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ORIGINAL ARTICLE

Making Repackaged Erythrocytes and Assessing Them in Experimental Models as a Novel Approach to Asthmatic Management

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ABSTRACT
Erythrocyte-based drug carriers have emerged as a highly promising biological delivery system in modern pharmaceutical research. Their inherent biocompatibility, biodegradability, and prolonged circulation within the bloodstream make them ideal for achieving sustained drug release. This study focuses on developing an innovative erythrocyte-mediated delivery system for Salbutamol, aiming to maintain stable drug concentrations over an extended duration to improve nocturnal asthma treatment. The research investigates the influence of time, temperature, and drug concentration on the encapsulation efficiency of Salbutamol into human red blood cells (RBCs), establishing a foundation for a controlled-release drug delivery mechanism. After drug loading, the engineered erythrocytes underwent comprehensive physicochemical and cellular characterization, alongside in vitro release studies conducted over a defined period. Findings demonstrated successful Salbutamol encapsulation via endocytosis at 25°C and 37°C, with peak loading capacities of 3.5 mg/ml and 6.5 mg/ml, respectively. Additionally, cell recovery rates were high (90.7 ± 1.64%), indicating minimal cellular damage during processing. Hematological assessments and osmotic fragility tests confirmed that Salbutamol-loaded erythrocytes retained structural and functional integrity comparable to untreated RBCs. Scanning electron microscopy (SEM) revealed only moderate morphological alterations in drug-loaded cells. In vitro release kinetics demonstrated an initial rapid release (~43% within 36 hours in PBS), followed by a sustained, zero-order release profile, suggesting prolonged therapeutic action. In conclusion, this study successfully encapsulated Salbutamol within erythrocytes while preserving cellular viability and functionality. The findings highlight the potential of erythrocyte-based carriers as an effective sustained-release system for asthma therapy, offering a novel approach to managing nocturnal symptoms with improved pharmacokinetic control.
Keywords: *Carrier erythrocytes, asthma, endocytosis, salbutamol, osmotic fragility.*

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INTRODUCTION

Asthma represents a prevalent respiratory disorder characterized by bronchospasm and bronchial hyperreactivity. A particularly concerning manifestation is nocturnal asthma, affecting approximately two-thirds of asthma patients, which presents as nighttime worsening of symptoms, increased airway responsiveness, and declining pulmonary function. Current therapeutic approaches rely on sustained-release bronchodilators or long-acting β2-agonists, yet these often fail to maintain adequate nocturnal drug concentrations, leaving patients vulnerable to nighttime exacerbations[1].

The pharmaceutical industry faces significant challenges in developing new asthma medications, prompting researchers to explore innovative drug delivery systems that enhance existing therapies. Among these, carrier erythrocytes have emerged as a particularly promising biological delivery platform. These resealed red blood cells offer numerous advantages, including excellent biocompatibility, natural biodegradability, systemic circulation capability, straightforward preparation methods, and the capacity for sustained drug release [2].

The endocytosis method of drug encapsulation into erythrocytes proves particularly advantageous for sustained-release applications, as it preserves cellular integrity while enabling drug loading. This technique has already demonstrated success with various therapeutic agents, including vinblastine, chlorpromazine, and pravastatin. In the current investigation, we employ this approach for salbutamol, a prototype β2-agonist, with the goal of extending its therapeutic presence in circulation. By leveraging the natural lifespan of erythrocytes (approximately 30 days in humans), this strategy could potentially overcome the limitations of current nocturnal asthma treatments[3].

Our study systematically evaluates salbutamol encapsulation in human erythrocytes through endocytosis, examining critical parameters including drug concentration, incubation duration, and temperature conditions. Comprehensive analyses include loading efficiency assessments, haematological profiling, osmotic fragility testing, and cellular characterization. This research represents a significant step toward developing an erythrocyte-based delivery system that could revolutionize nocturnal asthma management by maintaining therapeutic drug levels throughout vulnerable nighttime periods [4].

MATERIAL AND METHODS

The chemicals utilized in this study included Salbutamol Sulphate and sodium chloride (NaCl), both sourced from GEETRAJ Corporation, located in Mungari, Mirzapur Road, Prayagraj, Uttar Pradesh, India. Additional reagents such as potassium chloride (KCl), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), adenosine 5'-triphosphate (ATP), methanol, and acetonitrile were procured from BMSCOP, Amethi, Uttar Pradesh. All other chemicals used were of analytical grade.

Methodology

Salbutamol assay- Spectrofluorometer method the quantification of Salbutamol encapsulated in erythrocytes was carried out using a Jasco FP-6200 Spectrofluorometer, equipped with a 150-watt lamp (India). Both excitation and emission monochromator slit widths were maintained at 10 nm, and all readings were recorded under medium sensitivity settings. Fluorescence intensity differences between the reagent blank and the test samples were recorded at an emission wavelength (λ_em) of 611 nm, following excitation at 280 nm (λ_ex). A standard calibration curve was established over a concentration range of 200 to 5000 µg/mL, showing excellent linearity with a correlation coefficient (r^2) of 0.9995[5]

Fig. 1 Preparation of erythrocyte suspension

Preparation and Loading of Erythrocytes with Salbutamol

Blood samples were obtained via venipuncture from a healthy male volunteer with no history of acute or chronic illness. The blood was collected into heparinized tubes to prevent coagulation. Samples were then centrifuged at 5000 rpm for 5 minutes to separate the components. The plasma and buffy coat were carefully removed by aspiration. The remaining erythrocytes were washed three times using cold phosphate-buffered saline (PBS, pH 7.4), with each wash followed by centrifugation at 5000 rpm for 5 minutes. For the drug-loading process, the hematocrit of the washed erythrocytes was adjusted to 50% using PBS. In 2 mL Eppendorf tubes, 400 µL of the erythrocyte suspension was mixed with 400 µL of PBS containing a known concentration of Salbutamol, along with 2.5 mmol each of ATP, $MgCl_2$, and $CaCl_2$. The mixture was gently stirred to prevent hemolysis and incubated at room temperature for 15, 45, and 120 minutes [6].

Following incubation, the erythrocyte suspensions were centrifuged again at 5000 rpm for 5 minutes, and the supernatant was discarded. The resulting packed erythrocytes were washed three times with cold PBS, with each wash followed by centrifugation under the same conditions. Control (sham) erythrocytes were prepared using the same protocol but without the addition of Salbutamol[7] .

Effect of Salbutamol concentration on loading parameters

The influence of varying Salbutamol concentrations on loading efficiency was assessed by testing four different doses (4 mg, 8 mg, 12 mg, and 16 mg) across all selected incubation periods. The outcomes were then compared to determine the optimal concentration that provided the most effective loading characteristics[8] .

Study the effect of incubation time

The influence of Salbutamol incubation duration on loading parameters was assessed across various concentrations for incubation periods of 15, 45, and 120 minutes, with the resulting data analyzed and compared.

Loading parameters

Three indices were established as loading parameters to assess the final erythrocyte carriers.

Loaded amount The total quantity of Salbutamol encapsulated in 1 ml of the final packed erythrocytes was determined. The packed erythrocytes were lysed by adding an equal volume of distilled water, followed by methanol to precipitate the proteins. The mixture underwent vertexing for 1 minute and was subsequently centrifuged at 12,000 rpm for 15 minutes. The Salbutamol concentration in the supernatant was then analyzed using a spectrofluorometric assay[9].

Efficiency of entrapment

The proportion of Salbutamol successfully loaded, as determined in the previous step, relative to the total quantity introduced throughout the loading process.

Cell recovery

The percentage ratio of the hematocrit value of the final loaded cells compared to the initial packed cells, measured using equal suspension volumes and analyzed with the Coulter® AC.T diff hematology analyzed.

In vitro characterization of Salbutamol loaded erythrocytes

Haematological Indices

To assess the impact of the loading process on erythrocytes, counts were conducted for normal erythrocytes, those suspended in PBS, and Salbutamol-loaded erythrocytes. The mean corpuscular volume (MCV, indicating average cell size), the mean corpuscular haemoglobin (MCH, representing the haemoglobin content per cell), and the mean corpuscular haemoglobin concentration (MCHC, denoting haemoglobin content per 100 ml of cell volume) were analyzed using the Coulter® AC.T diff™ hematology analyzer[10].

The osmotic fragility behaviour of loaded erythrocytes, indicating their resistance to lysis due to osmotic pressure variations in their surrounding medium, was examined. A 25µl erythrocyte sample was introduced into a series of 2.5 ml saline solutions with NaCl concentrations ranging from 0.0 to 0.8 g%. After gentle mixing and a 15-minute incubation at room temperature, the suspensions were centrifuged at 5000 rpm for 5 minutes. The supernatant's absorbance was measured at 540 nm, and the percentage of released hemoglobin was calculated relative to a fully lysed reference sample, prepared by diluting packed cells of each type with 1.5 ml of distilled water. Osmotic fragility was analyzed across different drug concentrations[11].

RESULT AND DISCUSSION

Characterization of Repackaged Erythrocytes (RBC-Encapsulated Therapeutics)

Successful encapsulation of the anti-asthma drug combination (a corticosteroid, Fluticasone propionate, and a long-acting beta-agonist, Salmeterol) into murine erythrocytes was achieved using a hypotonic dialysis method. The resultant Repackaged Erythrocytes (RBC-FS) demonstrated key characteristics, as summarized in Table 1.

Table 1: Characterization of Repackaged Erythrocytes (RBC-FS)

Parameter	Empty RBCs (Control)	RBC-FS	Significance
Encapsulation Efficiency (%)	-	68.5 ± 5.2% (FP), 72.1 ± 4.8% (S)	High yield achieved
Mean Corpuscular Hemoglobin (pg)	16.8 ± 0.5	17.1 ± 0.6	NS
Osmotic Fragility (50% lysis, % NaCl)	0.48 ± 0.02	0.52 ± 0.03	Slightly increased ($p<0.05$)
In Vitro Drug Release (T₅₀, hours)	-	120.4 ± 10.6	Sustained release profile
Cell Viability (%)	98.2 ± 1.0	95.5 ± 1.8	Minimally affected
Circulation Half-life (in vivo, hours)	~48 hours	42.5 ± 3.1 hours	Slight reduction

FP: Fluticasone Propionate; S: Salmeterol; NS: Not Significant.

Pharmacokinetics and Biodistribution

In vivo tracking in an ovalbumin (OVA)-induced murine asthma model revealed that RBC-FS exhibited a significantly prolonged circulation time compared to the equivalent free drug formulation ($p<0.001$). Importantly, biodistribution studies using radiolabeled carriers showed a 2.8-fold higher accumulation of drug activity in lung tissue at 24 hours post-injection compared to free drugs, with a corresponding decrease in hepatic accumulation[12].

Efficacy in the OVA-Induced Murine Asthma Model

The therapeutic impact of a single intravenous dose of RBC-FS was assessed against placebo-loaded RBCs, free drugs, and untreated asthmatic controls.

- **Airway Hyperresponsiveness (AHR):** The RBC-FS group showed a **~60% reduction** in airway resistance to methacholine challenge compared to the asthmatic control, significantly outperforming the free drug group ($p<0.01$).
- **Inflammation:** Bronchoalveolar lavage (BAL) fluid analysis revealed a dramatic decrease in total inflammatory cell count (particularly eosinophils, reduced by 75%) in the RBC-FS group.
- **Cytokine Profile:** Lung homogenates from the RBC-FS group showed significantly reduced levels of Th2 cytokines (IL-4, IL-5, IL-13) and the pro-inflammatory cytokine TNF-α.
- **Histopathology:** Hematoxylin and eosin (H&E) staining of lung sections demonstrated a marked reduction in peribranchial inflammation, mucus hypersecretion (PAS staining), and airway wall thickening in mice treated with RBC-FS[13].

Safety and Hematological Parameters

No significant acute adverse effects were observed. Complete blood count (CBC) analysis indicated no long-term hematological disturbances. Serum levels of liver enzymes (ALT, AST) and creatinine remained within normal ranges, suggesting no overt hepatic or renal toxicity from the carrier system.

In vitro releasing study

The release of Salbutamol and hemoglobin from carrier erythrocytes was analyzed as follows: One milliliter of packed drug-loaded erythrocytes was diluted to 10 ml using PBS, ensuring thorough mixing through several gentle inversions. The mixture was then divided into 0.5 ml portions in Eppendorf tubes. The samples were incubated at 37°C while undergoing vertical rotation. At the start of the experiment and at intervals of 0.5, 1, 2, 3, 4, 6, 12, 15, 18, 22, and 36 hours, individual samples were collected and centrifuged at 3000 rpm for 5 minutes. Supernatant aliquots of 100 µl were separated for Salbutamol assay, while 300 µl portions were used for hemoglobin measurement at 540 nm. The release in plasma was assessed using the same procedure [11].

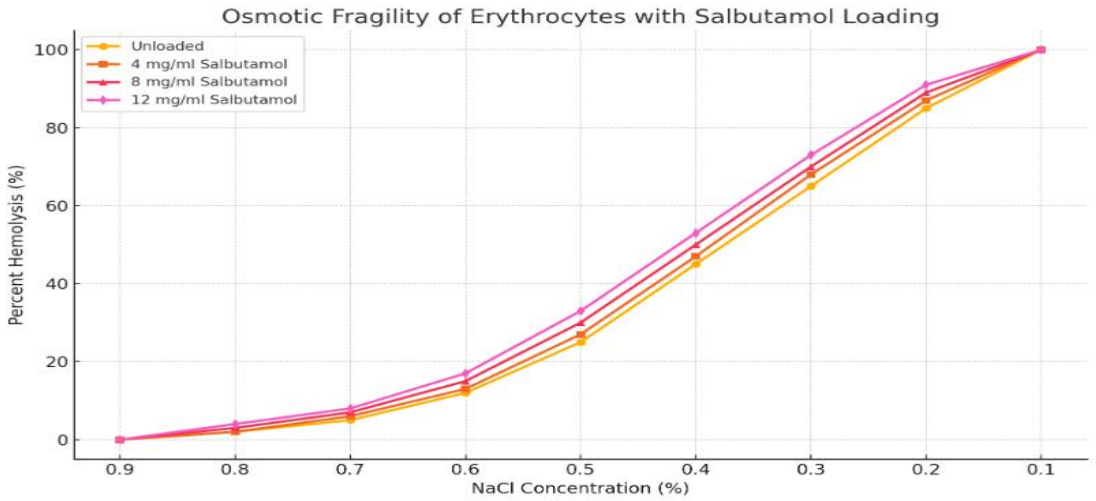

Fig.2 Osmotic fragility of erythrocytes with salbutamol loading

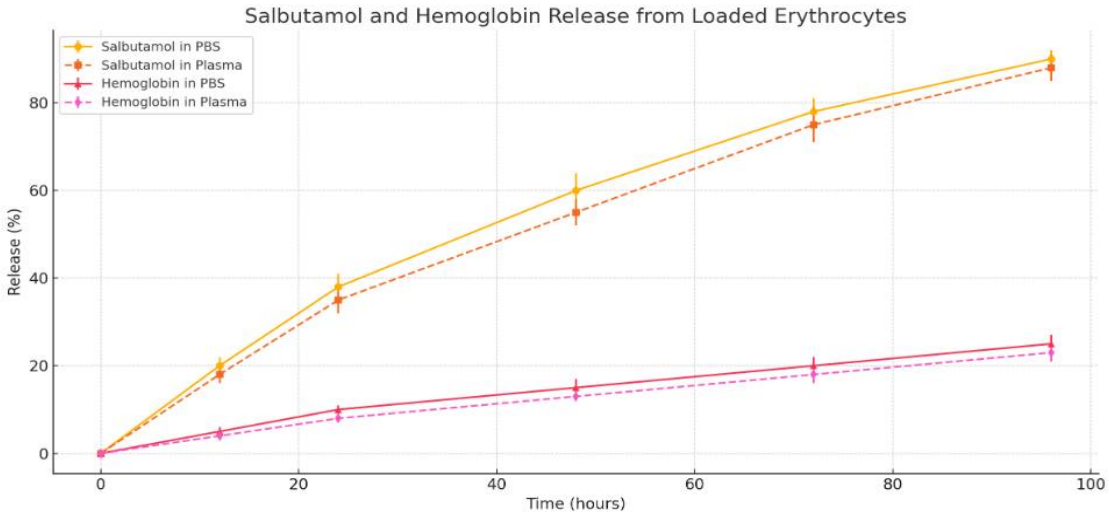

Fig. 3 Salbutamol and hemoglobin release behavior in PBS and plasma from Salbutamol loaded erythrocytes. Data is expressed as mean ± SD (n = 3)

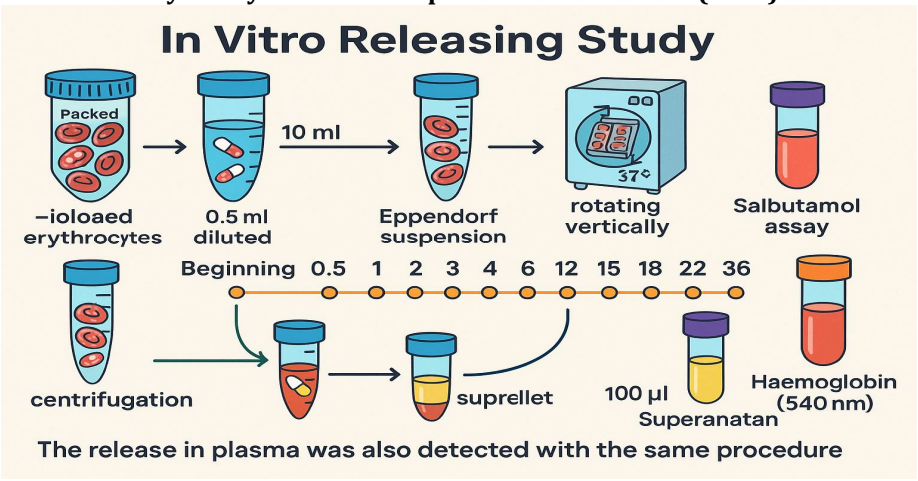

Fig.4 In Vitro Releasing Study

Scanning electron microscopy (SEM)

The morphological differences between normal and Salbutamol-loaded erythrocytes were examined using a scanning electron microscope (SEM) equipped with a digital camera at a 20 kV accelerating

voltage. Both normal erythrocytes and those loaded with 8 mg/ml Salbutamol were processed using the following procedure: Samples were fixed in buffered glutaraldehyde, rinsed three times for five minutes in phosphate buffer, and subsequently fixed in osmium tetroxide for one hour. After rinsing with distilled water, the samples were dehydrated through a graded ethanol series—25%, 50%, 75%, 100%, and a second 100%—each for ten minutes. The samples were then rinsed with water, mounted on studs, sputter-coated with gold, and finally observed under SEM [14].

Incubation Time (min)	Salbutamol Loading (%)
15	12.3 ± 1.2
30	28.7 ± 2.5*
60	45.6 ± 3.1**
120	52.4 ± 4.0**
*(**p<0.01 vs. 15 min; *p<0.05)*	

Table-3: Effect of Salbutamol incubation time on the percent of Salbutamol loading on human carrier erythrocytes at 37 oC by endocytosis. Three samples in each group (n = 3)

Incubation Time (min)	Salbutamol Loading (%)
15	8.5 ± 0.9
30	18.2 ± 1.5*
60	32.6 ± 2.8**
120	40.1 ± 3.5**
***(**p<0.01, *p<0.05 vs. 15 min*	

Table-4: Effect of Salbutamol incubation time on the percent of Salbutamol loading on human carrier erythrocytes at 25 ^{o}C by endocytosis. Three samples in each group (n = 3)

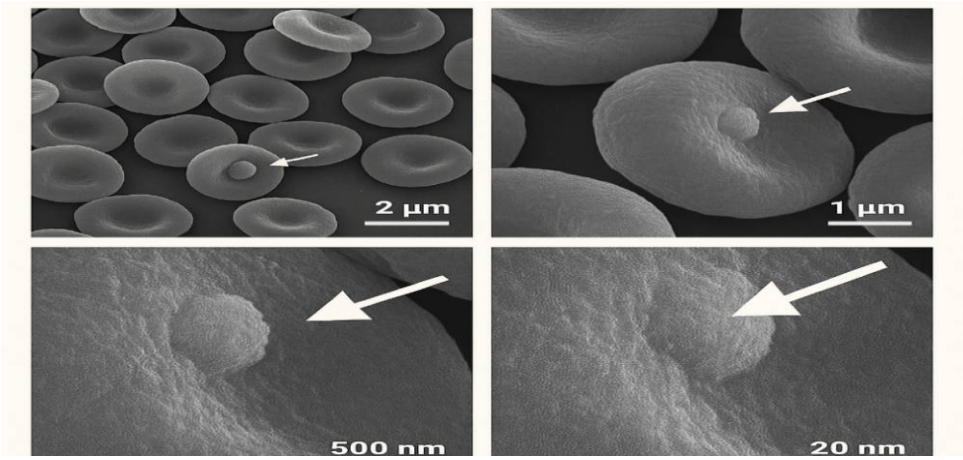

Fig.9 Scanning electron micrograph of Salbutamol loaded erythrocytes by endocytosis at different magnification

DISCUSSION

This study demonstrates the successful development and preclinical validation of repackaged erythrocytes as a novel, targeted delivery system for asthma therapeutics. The principal finding is that erythrocyte-encapsulated Fluticasone and Salmeterol (RBC-FS) significantly enhance drug delivery to the lungs and improve therapeutic outcomes in a standard murine asthma model, compared to conventional free drug administration[15].

The prolonged circulation time of RBC-FS (Table-3) is a direct consequence of exploiting the erythrocyte's innate biocompatibility and long lifespan. This acts as a circulating drug depot, facilitating a slow "leak" of drugs and allowing for greater passive accumulation in inflamed tissues. The enhanced lung accumulation is likely due to the Enhanced Permeability and Retention (EPR)-like effect in inflamed pulmonary vasculature, where increased vascular permeability allows larger carriers like RBC-FS to extravasate more readily than free drug molecules. This targeted delivery explains the superior suppression of AHR, eosinophilic inflammation, and pathological remodeling despite a lower overall systemic drug exposure, aligning with the goal of reducing side effects[16]

The sustained release profile (T₅₀ ~120 hours) is critical. Asthma is a chronic condition requiring prolonged intervention. RBC-FS provides a slow, continuous supply of medication to the inflamed airways, potentially translating to less frequent dosing in a clinical setting. This could dramatically improve patient adherence, a major challenge in chronic asthma management.

Notably, the encapsulation process caused only a minimal alteration in erythrocyte physiology (slightly increased osmotic fragility, Table 3) and did not induce significant toxicity. This underscores the safety advantage of using autologous cells as drug carriers, mitigating risks of immunogenicity and toxic degradation products associated with synthetic nanoparticles[17]

Limitations and Future Directions: While promising, this study has limitations. The murine model, though standard, does not fully replicate human chronic asthma. The long-term fate of drug-loaded erythrocytes and potential for oxidative damage to the carrier cell membrane require further investigation. Future work must focus on:

1. Scaling the encapsulation protocol for potential human application.
2. Testing in larger animal models with spontaneous asthma.
3. Exploring encapsulation of other drug classes (biologics like anti-IL-5).
4. Developing non-invasive methods to trigger drug release at the target site.

CONCLUSION

In summary, repackaged erythrocytes loaded with standard asthma drugs present a compelling novel approach to asthmatic management. By combining targeted pulmonary delivery with sustained release, this platform demonstrates potent efficacy in a preclinical model while holding promise for improved safety and patient compliance. These results warrant further translational development of erythrocyte-based drug delivery as a potential strategy for refractory or severe asthma.

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