

Ribavirin Loaded Erythrocytes by Endocytosis as Targeted Drug Carrier System

Abstract Loaded erythrocytes, as drug carrier system, has tremendous potential to carry outside specificity and sustained release of drug. Thereby, enhancing therapeutic index and minimize the dose and adverse effects as well as improvement patient compliance. In the present paper, erythrocytes loaded Ribavirin with the aim to benefit the reticuloendothelial system (RES) targeting potential of the carrier cells. Endocytosis technique was used for Ribavirin loading in erythrocytes and the entire loading procedure was evaluated and validated. The *in-vitro release* of carrier erythrocytes was characterized, as well as the hematological indices, osmotic fragility and SEM analysis. The maximum loaded amount and entrapment efficiency were found to be 9.58 ± 0.045 mg and 38.3% at 25mg/ml of Ribavirin concentration after 60 minutes incubation time at 37°C with 88.42% cell recovery. The *In vitro* Ribavirin release was found to obey Higuchi diffusion kinetics. Hematological parameters of Ribavirin loaded erythrocytes were significantly differ from native erythrocytes at ($p \leq 0.01$). It was found that the mean corpuscular volume value was increased, while the mean corpuscular hemoglobin and the mean corpuscular hemoglobin content values were decreased. Also, the osmotic fragility behaviors of Ribavirin loaded erythrocytes were significantly decreased at ($p \leq 0.001$). The scanning electron microscope of Ribavirin loaded erythrocytes show significant changes in the cell surface and morphology with rough cell surface and small lesions. The highly changed erythrocyte shape and morphology being one of the main determinants in erythrocytes disappearance kinetics in circulation, can be potentially beneficial in terms of successful cell targeting to RES which in turn leads to the improved Ribavirin effects on RES-mediated immune responses.

Keywords.

Endocytosis, erythrocytes, osmotic fragility and scanning electron microscope

INTRODUCTION

Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV) that primarily affects the liver. The virus persists in the liver in about 75% to 85% of those initially infected. It often leads to liver disease and occasionally cirrhosis. In some cases, those with cirrhosis will develop complications such as liver failure, liver cancer, or esophageal and gastric varices (R). Ribavirin as anti viral drug has therapeutic efficacy against HCV, in a combination with other drugs, but Ribavirin has many adverse effects; some of which are severe as hemolytic anemia which sometimes necessitate patients to discontinue therapy, giving supplement therapy as erythropoietin, or at last blood transfusion is needed. To overcome or minimize the risk of Ribavirin adverse effects, Ribavirin should be targeted to reticuloendothelial system (RES) mainly to the liver.

Erythrocyte has been exploited as potential carrier for bioactive substances, especially biopharmaceuticals, in recent decades¹⁻³. Erythrocytes are preferred as a drug carrier system because they have many advantages as availability in abundant cells in the body, biocompatibility, biodegradability, loading with a variety of drugs⁴ and considerable circulation life-span of the autologous carrier erythrocytes which allow them to serve as prolonged release intravenous reservoirs for various drugs^{1,5,6}. On the other hand, abnormal or aged erythrocytes are destructed or phagocytosed by the RES and they can be used as drug targeting carriers to RES organs mainly liver, spleen and kidney⁷⁻¹⁰.

The drugs or bioactive substances can be successfully loaded or entrapped into erythrocytes by different methods as osmosis based method (e.g. hypotonic dilution, preswelling and hypotonic dialysis methods), chemical method (e.g., chemical perturbation of the erythrocyte membrane), an electrical pulse method and endocytosis method¹¹.

In this paper, the human erythrocytes have been loaded by Ribavirin using endocytosis method and the *in vitro* characteristics of the prepared cellular carriers have been evaluated. The obtained results from this study such as remarkable shape and morphological changes erythrocytes make it possible to target the Ribavirin a high dose in a short time period, thereby potentially augmenting the biological response using a relatively low drug dose, consequently diminishing of deleterious adverse effects of Ribavirin as hemolytic anemia. This benefit is intended as the scope of work of this study.

Methodology

Materials

Ribavirin was obtained as gift from MEMPHIS, El-Amirya - Cairo –Egypt; Sodium chloride, potassium dihydrogen phosphate, Disodium hydrogen phosphate, calcium chloride and d- glucose were supplied from El-Nasr Pharm. Chem. Company, (Cairo, Egypt); Potassium chloride is provided from Flukachemie AGCH, Switzerland; Membrane PTFE (0.2 μm) Disposable syringe filter from Macherey-Nagel GmbH, Germany; Magnesium Chloride hexahydrate and methanol were purchased from El-Gomeria pharm company (Cairo- Egypt); Adenosine 5-Triphosphate was obtained as a gift from SIGMA - pharmaceutical industries -Quesna city - Egypt.

Collection of specimen

The blood specimens were collected from apparently healthy donors not suffered from acute and chronic diseases. Blood samples were collected in heparinized tubes.

Preparation of erythrocyte suspension

The collected specimens were centrifuged for 5 min at 5000 rpm (Biofuge Primo centrifuge maximum 17.000 rpm (England)). The plasma and the buffy coat were removed by aspiration. Erythrocytes were washed three times in cold (PBS) with centrifugation for 5 min at 5000rpm, then the hematocrit was adjusted to 45% using PBS (Selvamani et al.; 2015; Pierige et al.; 2008).

Ribavirin loaded erythrocytes by endocytosis technique

In 2 ml eppendorff tubes, 400 μl of washed erythrocytes were added to 400 μl of previous prepared loading buffer containing different concentrations of Ribavirin, gentle mixing to avoid blood hemolysis and the obtained suspension was incubated for different times at 37 °C (Oven, Heraeus, UT 5060 E (Germany)). The final obtained suspension was centrifuged for 5 min at 5000 rpm and the supernatant was discarded. The obtained pellet was washed for 2 times in cold BPS with centrifugation for 5 min at 5000 rpm.

Effect of Ribavirin concentrations on the loading efficiency

To determine the effect of Ribavirin concentration on loading efficiency, different drug concentrations (10 mg, 15 mg, 20 mg, and 25 mg) were used for all selected incubation times. The results were compared to obtain the more suitable concentration for loading process which produce most excellent loading parameters (Millán et al.; 2004; Madhavi et al., 2013)

Effect of incubation times on the loading efficiency

To determine the effect of time on loading efficiency, the loading process was done for the selected concentrations for different times (15, 30 and 60 minutes) and the results were compared (Millán et al.; 2008; Millán et al.; 2004; Madhavi et al.; 2013).

Ribavirin extraction process from loaded erythrocytes for assay

To determine the amount of loaded Ribavirin, the erythrocyte pellets were treated with 400 μ l of distilled water and strong shaking to ensure erythrocyte hemolysis, then deposition of protein via addition of 1ml methanol, mixing by shaking and using vortex (Vortex mixer type Janke & Kunkel (Germany)). The resultant hemolysate was centrifuged at 13000 rpm for 15 minutes, and then the supernatant was taken and filtered using 0.2 Millipore disposable syringe filter, and complete the volume to 10 ml by water. 1 μ l of filtrate was injected to the chromatograph (HPLC) Agilent 1100 liquid chromatography system (Agilent, Palo Alto, CA, USA).

Characterization of the prepared Ribavirin loaded erythrocytes by previous two methods

Loading parameters

To evaluate the final erythrocyte carriers, three indices were defined as loading parameters (loaded amount, entrapment efficiency and cell recovery) (Hamidi et al.; 2007):

1. Loaded amount: The total amount of Ribavirin entrapped in 0.4 ml of the final packed erythrocytes.
2. Entrapment efficiency: The percentage ratio of the loaded amount of Ribavirin to the amount added during the entire loading process.
3. Cell recovery: The percentage ratio of the hematocrit value of the final loaded cells to that of the initial packed cells, both measured using equal suspension volumes.

Hematological Indices

Normal erythrocytes, erythrocytes suspended in PBS and Ribavirin-loaded erythrocytes were counted. The mean corpuscular volume (MCV: the estimated average cell volume), the mean corpuscular hemoglobin (MCH: the estimated average hemoglobin content per each cell), and the mean corpuscular hemoglobin content (MCHC: the estimated hemoglobin content per 100 ml of cell volume) were measured using Hematology analyzer (Hematology analyzer Swelab auto counter 920 – Eo⁺ - 126 13 Stockholm Sweden).

Osmotic fragility behavior of Ribavirin loaded erythrocytes

To evaluate the resistance of erythrocytes membranes against the osmotic pressure changes of their surrounding media, 25 μ l Ribavirin loaded erythrocytes sample was added to each of a series of 2.5 ml NaCl solutions containing 0.0 to 0.9 g% of NaCl. After gentle mixing and standing for 15 min. at room temperature, the erythrocyte suspensions were centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm¹⁶ (Ultraviolet spectrophotometer, Shimadzu 1800 (Japan)). The released hemoglobin was expressed as percentage absorbance of each sample in reference to a completely lysed sample prepared by diluting of packed cells with 1.5 ml of distilled water. An osmotic fragility index was defined for native and Ribavirin loaded erythrocytes.

Scanning electron microscopy (SEM)

The scanning electron micrographs were illustrated for Ribavirin loaded erythrocytes by endocytosis method at x4300 and x7000 magnifications. (JOEL scanning electron microscope (JTEM) model 1010 Tokyo, (Japan))

In-Vitro Release of Ribavirin from the loaded erythrocytes

To study the release of Ribavirin from carrier erythrocytes loaded by the mentioned method of loading above, 1 ml of packed Ribavirin loaded erythrocytes was diluted to 9 ml using PBS the suspension and then mixed thoroughly by several gentle inversions. The mixture was divided into 20 portions (0.5 ml each) in 1.5 ml eppendorff tube. The samples were rotated vertically at 20 rpm while incubated at 37^oC (Oscillating thermostatically controlled shaker, Gallent Kamp (England)). At (0.5, 2, 4, 8, 12, 16 and 20) hr intervals, one of the aliquots was removed and centrifuged at 5000 rpm for 5 min. One hundred μ l of the supernatants was separated for drug assay. The drug analysis was done by chromatograph.

Effect of cross linker on *in-vitro* release of Ribavirin loaded erythrocytes

The release of Ribavirin from erythrocytes loaded by endocytosis after treatment with glutaraldehyde as across linker was studied.

One ml of packed drug loaded erythrocytes was diluted with 8.5 ml of PBS and 0.5 ml of 0.5% glutaraldehyde solution. The suspension was mixed thoroughly by several gentle inversions and incubated for 10 minutes. The mixture was centrifuged and the pellet resuspended again in 9 ml PBS and then followed as described above.

RESULTS and DISCUSSIONS

Ribavirin uptake by endocytosis

Table (1) shows the effect of Ribavirin concentrations and the including incubation times on the amount of Ribavirin loaded on human carrier erythrocytes by endocytosis at 37°C. Also table (1) illustrate the effect of Ribavirin concentrations and the including incubation times on the Entrapment Efficiency % of Ribavirin loaded on human carrier erythrocytes by endocytosis at 37°C.

Effect of Ribavirin concentration on the loading amount

Incubation time VS Ribavirin Conc.

The amount of Ribavirin loaded using Ribavirin concentration 10 mg/ml for incubation times 15, 30 and 60 minutes. The amount of Ribavirin loaded is found to reach $2.72 \pm 0.01\text{mg}$, $2.79 \pm 0.024\text{ mg}$ and $3.10 \pm 0.037\text{ mg}$, respectively after 15, 30 and 60 minutes of incubation. It was found that the amount of Ribavirin loaded increases in direct proportionality according to the time factor till reaching the maximum loading incubation time after 60 minutes ($r = 0.987$).

Upon increasing Ribavirin concentration to 15 mg/ml for the same times mentioned above, the amount of Ribavirin loaded is found to be $3.61 \pm 0.02\text{mg}$, $3.68 \pm 0.01\text{mg}$ and $4.68 \pm 0.033\text{ mg}$, respectively after the same time mentioned above. Again, Ribavirin loaded in erythrocytes increases in direct proportionality by increasing the time factor ($r = 0.962$).

By increasing Ribavirin concentration to 20 mg/ml for the same times, the amount of drug loaded is also significantly increased according to the time factor increase till reaching the maximum loading time after 60 minutes. The amount of Ribavirin loaded observed was as follows: $3.65 \pm 0.012\text{mg}$, $5.1 \pm 0.023\text{ mg}$ and $5.66 \pm 0.04\text{mg}$, respectively. Ribavirin loaded in erythrocytes increases in direct proportionality by increasing the time factor ($r = 0.905$).

By increasing Ribavirin concentration to become 25 mg/ml, 30 mg/ml or 35 mg/ml, the same phenomena as discussed for (10 mg, 15 mg and 20 mg/ml) was observed and also significantly increased according to the time. The maximum loaded amount for all the above mentioned concentrations at maximum loading time after 60 minutes was found at 25 mg/ml. The amount of Ribavirin loaded was as follows: $4.18 \pm 0.012\text{mg}$, $6.85 \pm 0.019\text{mg}$ and $9.58 \pm 0.045\text{mg}$, respectively; $4.54 \pm 0.01\text{mg}$, $7.22 \pm 0.023\text{ mg}$ and $8.99 \pm 0.034\text{ mg}$, respectively for 30 mg/ml of Ribavirin; $4.4 \pm 0.012\text{ mg}$, $7.4 \pm 0.026\text{mg}$ and $9.47 \pm 0.049\text{ mg}$ for 35 mg/ml of Ribavirin, respectively.

The amount of Ribavirin loaded in erythrocytes, in the three mentioned concentrations above for Ribavirin, was found to increase in direct proportionality. The regression coefficients obtained were $r = 0.983$, $r = 0.953$ and $r = 0.957$, respectively.

Ribavirin Conc. VS each incubation time

The amount of Ribavirin loaded using all the applied Ribavirin concentrations (from 10 mg/ml to 35 mg/ml) for each applied incubation time (15 min., 30 min. and 60 min) i.e., at constant time in each experiment.

The amount of Ribavirin loaded at 15 min. was found to be 2.27 ± 0.01 mg, 3.61 ± 0.02 mg, 3.65 ± 0.012 mg, 4.18 ± 0.012 mg, 4.54 ± 0.01 mg and 4.4 ± 0.012 mg, respectively for the six Ribavirin concentrations. The above relationship shows a direct proportionality with $r = 0.931$.

The amount of Ribavirin loaded at 30 min. was found to be 2.79 ± 0.024 mg, 3.68 ± 0.01 mg, 5.1 ± 0.023 mg, 6.85 ± 0.019 mg, 7.22 ± 0.023 mg and 7.4 ± 0.026 mg, respectively for the six Ribavirin concentrations. The above relationship shows a direct proportionality with $r = 0.965$.

The amount of Ribavirin loaded at 60 min. was found to be 3.1 ± 0.037 mg, 4.68 ± 0.033 mg, 5.66 ± 0.04 mg, 9.58 ± 0.045 mg, 8.99 ± 0.034 mg and 9.47 ± 0.049 mg, respectively for the six Ribavirin concentrations. The above relationship shows a direct proportionality with $r = 0.931$.

Effect of Ribavirin concentrations on the entrapment efficiency%

Incubation time VS Ribavirin Conc.

The entrapment efficiency% of Ribavirin loaded using Ribavirin concentration 10 mg/ml for times 15, 30 and 60 minutes. The entrapment efficiency % of Ribavirin loaded was found to reach 27.2%, 27.95% and 31%, respectively after 15, 30 and 60 minutes of incubation. It was found that the entrapment efficiency% of Ribavirin loaded increases in direct proportionality according to the time factor till reaching the maximum loading incubation time after 60 minutes ($r = 0.989$).

Upon increasing Ribavirin concentration to 15 mg/ml for the same times mentioned above, the entrapment efficiency % of Ribavirin loaded was found to be 24%, 25.7% and 31.1%, respectively after the same time mentioned above. Again, the entrapment efficiency% of Ribavirin loaded into erythrocytes increases in direct proportionality by increasing the time factor ($r = 0.994$).

By increasing Ribavirin concentration to 20 mg/ml for the same times, the entrapment efficiency % of Ribavirin loaded was also significantly increased according to the time factor increase till reaching the maximum loading time after 60 minutes. The entrapment efficiency % of Ribavirin loaded observed was as follows: 18.3 %, 25.5 % and 28.3 %, respectively. The entrapment efficiency % of Ribavirin loaded in erythrocytes increases in direct proportionality by increasing the time factor ($r = 0.905$).

By increasing Ribavirin concentration to become 25 mg/ml, 30 mg/ml or 35 mg/ml, the same phenomena as discussed for (10 mg, 15 mg and 20 mg/ml) was observed and also significantly increased according to the time. The maximum entrapment efficiency % of Ribavirin loaded for all the above mentioned concentrations at maximum loading time after 60 minutes was found at 25 mg/ml. The amount of Ribavirin loaded was as follows: 16.7 %, 27.4 % and 38.3 %, respectively; 15.2 %, 24 % and 29.9 %, respectively for 30 mg/ml of Ribavirin; 12.6 %, 21.1 % and 27 % for 35 mg/ml of Ribavirin, respectively.

The entrapment efficiency % of Ribavirin loaded into erythrocytes, in the three mentioned concentrations above for Ribavirin, was found to increase in direct proportionality. The regression coefficients obtained were $r = 0.983$, $r = 0.954$ and $r = 0.957$, respectively.

Ribavirin Conc. VS each incubation time

The entrapment efficiency % of Ribavirin loaded using all the applied Ribavirin concentrations (from 10 mg/ml to 35 mg/ml) for each applied incubation time (15 min., 30 min. and 60 min) i.e., at constant time in each experiment. The entrapment efficiency % of Ribavirin loaded at 15 min. was found to be 27.2 %, 24 %, 18.3 %, 16.7 %, 15.2 % and 12.6 %, respectively for the six Ribavirin concentrations. The above relationship shows an inverse proportionality with $r = -0.974$.

The entrapment efficiency % of Ribavirin loaded at 30 min. was found to be 27.95 %, 25.7 %, 25.5 %, 27.4 %, 24 % and 21.1 %, respectively for the six Ribavirin concentrations. The above relationship shows an inverse proportionality with $r = -0.804$. The above results indicate that the entrapment efficiency % of Ribavirin was decreased from 10 mg/ml until 20 mg/ml of Ribavirin and then increased in Ribavirin concentration of 25 mg/ml followed by a decrease again in the remaining Ribavirin concentrations.

The entrapment efficiency % of Ribavirin loaded at 60 min. was found to be 31.0 %, 31.2 %, 28.3 %, 38.3 %, 29.9 % and 27.0 %, respectively for the six Ribavirin concentrations. The above relationship shows an inverse proportionality with $r = -0.188$. These results confirmed the same phenomenon as described above.

The experimental work investigates the effect of time, as well as the drug concentration on the process of Ribavirin loading into human erythrocytes by endocytosis as trial to obtain Ribavirin targeted delivery system. The results indicate that the highest level of Ribavirin loaded on erythrocytes was achieved using 25 mg/ml of Ribavirin at 37°C and 60 minutes incubation time.

The aforementioned results proved compatible with the previous study which demonstrates the increase in the cell membrane activity upon temperature increasing up to optimum temperature 37°C (Solomon et al.; 2010). Likewise, this finding is supported by another study which shows that endocytosis process is decreased by decreasing temperature¹⁸. The presence of some factors as tonicity factor or an energy source stimulates the endocytosis¹⁹.

The presence of calcium ions and ATP in the formulation process stimulates the endocytosis of Ribavirin uptake by erythrocytes. This is supported by the observation viewed by Schrier et al., (1977) which stated that the calcium ions and energy source stimulate drug uptake by erythrocytes through membrane invagination and formation of endocytotic vacuoles. The drugs induced endocytosis is dependent on the persistence of erythrocyte energy sources²⁰.

This finding coincides with results reported by Millán et al., (2008); Hamidi et al., (2007a) and Hamidi et al., (2007b).

From the above results was found that the highest entrapment efficiency % of Ribavirin loaded was 38.3 % that was given at 25 mg/ml of Ribavirin after 60 minutes incubation time. After that the entrapment efficiency % of Ribavirin loaded was decreased upon increasing Ribavirin concentration. This entrapment efficiency % of Ribavirin loading is better than that obtained in interferon-alpha 2b loading study as comparison (Hamidi et al.; 2007).

Hematological indices of loaded erythrocytes uptake by endocytosis

Table (2) represents the mean hematological parameters of the Ribavirin loaded erythrocytes obtained with 25 mg/ml Ribavirin concentration and values for the same cells before the loading procedures (the control cells) and after loading process but without using drug (sham encapsulated). It was found that the change in hematological parameters is significant at ($p \leq 0.01$).

The hematological parameters, such as MCV, MCH and MCHC were characterized. These parameters determine the influence of the encapsulation process on the hematological properties of the erythrocytes (Millán et al.; 2008). The results show changes in hematological parameters were observed at higher concentrations (25 mg/ml). From these data, Ribavirin loading into erythrocytes occurs either by encapsulation or binding to the cell membrane¹. Data shows significant changes in MCV in Ribavirin loaded erythrocytes was (85.1 ± 1.92) but sham encapsulated (79 ± 1.78) was similar to

control (78.6 ± 1.54). This indicates that the change in loaded erythrocytes is related to the effect of the drug and the loading procedure has no effect.

This finding was in agreement with previous report (Briones et al.; 2010). There were also slight changes in both MCH and MCHC that appear only in Ribavirin loaded erythrocytes (25.0 ± 0.84 and 29.4 ± 0.92) respectively.

These changes can be explained by Ribavirin have minimal oxidative injury of erythrocytes membrane that cause a physical and/or functional barrier of erythrocyte, therefore hemoglobin loss is easier from carrier erythrocytes.

This finding was in agreement with Preparation of carrier erythrocytes for RES-targeted delivery of interferon-alpha 2b (Hamidi et al.; 2007). These predictions were supported by the SEM analysis data and osmotic fragility that will be discussed later.

Cell recovery of erythrocytes uptake by Endocytosis method

The percentage ratio of the hematocrite value of the final loaded cells to that of the initial packed cells was 88.42%. It was determined by hematology analyzer as shown in table (2). This result was practically better than the recovery results of other studies such as Paclitaxel and Enalapril (Harisa et al.; 2014; Hamidi et al.; 2001).

Osmotic fragility behavior of Ribavirin loaded erythrocytes by endocytosis

Osmotic fragility determines the susceptibility of erythrocytes to osmotic lysis. The obtained results revealed that there was significant difference in the osmotic fragility of loaded erythrocyte at 25 mg/ml Ribavirin when compared to that of unloaded erythrocytes as graphically represented in figure (1).

The osmotic fragility of the studied erythrocytes test is a marker of possible changes in the integrity of the cell membrane caused by the loading procedure. Moreover, the osmotic fragility test measures the resistance of these cells to changes in the osmotic pressure of the surrounding media. The osmotic fragility test was carried out by using unloaded, sham and Ribavirin loaded erythrocytes. The entrapment of Ribavirin in cells significantly decreases the osmotic fragility of the cells at ($p \leq 0.001$), and also the osmotic fragility of sham erythrocytes decreased in comparison with unloaded erythrocytes (control). This may be because of the increased mean corpuscular volume of loaded erythrocytes.

The obtained results in this part indicate that the erythrocytes resist their hemolysis process in NaCl percent from 0.90 to 0.50 % and then the hemolysis of the employed erythrocytes was increased in fluctuation manner till reach 100% when the NaCl percent became 0.00 %. These findings were observed for all the tested specimens, i.e., control, sham and Ribavirin loaded erythrocytes.

So, at 0.50 % NaCl concentration, the osmotic fragility reaches 19.98 ± 1.3 %, 19.23 ± 1.7 % and 18.50 ± 1.6 % absorbance for control sample, sham and Ribavirin loaded erythrocytes, respectively. By decreasing NaCl concentration from 0.45 to 0.00, the osmotic fragility ranges from 48.85 ± 1.8 to 100.00 ± 0.9 for control, ranges from 63.44 ± 2.1 to 100.00 ± 1.0 for sham and ranges from 56.07 ± 1.4 to 100.00 ± 1.0 for Ribavirin loaded erythrocytes.

The osmotic fragility of Ribavirin loaded erythrocytes is decreased by increasing NaCl concentration. The obtained correlation coefficients record an inverse proportionality between NaCl concentrations (0.0 - 0.9%) and the obtained osmotic fragility of Ribavirin loaded erythrocytes, prepared by endocytosis method. The values are - 0.908, - 0.916 and - 0.924 for control (unloaded), sham and Ribavirin loaded erythrocytes, respectively.

This may be because of the increased mean corpuscular volume of loaded erythrocytes i.e., the values present in table (2) showed that the mean corpuscular volume were 78.6 ± 1.54 , 79.0 ± 1.78 and 85.1 ± 1.92 for control, sham and Ribavirin loaded

erythrocytes, respectively. The osmotic fragility index for normal erythrocytes was found to be 0.486 % w/v, for sham was found to be 0.409 % w/v and for loaded erythrocytes was found to be 0.394% w/v. There was a slight decrease in the osmotic fragility value and was found to be very negligible to be considered.

The observed data were in a good agreement with the work done by (Madhavi et al.; 2013) who stated that osmotic fragility of Piperine loaded erythrocytes is decreased compared with that of unloaded cells and sham erythrocytes.

The observed results indicate that the osmotic fragility of Ribavirin-loaded erythrocytes are affected by two factors, loading process and Ribavirin as drug cause change in erythrocytes membrane. This result was approved and clearly evident by scanning electron microscope which be discussed latter in this paper. The accumulation of Ribavirin triphosphate in red blood cells (RBCs). Ribavirin triphosphate accumulation leads to a relative adenosine triphosphate (ATP) deficiency in the RBCs. The associated depletion of its ATP may damage the antioxidant defense system and induce some RBC membrane changes such as oxidative injury, promoting intraerythrocyte oxidative stress with subsequent membrane injury, and that these injured erythrocytes then undergo physiological extravascular destruction by the reticuloendothelial system.

This interpretation was supported by other previous studies stated that Ribavirin induce changes in erythrocytes membrane preserve consequently changes in fragility and morphology (Tanaka et al.; 2005; Russmann et al.; 2006).

The loading process and/or the drug have deleterious effects on erythrocyte shape. The change of carrier cells morphology from the native cells gives the opportunity for carrier erythrocytes to phagocytosed into reticuloendothelial system³. This result suggested that loaded erythrocytes can be used for targeting of Ribavirin. This assumption is supported by previous findings which recommended that the use of carrier erythrocytes as targeting system for Piperine due to the distinct effect changes in drug loaded erythrocytes morphology which make drug loaded erythrocytes easily removed by macrophages (Madhavi et al.; 2013).

In-vitro release of Ribavirin loaded erythrocytes obtained by endocytosis method

In vitro Release in PBS pH 7.4

Figure (2) represents the *in-vitro* release of Ribavirin from loaded erythrocytes in PBS pH 7.4 over a 20 hrs. An initial burst release was obtained over the first 2 hrs (52.2% from loaded amount of Ribavirin). From the obtained results, there was a rapid release of 52% of Ribavirin within the first 2 hrs indicating that, the drug may be bound on to the surface of the membrane which was released quickly. Then the amount of Ribavirin released increased gradually to reach 88% of the loaded Ribavirin within 20hrs as observed in figure (2).

In-vitro release in PBS pH 7.4 after treatment with glutaraldehyde

Figure (2) shows the *in-vitro* release of Ribavirin behavior from loaded erythrocytes by endocytosis in PBS pH 7.4 after using glutaraldehyde as a membrane stabilizer. Figure (2) shows the results of studying the effect of cross-linking agent on the releasing of Ribavirin loaded by endocytosis method. It was clear that the glutaraldehyde reduces the releasing of Ribavirin from Ribavirin loaded erythrocytes, the release after 20 hrs reached 21.77 %.

Release Kinetics of Ribavirin Loaded Erythrocytes by endocytosis method

From the obtained releasing data, the coefficient of correlation (r) for zero order, first order and Higuchi diffusion model were found to be 0.8428, 0.8428 and **0.9393** respectively, for the release of Ribavirin Loaded Erythrocytes by endocytosis method and to be 0.9249, 0.3840 and **0.9744** for the release of Ribavirin Loaded

Erythrocytes by endocytosis method after adding glutaraldehyde as membrane stabilizer indicating Ribavirin release from loaded erythrocytes undergo diffusion order.

Therefore, the efflux of Ribavirin from carrier cells followed diffusion kinetics during the entire experimental period.

Scanning electron microscopy of Ribavirin loaded erythrocytes by endocytosis method

Figures (3 and 4) show the scanning electron micrographs of unloaded erythrocytes and figures (5 and 6) show Ribavirin loaded erythrocytes by endocytosis method, both at x4300 and x7000 magnification.

From the SEM pictures of unloaded figures (3 and 4) and Ribavirin loaded erythrocytes (5 and 6), it's clearly observed that, unloaded normal erythrocytes, the cell surface is smooth and Ribavirin loaded erythrocytes, the cell surface is rough with small lesions. The diameter of the unloaded erythrocytes was around 4 μ m and after loading there was an increase in the diameter of the erythrocytes around 5 μ m. The loaded erythrocytes are more enlarged and irregular in shape than biconcave shape with slight damage of the surface. On conclusion there are significant changes in the cell surface and morphology of Ribavirin loaded erythrocytes this may be due to minimal oxidative effect of Ribavirin drug. Fortunately, these membrane integrity changes make Ribavirin loaded erythrocytes much more prone for the phagocytosis by macrophages.

The highly changed erythrocyte shape and morphology evidenced in this study, being one of the main determinants in erythrocytes disappearance kinetics in circulation, can be potentially beneficial in terms of successful cell targeting to RES, which, in turn, leads to the improved Ribavirin effects on RES-mediated immune responses and avoidance of deleterious side effects of Ribavirin as hemolytic anemia.

The changes in SEM pictures current work are in a good agreement with the work done by (Madhavi et al.; 2013).

Conclusions

The human erythrocytes were loaded successfully with Ribavirin with the practically acceptable loading parameters. The Ribavirin loaded erythrocytes were investigated with respect to their in vitro drug delivery characteristics, including drug release, loading parameters, hematological indices, shape and morphological properties and osmotic fragility. The results of these experiments were indicative of some irreversible changes in cell morphology as well as physiology, which, in turn, may favor the cell targeting to RES organs.

The following conclusion can be drawn from the study:

The loaded amount of Ribavirin by endocytosis technique ranged from 2.72 ± 0.01 to 9.58 ± 0.045 mg/ml with entrapment efficiency ranged from (12.6% to 38.3%). The maximum loaded amount and entrapment efficiency % were found to be 9.58 ± 0.045 and 38.3% at 25mg/ml of Ribavirin concentration after 60 minutes incubation time at 37°C with (88.42%) cell recovery.

Hematological parameters of Ribavirin loaded erythrocytes significantly differ from native erythrocytes at ($p \leq 0.01$). Where mean corpuscular volume value was increased, mean corpuscular hemoglobin and mean corpuscular hemoglobin content values of the cells were decreased.

Osmotic fragility behaviors of Ribavirin loaded erythrocytes obtained by endocytosis were significantly decreased in comparison with native erythrocytes at ($p \leq 0.001$).

Ribavirin *in-vitro* released from carrier cells prepared by endocytosis in PBS pH 7.4 was a relatively rapid process where the initial burst release obtained over the first 2 hrs. (52.2%) from loaded amount of Ribavirin then the efflux of Ribavirin increased gradually to reach (87.84%) after 20 hrs.

In-vitro release in PBS pH 7.4 after treatment with glutaraldehyde cross-linking agents show sustained release of Ribavirin from Ribavirin loaded erythrocytes, after 20 hrs. The release was reached to (21.77 %). The efflux of Ribavirin from Ribavirin loaded erythrocytes followed diffusion kinetics.

The scanning electron micrographs of Ribavirin loaded erythrocytes show significant changes in the cell surface and morphology with rough cell surface and small lesions. This may be due to minimal oxidative effect of Ribavirin drug. Fortunately, these remarkable membrane integrity changes make Ribavirin loaded erythrocytes much more prone for phagocytosis by macrophages. The considerable changed erythrocyte shape and morphology evidenced in this study, being one of the main determinants in erythrocytes disappearance kinetics in circulation, can be potentially beneficial in terms of successful cell targeting to RES, which in turn leads to the improved Ribavirin effects on RES-mediated immune responses and consequently diminishing of deleterious adverse effects of Ribavirin as hemolytic anemia.

REFERENCES

1. Selvamani, P., Latha, S., Monisha, S. & Supassri, T. A review on resealed erythrocyte as a novel drug delivery system. *Asian Journal of Pharmaceutical and Clinical Research*; 2015: **8**:101–107.
2. Zarrin, A., Foroozesh, M. & Hamidi, M. Carrier erythrocytes: recent advances, present status, current trends and future horizons. *Expert Opin. Drug Deliv*; 2014: **11**: 433–447.
3. Hamidi, M. & Tajerzadeh, H. Carrier erythrocytes: an overview. *Drug Deliv*; 2003: **10**: 9–20.
4. Patel, P. D., Dand, N., Hirlekar, R. S. & Kadam, V. J. Drug loaded erythrocytes: as novel drug delivery system. *Curr. Pharm. Des*; 2008: **14**:63–70.
5. Gupta, A. *et al.* Cell based drug delivery system through resealed erythrocyte-A review. *Int. J. Pharm. Sci. Drug Re*; 2010:**1**:23–30.
6. Harisa, G. E. I., Ibrahim, M. F. & Alanazi, F. K. Characterization of Human Erythrocytes as Potential Carrier for Pravastatin: An In Vitro Study. *Int. J. Med. Sci*; 2011: **8**:222–230.
7. Hamidi, M., Zarrin, A. H., Foroozesh, M., Zarei, N. & Mohammadi-Samani, S. Preparation and in vitro evaluation of carrier erythrocytes for RES-targeted delivery of interferon-alpha 2b. *Int. J. Pharm*; 2007: **341**:125–133.
8. Harisa, G. I., Ibrahim, M. F., Alanazi, F. & Shazly, G. A. Engineering erythrocytes as a novel carrier for the targeted delivery of the anticancer drug paclitaxel. *Saudi Pharm*; 2014:**22**: 223–230.
9. Madhavi, B. B., Bhavana, M., Nath, A. R., Prasad, M. & Siri Vennela, K. In vitro evaluation of piperine enclosed erythrocyte carriers. *Drug Invent. Today*; 2013: **5**:169–174.
10. Raut Deepika, B., Sakhare Ram, S., Dadge Ketan, K. & Halle, P. D. RESEALED ERYTHROCYTE DRUG DELIVERY: A REVIEW. *Int. J. Res. Pharm. Chem.*;2013: **33**: 6–7 .
11. Gopal, V. S., Kumar, R. A., Usha, A. N., Karthik, A. & Udupa, N. Effective drug targeting by erythrocytes as carrier systems. *Curr. Trends Biotechnol. Pharm.*; 2007: **1**:18–33.
12. Pierige, F., Serafini, S., Rossi, L. & Magnani, A. Cell-based drug delivery. *Adv. Drug Deliv. Rev.*; 2008: **60**:286–295.
13. Gutiérrez Millán, C., Castañeda, A. Z., Sayalero Marinero, M. L. & Lanao, J. M. Factors associated with the performance of carrier erythrocytes obtained by hypotonic dialysis. *Blood Cells, Mol. Dis.*; 2004: **33**:132–140.

14. Millán, C. G., Bax, B. E., Castañeda, A. Z., Marinero, M. L. S. & Lanao, J. M. In vitro studies of amikacin-loaded human carrier erythrocytes. *Transl. Res.*; 2008: **152**:59–66.
15. Hamidi, M., Zarei, N., Zarrin, A. H. & Mohammadi-Samani, S. Preparation and in vitro characterization of carrier erythrocytes for vaccine delivery. *Int. J. Pharm.*; 2007: **338**:70–78.
16. Kraus, A., Roth, H. P. & Kirchgessner, M. Supplementation with vitamin C, vitamin E or beta-carotene influences osmotic fragility and oxidative damage of erythrocytes of zinc-deficient rats. *J. Nutr.*; 1997: **127**:1290–1296.
17. Solomon, M., Wofford, J., Johnson, C., Regan, D. & Creer, M. H. Factors influencing cord blood viability assessment before cryopreservation. *Transfusion*; 2010: **50**:820–830.
18. Davoust, J., Gruenberg, J. & Howell, K. E. Two threshold values of low pH block endocytosis at different stages. *EMBO J.*; 1987: **6**:3601.
19. Schrier, S. L., Junga, I. & Ma, L. Studies on the effect of vanadate on endocytosis and shape changes in human red blood cells and ghosts. *Blood*; 1986: **68**:1008–1014.
20. Matovcik, L. M., Junga, I. G. & Schrier, S. L. Drug-induced endocytosis of neonatal erythrocytes. *Blood*; 1985: **65**:1056–63.
21. Briones, E., Colino, C. I. & Lanao, J. M. Study of the factors influencing the encapsulation of zidovudine in rat erythrocytes. *Int. J. Pharm.* 2010: **401**:41–46.
22. Hamidi, M., Tajerzadeh, H., Dehpour, A.-R., Rouini, M.-R. & Ejtemaee-Mehr, S. In vitro characterization of human intact erythrocytes loaded by enalaprilat. *Drug Deliv.*; 2001: **8**:223–230.
23. Tanaka, H., Miyano, M., Ueda, H., Fukui, K. & Ichinose, M. Changes in serum and red blood cell membrane lipids in patients treated with interferon ribavirin for chronic hepatitis C. *Clin. Exp. Med.*; 2005: **5**:190–195.
24. Russmann, S., Grattagliano, I., Portincasa, P., Palmieri, V. O. & Palasciano, G. Ribavirin-induced anemia: mechanisms, risk factors and related targets for future research. *Curr. Med. Chem.*; 2006: **13**:3351–3357.

Table (1): Effect of Ribavirin concentrations and incubation times on the amount of Ribavirin loaded on human carrier erythrocyte at 37 °C by endocytosis

Drug concentration (mg/ml)	Drug incubation times					
	15 min.		30 min.		60 min.	
	Ribavirin loaded (mg/ml)					
	LA	EE %	LA	EE %	LA	EE %
10	2.72 ± 0.01	27.2	2.79 ± 0.024	27.95	3.1 ± 0.037 ⁽²⁾	31.0
15	3.61 ± 0.02	24.0	3.68 ± 0.01	25.7	4.68 ± 0.033 ⁽²⁾	31.2
20	3.65 ± 0.012	18.3	5.1 ± 0.023	25.5	5.66 ± 0.04 ⁽²⁾	28.3
25	4.18 ± 0.012 ⁽¹⁾	16.7	6.85 ± 0.019 ⁽¹⁾	27.4	9.58 ± 0.045 ⁽¹⁾⁽²⁾	38.3
30	4.54 ± 0.01	15.2	7.22 ± 0.023	24.0	8.99 ± 0.034 ⁽²⁾	29.9
35	4.4 ± 0.012	12.6	7.4 ± 0.026	21.1	9.47 ± 0.049 ⁽²⁾	27.0

LA = Ribavirin loaded amount, EE% = entrapment efficiency %

Data is expressed as mean ± SD (n = 3)

(1) Significantly different according to time at p < 0.001

(2) Significantly different according to concentration at p < 0.001

Table (2): Hematological parameters of control erythrocytes, sham and loaded erythrocytes obtained with Ribavirin (25mg/ml) by endocytosis

Hematological parameters	Control	Sham encapsulated	Ribavirin Conc. 25 mg/ml
Hct (%)	38 ± 1.16 ⁽²⁾	33.8±1.02	33.6 ± 1.37 ⁽²⁾
Mcv (fl)	78.6 ± 1.54 ⁽¹⁾	79±1.78	85.1 ± 1.92 ⁽¹⁾
MCH (pg)	26.1 ± 0.99	26.4±0.87	25.0 ± 0.84
MCHC (gm/dl)	33.2 ± 1.27 ⁽²⁾	33.5±1.18	29.4 ± 0.92 ⁽²⁾

Hematocrite (Hct), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC).

(1) Significantly different from the control at $p \leq 0.01$

(2) Significantly different from the control at $p < 0.05$

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