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# Investigation on Resealed Erythrocytes as Carriers for 5-Fluorouracil

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**The present investigation reports the formulation of resealed erythrocytes (RBCs) as carriers for the anticancer agent, 5-fluorouracil (5-FU). The preswell dilution technique was adopted for loading and various formulation conditions like preswelling point, drug concentration, time of contact, point of lysis, resealing point, incubation period and crosslinking conditions were optimized. Nearly spherical RBCs with 6.9±1.014 µm size, 88% cell recovery and 43.84±1.54% drug loading were obtained after optimization. The 5-FU loaded RBCs were evaluated with respect to osmotic fragility, turbulence shock studies, osmotic shock studies, in vitro 5-FU and hemoglobin (Hb) leaching. It was concluded that the loading procedure had reduced the resistance of the RBCs to osmotic and turbulence shock but the cell shape and integrity remain unaffected. Due to crosslinking, stress resistance was improved and 5-FU and Hb leaching during storage were reduced. *In vivo* studies in healthy rats after i.v. administration of loaded RBCs showed a significant 5-FU accumulation in spleen and lungs as compared to that from plain 5-FU solution. These findings point to the potential utility of resealed RBCs as site specific carriers for improving therapy of 5-FU, especially in tumors of lungs.**

Drug targeting is a specific form of drug delivery where a pharmacological agent is directed selectively to its site of action. Drug targeting is achieved by either of two approaches, chemical modification or carrier system. Various drug carriers being investigated today include polymers, nanoparticles, cellular carriers and macromolecules, amongst which, cellular carriers offer many advantages that include biodegradibility, non-immunogenicity and ability to carry a large amount of drug in small volumes.

Ihler *et al*[1] and Zimmermann[2] independently suggested that erythrocytes (RBCs) could be potentially useful as drug carriers. When they are osmotically lysed and then resealed, there is an exchange of intracellular and extracellular solutes. Therefore, a drug present during the lysis procedure will be encapsulated within the membrane envelope of the erythrocytes. Such RBCs are referred to as Carrier RBCs[3]. The carrier RBCs have been used to target Reticulo Endothelial System (RES)[4-9] whereas, surface treated RBCs can escape detection by RES and have been used as long circulating carriers for controlled release[10-12] and targeted delivery[13-16].

The aim of the present research was to investigate the potential of resealed erythrocytes as carriers for the widely used anticancer agent, 5-fluorouracil (5-FU).

## EXPERIMENTAL

5-FU was gifted by BioChem Pharmaceuticals, Mumbai and Soft Bloom Gelatin was gifted by Gellikeps Pvt. Ltd., Baroda. Human Blood was collected from blood banks. All other chemicals and reagents used were of Analytical Grade. Albino rats (from M/s. Deep Biolab Animal Supply, Ahmedabad) of either sex weighing 150-250 g were used after overnight fasting.

### Isolation of erythrocytes and drug loading:

The whole human blood was centrifuged at 2750 rpm for 10 min and plasma and buffy coats were removed.

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The separated RBCs were washed thrice with phosphate buffer saline (PBS) and mixed with sufficient PBS to get haematocrit of 50%. Preswell dilution technique[13,17,18] was adopted for loading of 5-FU. The haematocrit was centrifuged at 2750 rpm and the packed RBCs were treated with 4 volumes of NaCl solution to bring about their preswelling. The contents were mixed gently by inversion and centrifuged. Cell hemolysate (1:1 combination of packed RBCs and distilled water) was layered over the packed RBCs and 5-FU solution in distilled water (10 mg/ml) was added dropwise with gentle mixing till the point of lysis. After allowing contact for specified time, resealing was done by adding resealing buffer, Hank's Balanced Saline Solution (HBSS). The RBCs were then incubated at 37° to harden the cell wall and washed thrice with PBS to remove unentrapped drug.

**Crosslinking of RBCs:**

The stabilization of RBCs was brought about by chemical croslinking with gluteraldehyde solution[19]. Different concentration and contact time were investigated. Then RBCs were washed thoroughly to remove traces of gluteraldehyde. Finally, the loaded RBCs were suspended in 1% soft bloom gelatin in HBSS.

**Evaluation of loaded RBCs:**

The drug inside the RBCs was evaluated by lysing the loaded RBCs with double its volume of distilled water and extracted with ethyl acetate:propane-2-ol (7:3) and absorbance was measured against similarly prepared blank at 272 nm using Hitachi Spectrophotometer. The cell size was measured using Neubaur's chamber at 45 X and its shape and integrity were observed microscopically.

***In vitro* characterization:**

The per cent cell recovery was determined by counting the number of RBCs per $mm^3$ before and after loading of 5-FU using a haemocytometer. The per cent hemoglobin (Hb) retained inside the carrier RBCs was determined spectrophotometrically. The carrier RBCs were lysed in distilled water and then centrifuged at 1000 rpm for 5 min and the absorbance of the supernatant was measured at 540 nm. Value obtained after similar treatment of normal RBCs, using the supernatant of RBCs washings as the background, was considered 100% Hb release.

*In vitro* release of 5-FU and Hb were monitored periodically from the loaded RBCs. The suspension was stored at 4° in a refrigerator and the clear supernatant was analysed for 5-FU and Hb content spectrophotometrically. Osmotic fragility studies were performed for normal as well as loaded RBCs by incubating them in NaCl solution of different strengths for 10 min at 37°. The supernatant was estimated for per cent Hb release spectrophotometrically. The turbulence shock studies were performed by extruding the erythrocyte suspension through a 22 gauge needle, centrifuging and estimating the supernatant for Hb content. Osmotic shock studies were conducted by diluting the erythrocyte suspension with different volumes of distilled water and measuring the Hb content of the supernatant.

***In vivo* studies:**

Rats were anaesthetized with urethane given i.p. and product equivalent to 1 mg 5-FU was injected via tail vein. The rats were sacrificed at intervals of 1, 2 and 4 h in case of 5-FU loaded RBCs and 1/2, 1 and 2 h in case of plain 5-FU drug solution. The liver, the lungs and the spleen were removed and homogenized in chilled PBS. The tissue homogenates were extracted with equal volumes of ethyl acetate:propane-2-ol (7:3) and absorbance was measured at 272 nm against similarly prepared blank.

## RESULTS AND DISCUSSION

An optimum preswelling point was decided on the basis of most dilute solution of NaCl which provided optimum swelling and drug entrapment without visible hemolysis. Data in Table 1 shows that optimum preswelling point was 0.65% NaCl as it could bring about visible hemolysis and optimum drug entrapment. Higher concentration (>0.7% NaCl) showed poor preswelling while drug entrapment was reduced at lower concentration (<0.65%) due to extensive lysis.

It was found that the drug entrapment was 41.86%±1.57 when 5 mg/ml 5-FU solution was used and 45.00%±3.75 when 10 mg/ml was used. But the drug entrapped per unit volume of packed RBCs was found 1.6 mg/ml in case of 5 mg/ml and 3.4 mg/ml in case of 10 mg/ml solution. Hence, 10 mg/ml of 5-FU solution was used for loading of RBCs as it gave nearly double loading as compared to 5 mg/ml solution.

Based on microscopic studies, we found that 10 min contact time provided optimum drug entrapment without affecting cell integrity, whereas at higher time periods,

TABLE 1: OPTIMIZATION OF PRESWELLING POINT-EFFECT OF NACL ON HB RELEASE AND 5-FU ENTRAPMENT

| NaCl(%) | Hb release (% ±S.D.)* | Drug entrapment (% ±S.D.)* |
|---|---|---|
| 0.9 | 0.25±0.012 | 12.01±1.598 |
| 0.85 | 0.59±0.021 | 12.63±1.492 |
| 0.8 | 1.56±0.006 | 13.25±1.866 |
| 0.75 | 3.08±0.016 | 29.10±2.904 |
| 0.7 | 5.33±0.061 | 41.74±4.033 |
| 0.65 | 21.52±1.66 | 45.02±5.283 |
| 0.6 | 29.79±2.230 | 38.02±2.85 |
| 0.5 | 36.65±4.55 | 26.75±1.590 |

* Mean of 3 readings

# % Hb release is calculated using the formula

$$\frac{A_{540} \text{ of sample} - A_{540} \text{ of background}}{A_{540} \text{ of 100\% Hb release}}$$

the RBCs shrunk in size. It was observed that as the volume of resealing solution increased from 5 to 15 ml, cell recovery was increased from 70 to 88% and remained at 88% when 20 ml was used. Hence, 15 ml resealing volume was considered optimum. It was also observed that incubation for longer than 15 min led to change in cell shape and integrity due to detrimental effect of incubation temperature.

The crosslinking conditions such as gluteraldehyde (glu) concentration and time of crosslinking were optimized on the basis of per cent drug release, Hb release and microscopy. The concentration of glu in the range of 0.1 to 0.5% and time of crosslinking in the range of 5 to 20 min were investigated. It was found that RBCs shrunk at higher than 10 min and at higher than 0.3% glu concentration. Lower concentration of 0.1% glu and 5 min contact were found inappropriate to stabilize the RBCs, as high Hb release was observed during storage. Hence, 0.2 and 0.3% glu concentration and 10 min crosslinking time were studied further. Of these, 0.3% glu was found better as it appreciably reduced Hb and 5-FU release from the RBCs during storage (Table 2)

The per cent drug entrapment was found 43.83±4.53 and particle size was found 6.9±1.04 μm in case of normal RBCs and 6.7±0.92 μm in case of loaded RBCs. The cell recovery was found 94.00%±11.58. The reason for

TABLE 2: EFFECT OF CROSSLINKING ON RBCS

| Gluteraldehyde Concentration (%) | Hb release (% ±S.D.)* | 5-FU release (% ±S.D.)* |
|---|---|---|
| 0.0 | 3.676±0.54 | 7.09±0.680 |
| 0.2 | 2.619±0.095 | 4.16±0.840 |
| 0.3 | 2.023±0.850 | 3.17±0.469 |

The effect of Crosslinking of RBCs with gluteraldehyde on the release of Hb and 5-FU from RBC was determined at different gluteraldehyde concentration. *Each value is a mean of three readings.

loss may be due to old fragile cells and damage to some RBCs during processing. However, cell integrity was not changed and shape of cells was found nearly spherical.

The data in Table 3 describes the effect of different concentrations of NaCl on the Hb release from 5-FU loaded RBCs. It was observed that 0.9% NaCl, which was found isotonic for normal RBCs, brought about significant lysis of the loaded RBCs but protection was observed in 1.8% NaCl. This drastic change in the tonicity of the loaded RBCs may be due to the presence of the drug within the cell. When Hb release was determined on

TABLE 3: OSMOTIC SHOCK STUDIES

| NaCl solution (%) | Hb release from loaded RBCs (%±S.D.)* |
|---|---|
| 1.8 | 2.05±0.067 |
| 1.7 | 2.20±0.070 |
| 1.6 | 6.62±1.050 |
| 1.5 | 8.82±1.005 |
| 1.4 | 15.44±2.225 |
| 1.3 | 22.79±2.020 |
| 1.2 | 31.60±3.120 |
| 1.1 | 37.50±2.950 |
| 1.0 | 44.26±4.567 |
| 0.9 | 48.97±5.754 |
| 0.8 | 49.26±3.120 |

The effect of different concentration of NaCl on the release of Hb from loaded RBCs was studied. * Each value is a mean of 3 determinations.

TABLE 4: EFFECT OF CROSSLINKING WITH GLUTERALDEHYDE-5-FU RETAINED AND HB RELEASE DURING STORAGE

| Time in Days | 5-FU retained in (%±S.D.)* | | Hb release from (% ±S.D.)* | |
|---|---|---|---|---|
| | Untreated RBCs | Glu-treated RBCs | Untreated RBCs | Glu-treated RBCs |
| 1 | 93.00±8.2 | 97.23±6.23 | 3.67±0.033 | 2.20±0.067 |
| 2 | 80.09±4.5 | 95.54±8.5 | 7.79±0.667 | 2.64±0.024 |
| 3 | 68.47±2.5 | 93.00±5.6 | 4.47±0.597 | 3.08±0.87 |
| 4 | 58.34±4.3 | 90.68±12.2 | 14.68±2.21 | 3.67±0.056 |
| 5 | 41.50±6.65 | 87.33±9.3 | 29.64±3.35 | 4.85±1.09 |
| 6 | 39.36±3.85 | 86.22±5.6 | 38.97±1.078 | 7.79±0.958 |

*Mean of 3 readings

addition of different volumes of distilled water, we found that 2 ml distilled water brought about lysis of loaded RBCs due to osmotic shock.

It was observed that hemoglobin is leached out from RBCs due to passage through 22 gauge needle. The size of cells was also found reduced from 6.70 µm to 5.54 µm after the fourth pass. Microscopic examination of the RBCs also revealed that upto second pass, the shape of RBCs remained intact, but thereafter it became more and more nonspherical. All these observations indicate that even syringeability damages loaded RBCs.

The data of 5-FU and Hb release during 6 days of storage for both glu-treated and untreated RBCs is shown in Table 4. It is clear that the untreated RBCs are unstable as appreciable amounts of 5-FU (60.64%) and Hb (38.97%) are released from them during storage. Crosslinking with glu drastically reduced this leaching (23.78 and 7.79% for 5-FU and Hb respectively), indicating its stabilizing effect on the RBCs.

The *in vivo* organ distribution studies (Table 5) show that the organ disposition pattern of 5-FU is significantly different in case of RBC-loaded and free drug formulations. Thus, peak concentration was observed at 2 h in case of loaded RBCs and 1 h in case of 5-FU solution. The total 5-FU accumulated in all three organs of the RES (liver, lungs, spleen) from loaded RBCs was double than that from plain 5-FU solution, with maximum values at peak levels in each case being 0.37±0.09 and 0.175±0.049 mg respectively. This indicates a preferential 5-FU accumulation in the organs of the RES due to entrapment in resealed RBCs.

When compared at peak levels using Student's t test, significant ($P<0.05$) drug accumulation was observed in lungs and spleen after administration of RBCs suspen-

TABLE 5: DISTRIBUTION OF 5-FU *IN VIVO*

| Time in hour | 5-FU from loaded RBCs (mg±S.D.)* | | | | Time in hour | 5-FU from free 5-FU solution (mg±S.D.)* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | spleen | liver | lungs | total | | spleen | liver | lungs | total |
| 1 | 0.019± 0.006 | 0.020± 0.007 | 0.015± 0.0023 | 0.054± 0.005 | 1/2 | 0.019± 0.006 | 0.054± 0.0070 | 0.011± 0.0019 | 0.084± 0.007 |
| 2 | 0.152± 0.047 | 0.127± 0.038 | 0.096± 0.026 | 0.37± 0.09 | 1 | 0.034± 0.0052 | 0.100± 0.0046 | 0.040± 0.008 | 0.175± 0.049 |
| 4 | 0.0355± 0.002 | 0.048± 0.0052 | 0.013± 0.0021 | 0.09± 0.008 | 2 | 0.022± 0.0054 | 0.053± 0.0085 | 0.013± 0.0057 | 0.088± 0.008 |

Comparative data of 5-FU distribution to Spleen, Liver, Lungs from free drug solution and drug-loaded RBCs.
* Each value is a mean of 4 readings.

sion as compared to plain 5-FU solution. These findings suggest the potential of resealed erythrocytes as site specific carriers for improving therapy of 5-FU especially in tumors of the lungs which would concomitantly reduce side effects.

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