RESEARCH ARTICLE
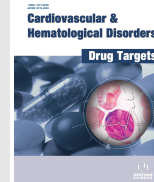
Red Blood Cells are Appropriate Carrier for Coagulation Factor VIII

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Abstract: *Aims*: Factor VIII (FVIII) replacement therapy remains a primary treatment for hemophilia A, however, the development of FVIII antibodies (inhibitors) and short half-life of the FVIII products are the major complications. Erythrocytes may prevent rapid removal of drugs from plasma. Erythrocytes are biocompatible and non-immunogenic drug delivery. In this study, *in vitro* activity of FVIII encapsulated by human erythrocytes was investigated.

Methods: FVIII was loaded into erythrocytes using the hypo-osmotic dialysis technique. FVIII activity assay has been analyzed using Activated Partial Thromboplastin Time (APTT). Presence of FVIII on erythrocytes was detected by western blotting and flowcytometry using specific monoclonal antibody (abcam, U.K) against FVIII. Moreover, the osmotic fragility and hematologic parameters of FVIII-loaded carrier erythrocytes were measured.

Results: Our results indicated that FVIII could not cross the membrane, where plenty of FVIII was found on the surface of the carrier erythrocyte. Flow cytometery results showed that 11% of the loaded carrier erythrocytes was positive for FVIII protein on their surface.

The greatest activation of FVIII in both groups including lysate and non-lysate FVIII-loaded RBCs was observed on the first day, and the coagulant activity of this factor was gradually reduced on days 3 and 5. In 1:50 dilution of both groups, significant differences in FVIII activity were observed in 1:50 dilution of both groups, especially on the 5th day.

Conclusion: This study aims to introduce erythrocytes as appropriate carriers for FVIII to prolong the dosing intervals in the effective and safe levels for a relatively longer time.

Keywords: Factor VIII, erythrocyte, drug delivery, hemophilia A, erythrocytes, nucleotide.

1. INTRODUCTION

Hemophilia A is one of the most common congenital severe bleeding disorders resulting from deficient activity of the coagulation factor VIII (FVIII). It is characterized with spontaneous and continues bleeding in the tissues including joints and muscles. Further, severe bleeding can occur externally, from minor cuts, dental procedures or trauma [1]. The severity of hemophilia A is associated with plasma levels of FVIII. The main method of the treatment of hemophilia A is based on recurrent infusions of coagulation factor VIII [2]. Although FVIII infusion significantly improves the life of patients suffering from hemophilia A, there are many risks for severe bleeding episodes because of rapid inactivation and production of antibody inhibitors. Indeed, frequent infusion of FVIII raises the chance of emerging inhibitors (25-30% of patients), which prevent the effect of FVIII

hemostatic actions. When this occurs, treatment becomes costlier and may lead to morbidity [3]. Therefore, different strategies might be tried to increase the durability of FVIII. Use of drug carriers promises to enhance the specificity, effectiveness and safety of drugs.

Erythrocytes or Red Blood Cells (RBCs) are the largest group of blood cells with 1 μL of human blood containing ~4-5 million RBCs. These cells have a longer circulation time (100 days to 120 days) *in vivo* than other blood cells [4]. RBCs can deliver a wide range of materials, such as nucleoside/nucleotide analogues, enzymes, glucocorticoid analogues, peptides, toxins and nanoparticles [4a, b]. Further, these cells can prolong the dosing intervals at effective and safe levels for a relatively long time. Erythrocytes possess certain remarkable properties that make them inherently suited for the drug delivery system. These erythrocyte properties include long half-life, high availability, delivery capability, a wide range of materials, reduced immuno-genicity, elevated drug circulation time, instinctive targeting carriers, reduced side effects and favorable biocompatibility [5].

*Address correspondence to this author at the Department of Laboratory Science, School of Medical Science, Kermanshah University of Medical Sciences, Kermanshah, Iran; Tel/Fax: +98 83-383582580;
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There are three major methods for drug encapsulation into RBCs including chemical perturbation of the membrane, electroporation and osmotic based methods [6]. Erythrocytes can swell during hypo-osmotic shock where drugs can enter into the cells through the pores generated in their membranes. These cells could increase their volume by 25-50% causing their morphology to change into spherical. In this way, erythrocytes retain their membrane integration. On the other hand, the cell morphology returns to normal in isotonic solutions [6]. In this study, we evaluated the FVIII activity loaded into RBCs by stepwise hypotonic dialysis. It is considered that the survival of erythrocyte-delivered FVIII is greater than that of free FVIII, suggesting the usefulness of RBCs in the delivery of this coagulation factor.

2. MATERIALS AND METHODS

2.1. Preparation of Human Erythrocytes

The blood samples were withdrawn from healthy volunteers with informed consent in heparin test tubes. The whole blood was centrifuged for 10 minutes at $4^{o}C$ at $1000\times$ g. After removal of plasma and the buffy coat, the erythrocytes were washed three times in cold ($4^{\circ}C$) phosphate-buffered saline (PBS) with centrifugation for 10 min at $1000\times$ g. The washed packed RBCs had a hematocrit of about 80%.

2.2. Dialysis Method

FVIII (Lyophilized powder from human plasma produced by Biotest, IBRF) was loaded into RBCs using the hypo-osmotic dialysis technique. Briefly, 2 ml of washed and packed RBCs was mixed with 250 units of FVIII into a dialysis bag (Sigma). The dialysis bag was immersed into 50 ml of PBS buffer containing 5 mM glucose for 4 h. The bag was placed in a beaker equipped with a magnetic stir bar. The beaker was filled with 100 ml of hypoosmotic buffer containing 0.0451 mM $KH2PO4/KOH$, 0.1 mM $MgCl2$, 0.22 mM glucose, and 0.2 mM of adenosine triphosphate; pH 7.45. At $4°C$, 30 ml of sterile distilled water was added into the outside buffer into 20 minutes times intervals and repeated 5 times to make. The dialysis bag was transferred into 100 ml of isoosmotic buffer; pH 7.45 and incubated at $37°C$ for 1 h. Pores were performed on the RBCs' membrane loaded with FVIII reversed into normal in isotonic media. The isoosmotic buffer used contained: 0.036 mM KH_2PO_4/KOH, 0.04 mM $MgCl_2$, 0.84 mM NaCl, 0.18 mM glucose, and 0.27 mM adenosine. The RBC-carriers were washed 3-4 times in sterile physiological saline by centrifugation (1250 g, 10 min) to remove hemoglobin and unloaded factor VIII.

2.3. Morphology and Hematological Indices

The hematological indices including Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Red Blood Cell Distribution Width (RDW) of native and FVIII-loaded RBCs were determined using a Sysmex KX-21N (Kobe, Japan). To investigate the morphology, blood smear of RBCs was prepared with Giemsa stain and analyzed using a light microscope.

2.4. Osmotic Fragility

The degree of membrane resistance of RBCs to lysis was determinded by the osmotic fragility test. 50 μL of washed and packed erythrocytes was suspended in 5 ml of NaCl aqueous solutions of varying concentrations between 1 to 9 g/dl. After incubation at room temperature ($25°C$) for 15 min, the suspension was centrifuged at $1000\times$g for 5min, and the absorbance of the supernatants was determined spectrophoto-metrically at 540 nm for hemoglobin. The hemoglobin released into distilled water was considered to completed (100%) hemolysis, and the hemoglobin released in the NaCl solutions was considered as a percentage of maximal hemolysis. The osmotic fragility was studied for each dialysis, and it was determined in triplicate for each dialysis time.

2.5. Coagulation Activity of FVIII Loaded into Erythrocytes by one Stage Clotting Assay

FVIII activity assay was analyzed using Activated Partial Thromboplastin Time (APTT). The APTT was determined according to the manufacturers' instructions (Fisher): 0.1 ml of citrated plasma (prewarmed for 1 min at $37^{o}C$), and 0.1 ml of APTT reagent (in part prewarmed to $37^{o}C$), incubation for 3 min at $37^{o}C$, addition of 0.1 ml of 25mmol/liter CaCl2 solution (simultaneous start of the stopwatch).

1:5, 1:10 and 1:50 dilutions of lysate and non-lysate FVIII-loaded RBCs and native red cells were prepared with buffer solution. 50 μL of prediluted samples and 50 μL of deficient FVIII plasma were incubated for 1 min at $37^{o}C$. After adding 100 ml of the APTT reagent, the mixture was incubated for exactly 3 min at $37^{o}C$. The stopwatch was started when 100 ml of 25 mmol/liter $CaCl_2$ solution ($37^{o}C$) were added. The FVIII activity assay was calculated from the standard curve.

The standard curve was prepared by making serial dilutions of a pooled normal plasma (n: 50 healthy individuals) as standard. 1:1, 1:2, 1:4, and 1:8 dilutions of pooled plasma with buffer solution indicated 100%, 50%, 25% and 12.5% of FVIII activation, respectively.

2.6. Western Blotting

Three types of the sample including recombinant FVIII, non-lysate and lysate FVIII-loaded RBC were run in discontinuous polyacrylamide gels. Plasma-derived FVIII (Lyophilized powder from human plasma produced by Biotest, IBRF) was used as a positive control. The SDS-PAGE gels were stained by Coomassie Brilliant Blue R-250 (Merck, Germany). The SDS-PAGE gels were transferred to polyvinylidene fluoride (PVDF) membrane (Roche, Germany). Sequentially, the membranes were incubated overnight at $4°C$ with 5% skim milk (Merck, Germany) in PBS as blocking solution. The membranes were over-laid with mouse monoclonal anti-human FVIII antibody (abcam, U.K) and incubated for 2h at room temperature (RT) and washed with 0.2% Tween 20/PBS three times, 5 min each. Incubation continued at RT for 1h with horseradish peroxidase (HRP)-anti mouse secondary antibody according to the manufacturer's protocol (abcam, U.K). After a final wash, separated proteins were visualized using ECL (Santa

Table 1.   Hematological indices of native and FVIII-loaded erythrocytes.

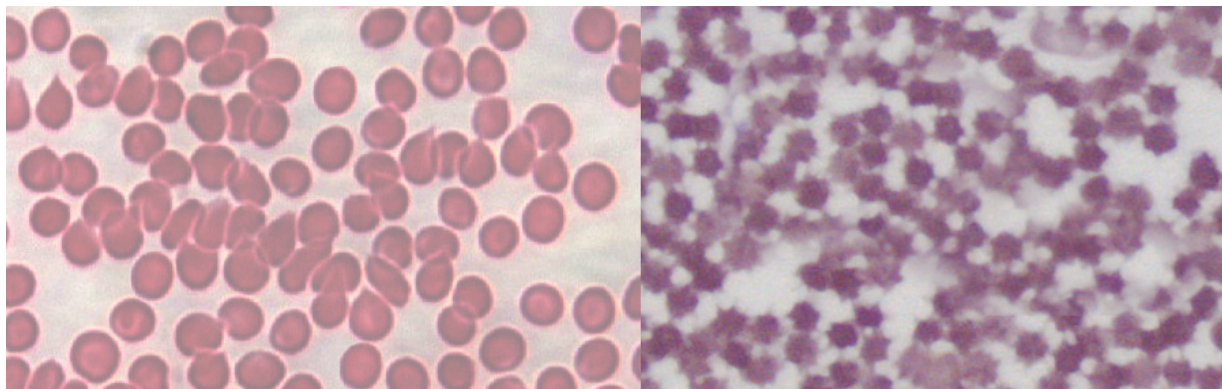| Hematological Index	Carrier Erythrocytes	Native Erythrocytes	Significant
MCV	84±3 fL	88±2 fl	$p<0.1$
MCH	23 ±2Pgr	28± 3 pgr	$p<0.05$
RDW	19±2%	14±3 %	$p<0.05$

Fig. (1). Morphology of loaded and unloaded erythrocytes on Giemsa stained slides using a light microscope. (**A**) Native erythrocytes. (**B**) FVIII- loaded erythrocytes. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

cruze, USA) substrate solution using chemiluminescense system (BioRad, USA).

2.7. Flow Cytometry

Presence of FVIII on erythrocytes was detected by flow cytometry using specific monoclonal antibody (abcam, U.K) against FVIII. 5 µl of washed and packed erythrocytes diluted with 100 µl of PBS. These cells were incubated at 4°C for 30 minutes with FVIII monoclonal antibody. Secondary antibody conjugated with FITC was added and incubated at 4°C for 30 minutes again. The cells were analyzed withPartec instrument.

2.8. Statistical Analyses

The data are expressed as the mean±SEM of at least three experiments. Statistical analyses were performed using a paired Student's t test and one-way Anova. The differences were considered statistically significant when $p<0.05$.

3. RESULTS

To determine the effect of FVIII-loaded on erythrocytes, we investigated RBC indices such as MCV, MCH, and RDW. The results demonstrated no significant decrease in MCV level of carrier RBCs as compared to unloaded erythrocytes. However, a significantly reduced MCH and increased RDW levels in both groups were detected (loaded- and unloaded erythrocytes) ($p<0.05$) (Table **1**). Furthermore, the morphology investigation revealed that erythrocytes containing FVIII changed in shape and crenated features were observed in stained slides (Fig. **1**).

The Osmotic Fragility (OF) curves of carrier erythrocytes are shown in Fig. (**2**). The graph of data indicated an increased index of erythrocytes containing FVIII compared with normal erythrocytes ($p<0.05$). The findings demonstrated hemolysis of carrier RBCs started at 0.9% NaCl compared to the hemolysis of unloaded erythrocytes at 0.7% NaCl concentration. Further, 100% hemolysis was observed in 0.2% and 0.1% NaCl for FVIII-loaded and unloaded erythrocytes, respectively (Fig. **2**).

After loading FVIII into erythrocytes *via* stepwise dialysis method, the FVIII activity was analyzed on the first, third, and fifth days. Based on the standard curve, coagulation times lower than 44 seconds revealed FVIII activity higher than 100% (Table **2**). Therefore, the samples containing 1, 1:5 and 1:10 dilutions of lysate and non-lysate FVIII-loaded erythrocytes mixed with FVIII plasma showed higher than 100% coagulant activity of FVIII during on days 1, 3, and 5 (Table **3**). The greatest activation of FVIII in both groups including lysate and non-lysate FVIII-loaded RBCs was observed on the first day, and coagulant activity of this factor was gradually reduced on days 3 and 5, especially in 1:50 dilutions. As the coagulation time in both groups of lysate and non-lysate FVIII-loaded RBCs in 1, 1:5 and 1:10 dilutions mixed with non-FVIII plasma was lower than 35 seconds, no significant difference was observed in FVIII activity. However, in 1:50 dilution, we observed a meaningful difference in FVIII activity, especially on the 5th day (Fig. **3**). This can be due to the important role of erythrocyte membrane on FVIII stability.

Presence of FVIII protein in non-lysate and lysate carrier cells was assessed by the western blotting method. These Our results indicated that FVIII could not cross the membrane, where plenty of FVIII was found on the surface
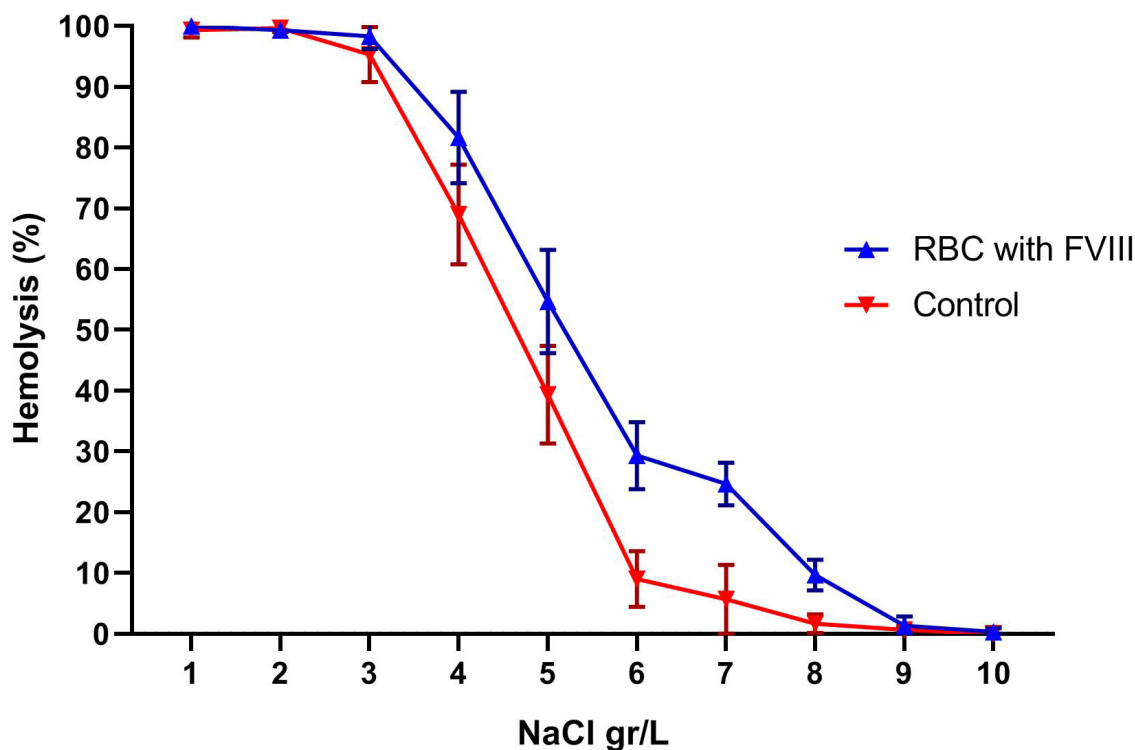
Osmotic Fragility Test

Fig. (2). Osmotic fragility curves of factor VIII-loaded erythrocytes and native erythrocytes prepared by stepwise dialysis method (n=3). 50 µL of washed and packed erythrocytes were suspended in 5 mL of a series of dilutions of a buffer. The supernatants were determined spectrophotometrically at 540 nm for hemoglobin. The hemoglobin released in the buffer solution was expressed as the percentage absorbance of each sample in reference to a completely lysed sample. Mean values ± standard deviation for 3 independent experiments are shown. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2.   APTT in different dilutions of pooled normal plasma to plot standard curve.

Dilutions of Pooled Normal Plasma	% Coagulant Activity	APTT
1/10	100%	44 sec
1/20	50%	52 sec
1/40	25%	60 sec
1/80	12.5%	71 sec
1/160	6.25%	79 sec

Abbrevation: sec (second).

Table 3.   APTT of lysate and non-lysate FVIII-loaded RBCs on 1, 3 and 5 days.

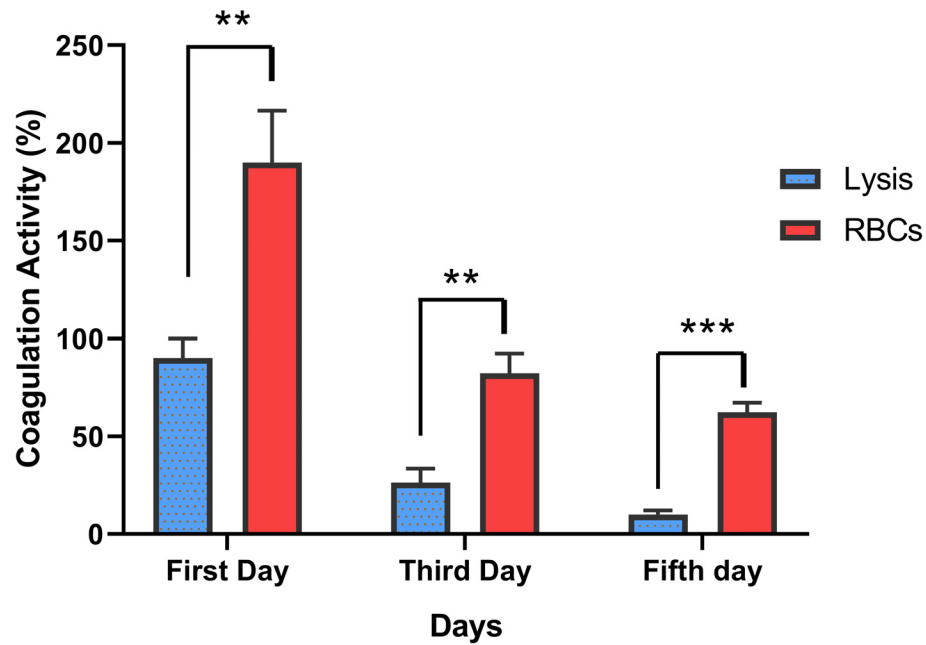| -	First Day		Third Day		Fifth Day	
Diluation	Membrane lysis	No Membrane lysis	Membrane lysis	No Membrane lysis	Membrane lysis	No Membrane lysis
1	3	3	5	5	5	5
1/5	5	4	12	7	20	14
1/10	29	23	33	25	36	32
1/50	45	34	59	47	63	51

Fig. (3). FVIII activity assay in 1/50 dilution of lysate and non-lysate FVIII-loaded RBCs on days 1, 3 and 5 using APTT. 50μL of FVIII-deficient plasma and 50μL of 1/50 dilution of lysate and non-lysate FVIII-loaded RBCs were mixed. On day 5, the difference in FVIII activity in both groups was more substantial. Mean values ± standard deviation for 3 independent experiments are shown.; bars, SEM. **p<0.01; ***p<0.001. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).
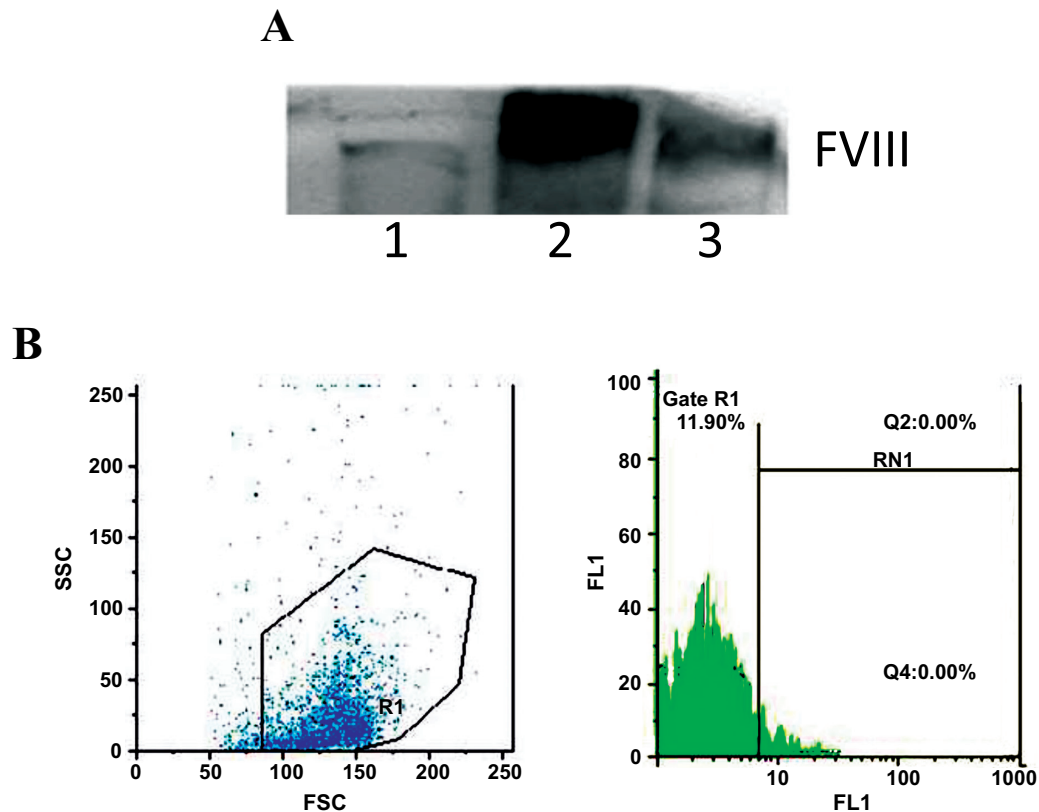

Fig. (4). Investigation of FVIII that was loaded on RBCs using western blotting and flow cytometry. **A**) Western blotting of FVIII-loaded erythrocytes. Recombinant FVIII (positive control), non-lysate and lysate of loaded RBC were run in discontinuous polyacrylamide gels. **1**) Lysate loaded erythrocytes **2**) Membrane loaded erythrocytes **3**) Positive FVIII control. **B**) Flow cytometry analysis of FVIII-loaded RBCs with FVIII monoclonal antibody. RN1 Region determined by the reaction of isotype antibody (negative control) with native RBCs. 11% of FVIII-loaded RBCs had a positive reaction with FVIII specific monoclonal antibody. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

of carrier erythrocyte. Indeed, due to high molecular weight (280 KD), FVIII was trapped in the membrane pores during the loading procedure (Fig. **4a**).

Flow cytomery results showed that 11% of the loaded carrier erythrocyte was positive for FVIII protein on their surface (Fig. **4b**). Flow cytometry and western blot results demonstrated that a large number of FVIIIs remained inside the membrane of erythrocytes where the antibody might not access to FVIII.

4. DISCUSSION

FVIII infusion significantly improves the life of patients with hemophilia A; however, there are high risks for severe bleeding episodes because of rapid inactivation and the production of antibody inhibitors [2, 3]. An estimated 30% of patients suffering from severe hemophilia develop an inhibitor. Patients with inhibitors are at increased risk for severe hemorrhagic events. The Extended half-life productsare known to extend the time in circulation of FVIII, leading to longer infusion intervals. Hence, we here studied erythrocytes as a carrier for FVIII to extend the half-life of FVIII due to unique characteristics of erythrocytes, where high levels of these cells are introduced as carriers for drug delivery.

In our study, RBC parameters revealed no substantial decrease in MCV. However, significantly reduced MCH and increased RDW levels of FVIII-loaded erythrocytes in comparison with native cells were seen. Hemoglobin release of carrier erythrocytes due to increased membrane permeability could be attributed to the reduction of MCH. The same changes of indices have been reported by other groups who examined the encapsulation of amikacin and phenytoin in carrier erythrocytes using an osmotic-based method [6, 7]. The morphological analysis of carrier erythrocytes showed echinocyte shape. Indeed, the morphological changes of erythrocytes and elevated RDW might be caused by osmotic pressure. However, these changes are reversible. Carrier erythrocytes revealed an altered osmotic fragility in comparison with the native erythrocytes curve ($p<0.05$). The osmotic fragility of erythrocytes is an index of the possible alterations in membrane integrity of cells as well as resistance of cells to osmotic pressure. As reported previously, osmotic fragility of carrier RBCs is higher than that of the control cells because of elevated intracellular osmotic pressure [8]. The less resistance to osmotic changes is a measure of the loss of integrity of the carrier erythrocyte membrane and its normal behavior in response to loading procedure [9].

The activity of erythrocytes-entrapped FVIII was assessed by APTT based method on days 1, 3, and 5. The highest FVIII coagulation activity of both lysate and non-lysate FVIII-loaded RBCs was observed on the first day. However, the coagulation activity was gradually diminished after days 3 and 5. Statistically, there is a significant difference between the coagulation activity of FVIII on different days for lysate and non-lysate samples ($p<0.01$).

Previous studies found no difference concerning hemolyzed and non-hemolyzed normal samples [10]. It can be deduced that the difference of APTT of lysate and non-lysate FVIII-carrier RBCs is not due to the release of

hemoglobin. Hence, membrane concentration of RBCs would shorten the clotting time due to a high level of FVIII.

The data collected from western and flow cytometry analysis also verified that FVIII remains into membranes of RBCs. A dense band was detected in the samples of the RBCs membrane in comparison with lysing erythrocytes. These data showed that there is a high level of FVIII on the membrane. Probably because of the large size, the amount of FVIII in cytoplasm of erythrocytes is low. Further, larger amounts of FVIII might trap into the pores in the membrane. Therefore, only 11% RBCs were positive for the antibody of FVIII using flow cytometry.

Elena I. Sinauridze *et al*. (2010) loaded factor IXbiot into erythrocytes by Stepwise dialysis method and showed that the lifetime of the erythrocyte-based form of FIXbiot in the circulation is significantly prolonged compared with its free form, suggesting that this form has potential clinical applications [11]. In our study, the function of FVIII-loaded erythrocytes was not investigated in patients with hemophilia A. However, FVIII-loaded RBCs infusion can be considered in future studies and carrier RBCs can be used to deliver FVIII with improved properties such as prolonged circulation half-life and sustained releases.

CONCLUSION

In conclusion, the present study demonstrates that erythrocytes can function as appropriate carriers for delivery FVIII. However, additional researches targeting mechanisms of action are required.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Blood samples were withdrawn from healthy volunteers with informed consent.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

REFERENCES

[1] Oldenburg, J. Optimal treatment strategies for hemophilia: Achievements and limitations of current prophylactic regimens. *Blood,* **2015**, *125*(13), 2038-2044.
http://dx.doi.org/10.1182/blood-2015-01-528414 PMID: 25712992

[2] Witmer, C.; Young, G. Factor VIII inhibitors in hemophilia A: Rationale and latest evidence. *Ther. Adv. Hematol.,* **2013**, *4*(1), 59-72. http://dx.doi.org/10.1177/2040620712464509 PMID: 23610614

[3] Porada, C.D.; Almeida-Porada, G. Treatment of hemophilia a in utero and postnatally using sheep as a model for cell and gene delivery. *J. Genet. Syndr. Gene Ther.,* **2012**, *S1*, S1.
PMID: 23264887

[4] (a) Muzykantov, V.R. Drug delivery by red blood cells: Vascular carriers designed by mother nature. *Expert Opin. Drug Deliv.,* **2010**, *7*(4), 403-427.
http://dx.doi.org/10.1517/17425241003610633 PMID: 20192900
(b) Saba, F.; Saki, N.; Khodadi, E.; Soleimani, M. Crosstalk between catecholamines and erythropoiesis. *Front. Biol.,* **2017**, *12*(2), 103-115.
http://dx.doi.org/10.1007/s11515-017-1428-4

[5] (a) Sun, Y.; Su, J.; Liu, G.; Chen, J.; Zhang, X.; Zhang, R.; Jiang, M.; Qiu, M. Advances of blood cell-based drug delivery systems. *Eur. J. Pharm. Sci.,* **2017**, *96*, 115-128.
http://dx.doi.org/10.1016/j.ejps.2016.07.021 PMID: 27496050
(b) Gupta, N.; Patel, B.; Ahsan, F. Nano-engineered erythrocyte ghosts as inhalational carriers for delivery of fasudil: Preparation and characterization. *Pharm. Res.,* **2014**, *31*(6), 1553-1565.
http://dx.doi.org/10.1007/s11095-013-1261-7 PMID: 24449438
(c) Parodi, A.; Quattrocchi, N.; van de Ven, A.L.; Chiappini, C.; Evangelopoulos, M.; Martinez, J.O.; Brown, B.S.; Khaled, S.Z.; Yazdi, I.K.; Enzo, M.V.; Isenhart, L.; Ferrari, M.; Tasciotti, E. Synthetic nanoparticles functionalized with biomimetic leukocyte membranes possess cell-like functions. *Nat. Nanotechnol.,* **2013**, *8*(1), 61-68.
http://dx.doi.org/10.1038/nnano.2012.212 PMID: 23241654
(d) Chessa, L.; Leuzzi, V.; Plebani, A.; Soresina, A.; Micheli, R.; D'Agnano, D.; Venturi, T.; Molinaro, A.; Fazzi, E.; Marini, M.; Ferremi Leali, P.; Quinti, I.; Cavaliere, F.M.; Girelli, G.; Pietrogrande, M.C.; Finocchi, A.; Tabolli, S.; Abeni, D.; Magnani, M. Intra-erythrocyte infusion of dexamethasone reduces neurological symptoms in ataxia teleangiectasia patients: Results of a phase 2 trial. *Orphanet J. Rare Dis.,* **2014**, *9*, 5.
http://dx.doi.org/10.1186/1750-1172-9-5 PMID: 24405665
(e) Anselmo, A.C.; Gupta, V.; Zern, B.J.; Pan, D.; Zakrewsky, M.; Muzykantov, V.; Mitragotri, S. Delivering nanoparticles to lungs while avoiding liver and spleen through adsorption on red blood cells. *ACS Nano,* **2013**, *7*(12), 11129-11137.
http://dx.doi.org/10.1021/nn404853z PMID: 24182189

[6] Hamidi, M.; Azimi, K.; Mohammadi-Samani, S. Co-encapsulation of a drug with a protein in erythrocytes for improved drug loading and release: Phenytoin and bovine serum albumin (BSA). *J. Pharm. Sci.,* **2011**, *14*(1), 46-59.

[7] Sparrow, R.L. Red blood cell storage and transfusion-related immunomodulation. *Blood Trans.,* **2010**, *8*(3), s26-30.

[8] Garín, M-I.; López, R-M.; Sanz, S.; Pinilla, M.; Luque, J. Erythrocytes as carriers for recombinant human erythropoietin. *Pharm. Res.,* **1996**, *13*(6), 869-874.
http://dx.doi.org/10.1023/A:1016049027661 PMID: 8792424

[9] 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*(1-2), 125-133.
http://dx.doi.org/10.1016/j.ijpharm.2007.04.001 PMID: 17512685

[10] (a) Arora, S.; Kolte, S.; Dhupia, J. Hemolyzed samples should be processed for coagulation studies: The study of hemolysis effects on coagulation parameters. *Ann. Med. Health Sci. Res.,* **2014**, *4*(2), 233-237.
http://dx.doi.org/10.4103/2141-9248.129049 PMID: 24761244
(b) Laga, A.C.; Cheves, T.A.; Sweeney, J.D. The effect of specimen hemolysis on coagulation test results. *Am. J. Clin. Pathol.,* **2006**, *126*(5), 748-755.
http://dx.doi.org/10.1309/03FK3378YTRA1FRF PMID: 17050072

[11] Sinauridze, E.I.; Vuimo, T.A.; Kulikova, E.V.; Shmyrev, I.I.; Ataullakhanov, F.I. A new drug form of blood coagulation factor IX: Red blood cell-entrapped factor IX. *Med. Sci. Monit.,* **2010**, *16*(10), PI19-PI26.
PMID: 20885362