after loading are characterized for following parameters

Erythrocyte characterization with their quality control assays

Characterization	**Instrumentation**
Physical characterization	
Shape and surface	Transmission electron microscopy
Vesicle size and distribution	Optical microscopy diffusion cell
Drug release	Diffusion dialysis
% Encapsulation	Deprotinisation of cell membrane
Cell related characterization	
% Hemoglobin volume	Deprotinisation of cell

Osmotic fragibility	Stepwise incubation with isotonic to hypotonic saline solution
Osmotic shock	Dilution with distilled water
Turbulent shock	Passing cell suspension through a 23 gauge hypodermic needle (10ml/min)

Drug content

Packed loaded erythrocytes (0.5 ml) are first deproteinised with acetonitrile (2.0ml) and subjected to centrifugation at 2500 rpm for 10 min. The clear supernatant is analyzed for the drug content. Erythrocyte is loaded with magnetite to make them magnoresponsive.[11] The magnetite concentration in drug loaded erythrocyte could be determined using atomic absorption spectroscopy.

In vitro drug and haemoglobin release

Normal and loaded erythrocyte are incubated at 37+/-20 C in phosphate buffer saline (ph 7.4) at 50% heamotocrit in a metabolic rotating wheel incubator bath. The samples are withdrawn with the help of a hypodermic syringe fitted with 0.8 micro spectropore membrane filters. The sample is estimated for the amount of drug released. Percent hemoglobin can similarly be calculated at various time intervals at 540nm spectrophotometrically. Percent heamolysis can also be determined by comparing the absorbance of supernatant with the absorbance obtained after hydrolysis of same number of cell in distilled water. Laser light scattering may also be used to evaluate hemoglobin content if individual resealed erythrocytes.

Osmotic fragility

It is parameter which simulates and mimics the bioenvironmental conditions that are encountered on In vivo administration, in vitro handling and the effect of loaded content on the survival rates of the erythrocytes. When RBCs are exposed to solution of varying tonicities their shape changes due to osmotic imbalance. To evaluate, drug loaded erythrocyte are incubated with saline solution of different tonicities.

Osmotic shock

Osmotic shock describes a sudden exposure of drug loaded erythrocyte to an environment, which is far from isotonic to evaluate the ability of released erythrocytes to withstand the stress and maintain their integrity as well as appearance.

Turbulance shock

The parameter indicates the effect of shear force and pressure by which resealed erythrocyte formulation are injected, on the integrity of the loaded cells. Loaded erythrocytes are passed through a 23-guage hypodermic needle at a flow rate of 10 ml/min. After every pass the suspension is centrifuged at 300g for 15 min and hemoglobin content leached out is estimated spectrophotometrically.

Morphology and percent cellular recovery

Phase-contrast optical microscopy transmission electron microscopy and scanning electron microscopy are the microscopic method used to evaluate the shape, size, and the surface feature of the loaded erythrocytes percent cell recovery can be determined by accessioning the number of intact erythrocytes remaining per cubic mm with the help of hemocytometer.

Different forms of drug loaded red blood cells

Normally, more than 80% of the erythrocyte ghost loaded with drug or enzyme appears as a biconcave disks when they are observed under electron microscope.[12]

Less than 20% cells show abnormal morphology. The rest appear as stomatocyte or spherocyte, cell with different infoldins and other abnormal or destroyed forms. This is because of osmotic balance.

On swelling, the cell gets converted from diskocyte to spherocyte and thus get compromised with a lower ratio of surface features. Further increased in hypotonicity may lead to the formation of echinocytes and cells with different in foldings and other damaged forms.

Erythrocytes on haemolysis and washing loose nearly all their hemoglobin and on resealing the resultant cells appear as pale or transparent in appearance and are referred to as erythrocytes ghosts or white resealable erythrocyte ghosts. However these ghosts contain 5-10% of their original hemoglobin. This can be overcome by using gel filtration method for producing ghost. The resealed ghosts have been used frequently to study various transport pathway found in the erythrocyte membrane including the anionic exchange system sodium pump, and the calcium-activated potassium channel.

Shelf and storage stability

Storage of erythrocyte place a major challenge in their practical utility as drug delivery system Lewis and Alphar 1984 have reported that encapsulated product and carrier both

exhibit satisfactory self-stability when stored in hank's balanced salt solution at 40 C for 2 weeks similar result were obtained by suspending cell in oxygenated hbss containing 1% soft bloom gelatin. Another method utilized for storage has been the cryopreservation of erythrocytes in liquid nitrogen temperatures.

Influence of membranolytic and membranotropic substrate

Certain substances influence the geometric balance of normal biconcave dissociates and in turn influences the functioning of erythrocyte in circulation. The parameter affected includes normal metabolism, deformability, viscosity and morphology and surface characteristic.[13]

It can be categorized as follows

(a) membrane active substrates

(b) membrane lytic substrates.

Some drugs like daunomycin, amphotericin b and primaquine affect the erythrocyte in different way altogether.

Biomedical application:

Erythrocytes have found a large number of possible applications in various fields of human and veterinary medicine.[14]

Erythrocyte as drug / enzyme carriers

Erythrocyte could be used as a circulating carrier to disseminate bioactive agent over a prolonged period of time in circulation or selectively to liver, spleen and lymph nodes.

Erythrocytes as carrier for enzymes

Erythrocytes served as an ideal carrier for enzyme in the treatment of inherited metabolic diseases. The entrapment of catalase, urease, uricase, invertase, arginase, asparagines, and beta-galactosidase in erythrocytes using hypotonic lysis method has proved that erythrocyte could operate as enzyme carriers.

Erythrocytes as carrier for drug

Various bioactive agents encapsulated in erythrocyte are developed for the slow and sustained release in circulation to allow effective treatment of parasitic diseases. Erythrocytes served as a an ideal carrier for antineoplastic agents like bleomycin, adriamycin, actinomycin-d, or cytosine arabinoside in erythrocyte ghost.

Erythrocyte as carriers for protein and macromolecules

Zolla and co-workers, 1991 reported RBCs as carrier for delivering of protein. Bird et al 1983 proposed erythrocytes as carrier for insulin for its sustained release. They have examined a number of low molecular weight compounds, inhibitor of insulin degrading system found in erythrocyte. Grain and co-worker 1996 reported that carrier RBC preparation may serve as a cellular sustained delivery system for In vivo administration of recombinant human erythropoietin.

Drug targeting

A drug delivery should be site specific and target oriented in order to exhibit maximal therapeutic index and minimum side and toxic effect. It has been observed that osmotically loaded erythrocyte can act as drug carrier in systemic circulation, where as chemically surface modified erythrocyte are targeted to organ of the mononuclear phagocytic system.[15]

Drug targeting to RES organs

The damaged erythrocyte is quickly removed from circulation by phagocytic kupffer cells located in liver and spleen. Lightly damaged or modified erythrocyte is sequestered by spleen similar to normal senescent erythrocytes, where liver sequestered heavily modified erythrocyte. Since, the liver is per fused 7 times greater than the spleen. Cross linking of loaded erythrocytes increases delivery of encapsulated to macrophages. The hypotonic ally loaded mouse erythrocyte showed a week recognisation by macrophages, similar to native erythrocytes.

Antibody anchored erythrocytes (immunoerythrocytes)

Antibody coating of released drug carrier erythrocyte may be useful for drug targeting to the res. Mouse erythrocytes coupled with an Anti-Thy 1.2 monoclonal antibody was preferred to biotynilation. Antibody – coupled erythrocyte were incubated with cytotoxic t-lymphocyte in vitro at a 20:1 ratio

Erythrocyte as circulating bioreactors

Erythrocytes have been realized as carriers for enzymes to serve as circulating bioreactor. The immobilization of enzyme which catalyses these reactions can be used as bioreactors. The efficacy of araginase loaded erythrocyte in reducing plasma araginine levels by 40% with in 2 hours of infusion into a patient with hyper argininaemia. Erythrocyte has also been used as a circulating bioreactor for the control delivery of antiviral drugs.

Erythrocytes in cell biological application

Biological function of the macromolecules like DNA, RNA, and protein are exploited for various cell biological application. The techniques involve loading of macromolecules into erythrocytes ghost using hypotonic haemolysis method.[16] Erythrocyte ghost cell fusion method mediated by HVJ is relatively easier, and has the special advantages that it can be used for micro injection into many cells at the same time.

This method has certain advantages and disadvantages as

Advantages

- ✓ It can be employed for quantitative injection of material in to cells.
- ✓ It does not require any special apparatus and or techniques.
- ✓ simultaneous introduction of material into a large number of cells is possible
- ✓ It permits introduction of materials into cell in suspension culture.
- ✓ The damage to the cell is minimal.

Disadvantages

Co introduction of the erythrocyte membrane, viral envelopes, viral rna and residual hemoglobin may have unpredicted effect on the cells. A comparatively large amount of test material is desired than that for the micro capillary method. Direct injection into the cell nucleus is not feasible.

The techniques have been used in several cell biological applications

(i) Yamaizumi 1978[36] demonstrated that fragment of diphtheria toxin can be introduced in to the target cells by the erythrocyte ghost cell fusion.

(ii) Yamaizumi et al 1979 examined the feasibility of using antibody that can function in a cell. The studies using antibody against diphtheria toxin fragment indicated that the antigen-antibody reaction occur in a living cell as effectively as in a cell free system. This indicates that the antibody against fragment a retains its function equally well in vitro and In vivo.

(iii) Various workers have demonstrated the mechanistic of protein import into the nucleus using erythrocyte ghost fusion method.

CONCLUSION

The concept of employing erythrocytes as drug or bioactive carrier still needs further optimization. A large amount of valuable work is needed so as to utilize the potential of erythrocytes in passive as well as active targeting of drugs. Diseases like cancer could surely

find its cure. Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug carrier concept.

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