In vivo reduction of erythrocyte oxidant stress in a murine model of β-thalassemia

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A B S T R A C T

Background and Objectives. Oxidant damage is an important contributor to the premature destruction of erythrocytes and anemia in thalassemias. To assess the extent of oxidant damage of circulating erythrocytes and the effects of antioxidant therapy on erythrocyte characteristics and anemia, we used a mouse model of human β-thalassemia intermedia (*b1*/*b2* deletion).

Design and Methods. Several parameters indicative of oxidant damage were measured at baseline and following administration of the semi-synthetic flavonoid antioxidant, 7-monohydroxyethylrutoside (monoHER), to β-thalassemic mice at a dose of either 500 mg/kg i.p. once a day (n=6) or 250 mg/kg i.p. twice a day (n=6) for 21 days.

Results. Significant erythrocyte oxidant damage at baseline was indicated by: (i) dehydration, reduced cell K content, and up-regulated K-Cl co-transport; (ii) marked membrane externalization of phosphatidylserine; (iii) reduced plasma and membrane content of vitamin E; and (iv) increased membrane bound IgG. MonoHER treatment increased erythrocyte K content, and markedly improved all cellular indicators of oxidant stress and of lipid membrane peroxidation. While anemia did not improve, monoHER therapy reduced reticulocyte counts, improved survival of a fraction of red cells, and reduced ineffective erythropoiesis with decreased total bilirubin, lactate dehydrogenase and plasma iron.

Interpretation and Conclusions. Antioxidant therapy reverses several indicators of oxidant damage *in vivo*. These promising antioxidant effects of monoHER should be investigated further.

Key words: monoHER, K-Cl co-transport, erythrocyte, vitamin E, phosphatidylserine.

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Ineffective erythropoiesis and reduced survival of circulating erythrocytes are responsible for the anemia of β-thalassemia.[1] The membrane damage induced by the excess of free α chains plays a crucial role in the shortening of erythrocyte life span.[2-6] Red cells from patients with thalassemia intermedia show membrane clusters of hemichrome and band 3, presumably as a result of oxidative injury.[7-9] Immunoglobulins and complement components localize at the membrane surface over these clusters, mediating the removal of the damaged β-thalassemia erythrocytes by macrophages.[7,8] Membrane lipid peroxidation, loss of phospholipid asymmetry, and externalization of phosphatidylserine (PS) have been demonstrated in β thalassemia erythrocytes.[7-10] PS externalization in these erythrocytes is believed to play a significant role in their premature removal and reduced survival, as has been demonstrated for dense sickle erythrocytes.[11,12] Oxidative damage plays a significant role in the upregulation of K-Cl co-transport and associated cell dehydration of β-thalassemia erythrocytes.[13-15] *In vitro* treatment of β-thalassemia erythrocytes with dithiothreitol reduced K-Cl co-transport activity to almost normal values, indicating that the functional changes induced by oxidation are reversible.[14] Studies have been published on the use of antioxidants such as vitamin E, flavonoid(s) and polyphenols in β thalassemia,[16-19] but limited data are available about their *in vivo* use in either animal models or patients.

Recently, a novel semi-synthetic flavonoid, 7-monohydroxyethylrutoside (monoHER), has been shown to protect against doxorubicin cardiotoxicity, which is caused by the formation of free radicals. In addition, monoHER exhibited minimal toxicity and did not

affect the anti-proliferative activity of doxorubicin.[20,21] The antioxidant properties of monoHER are mostly related to its ability to scavenge radicals causing peroxidized lipids, as shown in microsomal liver preparations deficient in α-tocopherol.[19] In this report, we characterize the ion transports and cell volume characteristics of a mouse model with deletion of both *b1* and *b2* mouse β globin genes; this mouse model exhibits clinical and biological features similar to those observed in human β – thalassemia intermedia.[22] We also examined the effects of *in vivo* administration of monoHER, examining several membrane parameters related to red cell membrane oxidative stress.

Design and Methods

Drugs and chemicals

Sodium chloride (NaCl), potassium chloride (KCl), okadaic acid (OA), staurosporine, N-ethylmaleimide (NEM), dithiothreitol (DTT), sulfamic acid (SFa), A23187, Tris(hydroxymethyl) aminomethane (Tris), 3(N-morpholino) propanesulfonic acid (MOPS), ouabain, bumetanide and nystatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $MgCl_2$, $Mg(NO_3)_2$, dimethylsulfoxide (DMSO), and n-butyl phthalate were purchased from Fisher Scientific Co. Choline chloride was purchased from Calbiochem-Boehring (San Diego, CA, USA). Bovine serum albumin fraction V was purchased from Boehringer Mannheim (Mannheim, Germany). Phycoerythrin-conjugated anti-TER119 was purchased from Pharmingen (San Diego, CA, USA). MonoHER was provided by Prof. A. Bast, Maastricht, The Netherlands. The Ca-dependent phospholipid binding protein, annexin V, was provided by Dr. Frans A. Kuypers, Children's Hospital Oakland Research Institute, (Oakland, CA, USA). All solutions were prepared using double-distilled water.

Animals

Mice heterozygous for deletion of both *b1* and *b2* were obtained from Jackson Laboratories (Bar Harbor, ME, USA).[22] Animals between 4 and 6 months of age, the females weighing 25 to 28 grams and the males weighing 28 to 30 grams, were selected for the study. C57BL/6J mice were used as controls. Institutional and national guidelines for the care and use of laboratory animals were followed. The study protocol was reviewed and approved by the Children's Hospital Animal Care and Use Committee.

Hematologic parameters and red cell cation content

Blood was collected from isofluorane-anesthetized mice by retro-orbital venipuncture into heparinized microhematocrit tubes. Hemoglobin (Hb) concentration was determined by the spectroscopic measurement of the cyanmet derivative. The hematocrit (Hct) was determined by centrifugation in a micro-hematocrit centrifuge. Erythrocyte and reticulocyte cellular indices were determined on an ADVIA 120 hematology analyzer, using a mouse-specific software program (Bayer Diagnostics, Tarrytown, NY, USA). Density distribution curves were obtained using phthalate esters in microhematocrit tubes, after washing the cells three times with mouse PBS (330 mOsm) at 25°C.[23] Density values that define the 20% most dense fraction of cells (D20) were determined for each curve.

Characterization of ion content and transport

Erythrocyte Na and K contents were determined after 5 washes in 172 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Tris-MOPS, pH 7.4 at 4°C (choline washing solution). K-Cl co-transport activity was measured in fresh erythrocytes as chloride- and volume-dependent K efflux. Net K efflux from fresh cells was measured in a hypotonic (260 mOsm) Na solution (340 mOsm is isotonic for mouse erythrocytes). Chloride dependent- and volume-dependent K effluxes were calculated as the difference between K efflux in chloride and sulfamate hypotonic media, and in hypotonic and isotonic chloride media, respectively. All media contained 1 mM $MgCl_2$, 10 mM glucose, 1 mM ouabain, 0.01 mM bumetanide, and 10 mM Tris-MOPS (pH 7.40 at 37°C). Efflux was calculated from the K concentrations in the supernatant at 5 and 25 min.[24,25] The effect of DTT on K-Cl co-transport was assessed by a pre-incubation in NaCl isotonic medium containing 10 mM DTT. K efflux was measured as described above in media containing 1 mM DTT.

MonoHER administration

MonoHER was dissolved in sterile water containing 36 mM NaOH to a final concentration of 33 mg/mL, pH 7.8-8, and injected intra-peritoneally (i.p.).[20,21] β-thalassemia mice were divided into 2 groups of six mice each and treated for 21 days using two different monoHER dosing protocols: either 250 mg/kg twice a day, i.p. or 500 mg/kg once a day, i.p. Control mice (C57BL/6J) were treated with monoHER at the dosage of 500 mg/kg a day, i.p. (n =6). Total bilirubin, serum LDH and serum iron, hematologic parameters, red cell cation content, red cell density profile, phosphatidylserine (PS) exposure, plasma and membrane vitamin E levels were determined at baseline and after 3 weeks of treatment with monoHER. For experiments on red cell survival, β-thalassemia mice were treated with monoHER (500 mg/kg once a day, i.p) for 57 days. After 20 days of treatment, red cells were labeled with biotin and administration of monoHER was continued for a further 37 days.

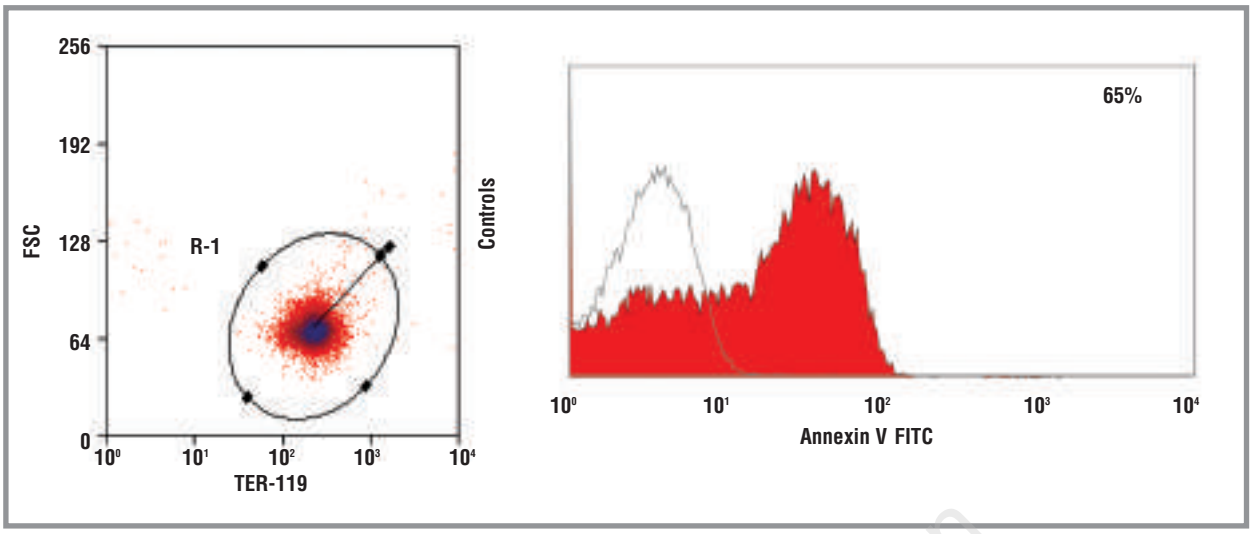


Figure 1. Gating strategy for analysis of the annexin-V positive mouse red cells. The erythrocyte population was identified based on forward and side scatter (gate R1) and staining for TER-119 (gate R2).[31] Expression of annexin-V was examined only on R1-gated cells.

Measurements of PS exposure in mouse red cells with annexin-V labeling

Erythroid PS exposure was assessed as previously described with minor modifications.[7] To generate *positive* control erythrocytes that expose PS on their outer surface, wild-type mouse red cells were incubated with NEM, which inhibits the aminophospholipid translocase by reacting with a sulfhydryl group necessary for its activity. Red cells from wild-type mice (Hct: 30%) were incubated in a buffer containing 10 mM Hepes, 170 mM NaCl, pH7.4 (HBS buffer) with 10 mM NEM for 30 min at 37°C and subsequently washed in buffer without NEM. Cells exposing PS were also generated by A23187 treatment in the presence of Ca: control mouse erythrocytes were equilibrated at 16% hematocrit in HBS buffer with 1 mM $CaCl_2$ for 3 min at 37°C. A23187 was then added to the red cell suspension to a final concentration of 4 mM and the suspension was incubated for 15 min at 37°C. Cells were washed with HBS buffer containing 1% bovine serum albumin, 5 mM EDTA, and re-suspended in HBS buffer.[7] PS positive control red cells and freshly drawn whole blood from control and β-thalassemia mice (1.5 mL, containing ≤ $1x10^7$ cells) were incubated for 30 min in ice in the dark with 1 μL of phycoerythrin-conjugated anti-TER119. TER119 is a cell-surface erythroid-specific antigen expressed in terminally differentiating erythroblasts and closely associated with glycophorin A.[26,27] Cells were then incubated in a binding buffer containing 10mM Hepes, 170 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4 and 2 μL of (FITC)-labeled annexin V for 30 min in the dark at room temperature.[11] Two-color immunofluorescence analysis was used to quantify the fraction of erythrocytes binding annexin V. Selection of initial events was based on the analysis of forward scatter (FSC) of the cells (set to include microcytic thalassemic red cells) and the expression of TER-119 marker (Figure 1). Only events that correlated with specific forward scatter and were positive for TER-119 were included in gate R1 for further analysis for annexin V binding. For each sample analyzed, 10,000 events were acquired. The threshold for annexin V positivity was based on the maximum fluorescence of the cells incubated without annexin V or in some cases, based on the maximum fluorescence of normal control cells incubated with annexin V. In the latter case, the threshold for defining annexin V positive cells was set so as to yield no more than 0.5% positivity in control cells. Stained cells that were brighter than the negative control (present on the right of the negative control histogram) were defined as positive for annexin V. The data were acquired using a MoFlo flow cytometer and analyzed using Summit software, both from Cytomation (Fort Collins, CO, USA). The cytometer was aligned using AlignFlow Beads (Molecular Probes, Eugene, OR, USA).

Flow-cytometric determination of maturation of bone marrow erythroblasts

Bone marrow cells were labeled with biotin-conjugated monoclonal antibody against CD71 and PE-conjugated antibody against TER-119, and analyzed as described by Socolovsky *et al.*[28]

Measurements of plasma and red cell membrane vitamin E levels in wild-type and β–thalassemia mice[29-31]

Analytical grade diethyl ether, HPLC grade methanol, chromatographically pure standards for α-tocopherol,

and α-tocopheryl acetate were obtained from Sigma. Stock standard solutions of α-tocopherol and α-tocopheryl acetate were obtained by dissolving the standards in methanol to a concentration of 15 g/L. A working standard solution containing 2 mg/L each of α-tocopherol and α-tocopheryl acetate in methanol was prepared by diluting 250-fold an intermediate solution containing 500 mg/L of each compound in methanol. Internal standard solution (ISTD), a solution with 2.5 g/L of α-tocopheryl acetate, was prepared by diluting the stock standard solution 6-fold with methanol. Stock and intermediate solutions were stored at 20°C for up to three months. The working standard solution was prepared fresh on the day of the experimental determination. In a 5 mL polypropylene tube, 500 μL of plasma or 100 μL membranes (20 mg/mL) and 10 μL of ISTD were combined, and after mixing, 3 mL of diethyl ether were added. The sample tube was vortexed vigorously for 1 min, then centrifuged at 3000 rpm for 3 min. The organic layer was transferred to another polypropylene tube and evaporated under a gentle stream of nitrogen. To dissolve the residue, 200 μL of methanol were added. The solution was transferred to a microcentrifuge 1.5 mL test tube. After vortexing for a few seconds, the tube was centrifuged at 14000 rpm for 5 min. A 20 μL aliquot of the supernatant was injected into the HPLC system. The HPLC system was a Hewlett-Packard series 1100 instrument with double binary pump, thermostated column compartment, variable wavelength UV-vis detector and Tecan MiniPrep 60 autosampler.

Data acquisition and analysis was performed on a standard PC with ChemStation software (Hewlett-Packard). The analysis was performed on a C_{18} reversed phase (3 μm,150×2.0 mm) column (Luna, Phenomenex) under isocratic conditions with methanol as the eluent. The chromatographic run was completed in 12 min at a flow rate of 300 μL/min. The detection wavelength used was 285 nm, response time was 4s and column temperature was 35°C. The calibration was performed by injecting the working standard solution separately immediately before and after each sample queue. A one-point calibration with origin included was used because the response is linear in the 2-20 mg/L range used for these measurements.

Calculations of vitamin E concentrations were made by the internal standard method (correction of peak areas based on ISTD areas). The accuracy of the method was above 95%; values of 98±1.7% and 97±2% for within and between assay accuracy, respectively, were obtained for three repetitions of two plasma and red cell membrane samples spiked with 5 and 10 mg/L of α-tocopherol. The recovery was 88±3% for α-tocopheryl acetate and 85±3% for α-tocopherol. The time for sample preparation was approximately 15 min.

Measurements of erythrocyte-bound IgG

Erythrocyte membrane-bound IgG was measured after labeling erythrocytes with rabbit anti-human IgG antibodies conjugated with alkaline phosphatase as described by Turrini *et al.*[32]

Measurements of erythrocyte in vivo survival

Mouse erythrocytes were labeled with NHS-biotin. (E-Z link, Pierce; 30-40 mg/kg of body weight, administered intravenously). The percentage of labeled cells in peripheral blood was quantified by flow cytometry using Streptavidin R-PE.[12,33]

Histology, electron microscopy and tissue iron content studies

Mouse tissues (spleen and liver) were fixed in phosphate-buffered formaldehyde (3.7%) prior to dehydration and paraffin embedding using standard techniques. Sections were cut at 4 μm and stained with hematoxylin and eosin or Perls' iron stain. For electron microscopy studies, washed erythrocytes were suspended in 2.5% glutaraldehyde (in Na-cacodylate buffer, pH 7.4), treated with 1% osmium tetraoxide solution, and embedded in Epon resin. Thin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy. Iron content was determined on freshly frozen liver and spleen samples: tissue samples were digested with 1 mL of 60% trace metal grade nitric acid at 80°C overnight. Digestion was followed by addition of 1 mL of hydrogen peroxide and double distilled water to a total final volume of 2 mL. The iron content of these extracts was measured by flame atomic absorption. Blank reagent was processed in parallel and its iron content was subtracted from that of the samples to correct for background iron.[34,35]

Statistical analysis

All values are presented as mean ± standard deviation (SD). For each group of mice, the statistical significance of changes in the variables measured after 21 days of treatment was assessed by one-way analysis of variance (ANOVA) with Tukey's test for *post hoc* comparison of the means.

Results

Characterization of β–thalassemia mouse erythrocytes: evidence for oxidant damage

Hematologic and biochemical features of β–thalassemia mice

Table 1 presents the baseline hematologic profile and erythrocyte cation content in wild type and β-thalassemia mice. β–thalassemia mice showed significant reductions in hematocrit, hemoglobin, mean corpuscu-

Table 1. Effects of monoHER administration on hematologic paramaters in wild-type and β-thalassemic mice.

	Wild type		*β-thalassemia*			
	Baseline	*monoHER 500 (21 days)*	*Baseline*	*monoHER 250 (21 days)*	*Baseline*	*MonoHER 500 (21 days)*
Hct (%)	49.9±0.8 (6)	48.6±1.5 (6)	28.6±1.3 (6)§	28.4±0.7 (6)	29.4±2.9 (6)§	29.8±3.0 (6)
Hb (g/dl)	15.8±0.2 (6)	15.7±0.8 (6)	8.9±0.3 (6)§	8.8±0.3(6)	9.0±1.1(6)§	9.1±1.0 (6)
MCV (fL)	47.6±0.7 (6)	47.8±0.4 (6)	36.3±2 (6)§	36.0±0.7(6)	35.4±0.8(6)§	36.4±1.1 (6)
RDW (%)	12.8±0.5 (6)	12.5±0.8(6)	36.3±2 (6)§	34.0±0.6 (6)*	35.0±2.2(6)§	31.7±1.9 (6)*
HDW (g/dL)	1.68±0.04 (6)	1.68±0.02 (6)	4.9±0.1(6)§	4.6±0.1(6)*	4.8±0.1(6)§	4.4±0.2 (6)
Retics ($\times 10^6$ cells/μL)	0.31±0.03 (6)	0.28±0.05(6)	1.6±0.19 (6)§	1.2±0.1(6)*	1.5±0.2 (6)§	1.3±0.23 (6)
MCVr (fL)	59.4±1.8 (6)	56.5±3.5 (6)	56.3±2.4 (6)§	54.0±0.8(6)*	55.5±1.0 (6)§	56.5±1.0 (6)
Cell Na (mmol/kg Hb)	22.9±3.8 (6)	20.7±4.6 (6)	25.9±2.5 (6)	25.6±2.9 (6)	26.7±2.46 (6)	27.4±4.5(6)
Cell K (mmol/kg Hb)	498.3±12.1(6)	491.6±33.4 (6)	414.6±9.6 (6)§	455.6±31.8 (6)*	410.6±20.5 (6)§	507±58 (6)*
D20 (n=6)	1.098±0.001	1.097±0.001	1.103±0.001§	1.101±0.001*	1.104±0.001§	1.101±0.001*
% PS-positive RBC	0.49±0.2 (8)	0.63±0.1 (8)	11.7±0.4 (8)§	1.13±0.5 (8)*	12.1±0.2 (8)§	0.26±0.3 (8)*
Membrane-bound IgG (abs/min)	0.012±0.004 (8)	0.016±0.002 (8)	ND	ND	0.088±0.003 (8)	0.047±0.002 (8)*

*Data are expressed as means ±SD; ()= n of determinations. §p<0.05 β-thalassemia compared to wild-type mice at baseline; *p<0.05 compared with baseline conditions. MonoHER 250: monoHER 250 mg/kg×2/day; monoHER500: monoHER 500 mg/kg/day.*

lar volume (MCV) and increases in red blood cell distribution width (RDW), hemoglobin concentration distribution width (HDW), and total reticulocyte count. D20 values were significantly higher in β-thalassemia mice than in normal controls indicating the presence of a fraction of dehydrated red cells. (Table 1). As in human β-thalassemia, erythrocyte K content was significantly lower in affected mice than in wild type animals, while the red cell Na content was similar. β-thalassemia mice showed a marked increase in serum total bilirubin and LDH, indicating hemolysis and/or ineffective erythropoiesis (Table 2). Decreased serum levels of vitamin E have been previously observed in untransfused human with β-thalassemia intermedia, suggesting that lipid oxidative damage induces a depletion of this antioxidant.[16] Compared with control mice, β-thalassemia mice exhibited a marked reduction in vitamin E levels, both in plasma (1.56±0.25 mg/L vs. 3.93±0.90 mg/L, $p < 0.05$), and in the erythrocyte membrane (14.25±0.23 mg/L vs. 22.61±0.33 mg/L, $p < 0.05$), suggesting marked oxidative stress. Studies in human β-thalassemia red cells have shown that membrane-bound hemichromes and co-clustering of band 3 promote binding of IgG and premature removal of erythrocytes. Membrane- bound IgG were measured in control and in β-thalassemia mouse red cells and were found to be significantly increased in mouse β-thalassemia erythrocytes compared to erythrocytes from control animals (0.088±0.003 abs/min vs. 0.012±0.004 abs/min, $p<0.05$).

PS exposure in normal and β-thalassemia mouse erythrocytes

Figure 1 shows the gating strategy for analysis of the annexin-V labeling of mouse erythrocytes. The erythrocyte population was selected based on the analysis of forward scatter, and the expression of TER-119 marker. Cells that correlated with the specific forward scatter (set to include microcytic thalassemic red cells) and were positive for TER-119 (gate R1) were further analyzed for annexin V binding. As shown in Table 1, the percentage of red cells exposing PS (annexin-V positive) was significantly higher in β-thalassemia mice than in wild-type mice (11.7±5.2% vs. 0.49+0.2%, n=8, $p < 0.05$), indicating a significant loss of phospholipid asymmetry, similar to that observed in human β-thalassemia and sickle cell disease.[7] In human β-tha-

Table 2. Effects of monoHER administration on biochemical parameters in wild-type and β-thalassemic mice.

	Wild type		*β-thalassemia*		
	Baseline	*monoHER 500 (21 days)*	*Baseline*	*monoHER 250 (21 days)*	*monoHER 250 (21 days)*
Total bilirubin (mg/dL)	0.2±0.06 (6)	0.1±0.06 (6)	0.7±0.2(6)§	0.4±0.04 (6)*	0.2±0.00 (6)*
Plasma LDH (U/L)	376.2±45.4 (6)	347.5±74.8 (6)	524.6±97.3(6)§	403±45.5 (6)*	341.1±43.2 (6)*
Plasma iron (μg/dL)	155.3±3.26 (6)	157.5±12.5 (6)	128.3±15.7 (6)	101.8±13.9 (6)*	109.3±11.2 (6)*

*Data are presented as mean± SD; ()=n of animals. §p<0.05; β-thalassemia compared to wild-type mice; *<0.05 compared to baseline conditions. MonoHER 250: monoHER 250 mg/kg twice a day; monoHER 500: monoHER 500 mg/kg once a day.*

lassemia, the proportion of PS-positive erythrocytes varies from patient to patient and the PS appears to be either distributed over the entire cell membrane or localized in areas with abundant deposits of α-globin chains.[7] The pattern of PS positivity observed in β-thalassemia mice exhibited a shift of the entire population of red cells toward increased annexin V binding, rather than the appearance of a distinct fraction of PS-positive cells on a background of PS-negative cells (Figures 1 and 3). This was not due to autofluorescence, since β thalassemic mouse erythrocytes without annexin V staining showed minimal fluorescence, accounting for a maximum of 0.2% of their annexin V positivity (*data not shown*). In addition to the uniform shift in annexin V positivity, the blood of β-thalassemia mice contained a subset of erythrocytes with very high annexin V binding, suggesting a gradation of PS externalization and oxidative damage, from low-moderate to high, with most cells exhibiting some degree of PS externalization.

Characterization of K-Cl co-transport in β-thalassemia mouse red cells

K-Cl co-transport activation and cell dehydration are characteristic features of erythrocytes in human β-thalassemia.[14] K-Cl co-transport activation can be reproduced *in vitro* in normal human erythrocytes with agents that mimic the oxidative damage seen in α and β-thalassemia.[13,14] As shown in Figure 2, β-thalassemia mouse erythrocytes exhibited a marked upregulation of K-Cl co-transport. K efflux mediated by this transporter was greatly increased in both isotonic and hypotonic conditions and was inhibited by the protein phosphatase inhibitor okadaic acid and stimulated by the protein kinase inhibitor staurosporine. Interestingly, only limited inhibition of K-Cl co-transport was observed when β-thalassemia erythrocytes were treated *in vitro* with 10 mM DTT (Figure 2), suggesting that reticulocytosis and the presence of younger cells play a significant role in the elevation of K-Cl co-transport, or that the oxidation-induced damage is not fully reversible *in vitro*.

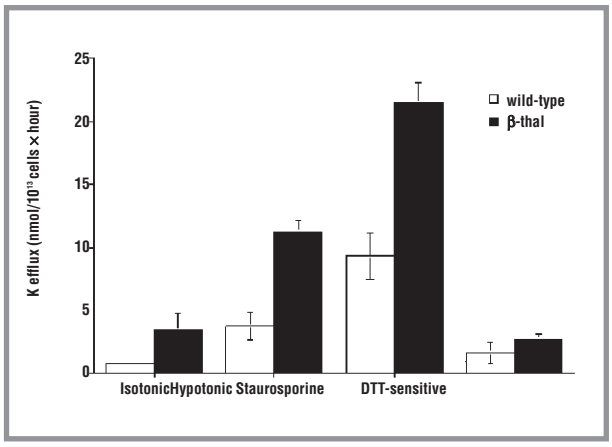


Figure 2. Functional characterization of K-Cl co-transport in β-thalassemia mouse erythrocytes. K-Cl co-transport was measured as okadaic acid-sensitive (100 nM) efflux in isotonic medium, as chloride-dependent K efflux in hypotonic conditions, and as K efflux in isotonic conditions stimulated by the presence of 2.5-μM staurosporine. The sensitivity of chloride-dependent K efflux to DTT treatment (10 mM preincubation, 1 mM in flux media) was assessed in hypotonic conditions. With ANOVA, significant differences between β-thalassemia and control mice were found for the hypotonic flux (p=0.0021), the staurosporine-stimulated flux (p=0.0031) and for the effect of DTT on hypotonic flux (p=0.0019).

Effects of in vivo administration of the antioxidant monoHER

Hematologic and biochemical effects of monoHER treatment on wild type and β-thalassemia mice

MonoHER was administered daily for 21 days: wild type mice were treated with 500 mg/kg i.p. once a day, while β-thalassemia mice were divided into two groups, one treated with 250 mg/kg i.p. twice a day and the other one with 500 mg/kg i.p., once a day. Table 1 presents the effects of monoHER treatment on the hematologic parameters of the wild type and β-thalassemia mice. No statistically significant changes were observed in either Hct or Hb levels with monoHER treatment. The reticulocyte count was significantly reduced in the β-thalassemia mice treated with monoHER at 250 mg/kg twice a day, while it showed a non-statistically significant trend for reduction in the β-thalassemia mice treated with 500 mg/kg once a day. HDW and RDW showed a significant decrease with monoHER treatment, most likely as a consequence of the reduced reticulocyte count and decreased dehydration (*see below*). Erythrocyte Na content was unchanged, while erythrocyte K content was significantly increased after 21 days of treatment with monoHER (Table 1). The improved hydration state of the β-thalassemia erythrocytes was associated with a reduction in dense cells, as estimated by the D20, which identifies the density value cut-off for the 20% most dense erythrocyte fraction (Table 1). MonoHER therapy resulted in a statistically significant decrease in total bilirubin and in plasma LDH in β-thalassemia mice, suggesting an improvement of hemolysis and/or reduction in ineffective erythropoiesis (Table 2). No significant changes in either hematological or biochemical parameters were noted in the wild type mice treated with monoHER at 500 mg/kg once a day (Tables 1 and 2).

Effects of monoHER treatment on membrane and plasma indicators of oxidative stress

In β-thalassemia mice, monoHER treatment caused a marked reduction in the percentage of PS-exposing ery-

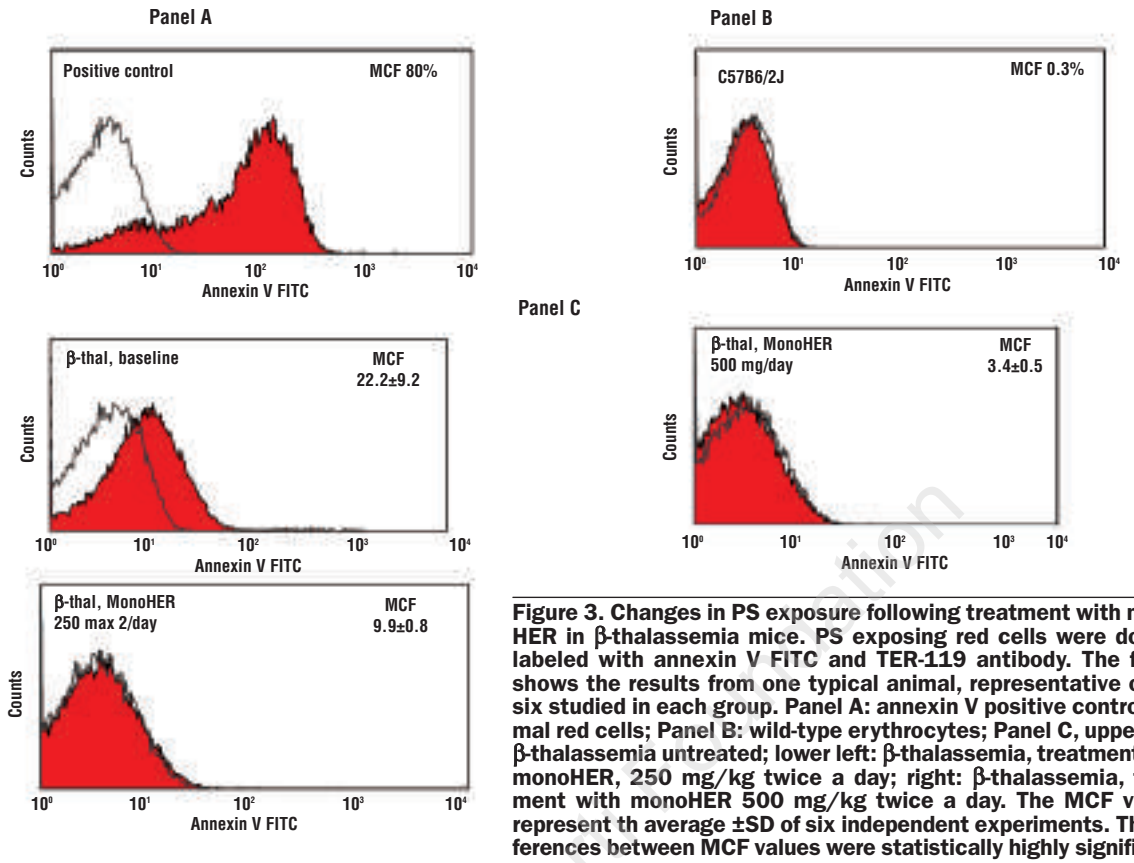


Figure 3. Changes in PS exposure following treatment with monoHER in β-thalassemia mice. PS exposing red cells were double-labeled with annexin V FITC and TER-119 antibody. The figure shows the results from one typical animal, representative of the six studied in each group. Panel A: annexin V positive control normal red cells; Panel B: wild-type erythrocytes; Panel C, upper left: β-thalassemia untreated; lower left: β-thalassemia, treatment with monoHER, 250 mg/kg twice a day; right: β-thalassemia, treatment with monoHER 500 mg/kg twice a day. The MCF values represent th average ±SD of six independent experiments. The differences between MCF values were statistically highly significant.

throcytes, to values essentially similar to those of wild-type control mice (Table 1 and Figure 3). The mean channel fluorescence (MCF) of untreated, PS-positive erythrocytes (mean±SD) was 22±9 and decreased significantly to 9.9±0.8 ($p<0.005$) and 3.4±0.1 ($p<0.0005$) after treatment with monoHER 250 mg/kg/twice a day and 500 mg/kg/day, respectively. Similarly, the median fluorescence (MF) changed from 15.5±0.5 before treatment to 7.1±0.6 ($p<0.005$) and 3±0.1 ($p<0.0005$) after treatment with monoHER 250 mg/kg/twice a day and 500 mg/kg/day, respectively. The subset of erythrocytes with very high annexin V positivity also disappeared with monoHER treatment (Figure 3). Control experiments showed that addition of monoHER to annexin V positive control cells did not affect annexin V binding, ruling out a direct effect of this compound on the binding of annexin V to β-thalassemia erythrocytes (*data not shown*). No changes in the percentage of PS-exposing red cells were detectable in the treated wild type group (Table 1). Plasma and the red cell membrane vitamin E levels were markedly lower at baseline in β-thalassemia mice than in wild type ones, indicating the presence of severe membrane oxidative stress. MonoHER treatment resulted in a marked increase in vitamin E in plasma (Figure 4A) and in erythrocyte membrane (Figure 4B), to values close to those observed in wild-type mice (Figures 4A-B). No changes in plasma and in membrane vitamin E levels were detectable in wild-type mice treated with monoHER (Figures 4A-B). MonoHER treatment caused a marked reduction in β-thalassemia membrane-bound IgG, while no significant changes were observed in treated control mice (Table 1).

Effects of monoHER treatment on erythrocyte morphology and liver-spleen histopathology

Transmission electron micrographs of peripheral blood demonstrated no difference in red cell membrane ultrastructure or the frequency or quality of intracellular globin precipitates after monoHER treatment. The frequency of erythrocytes containing residual organelles and/or abundant ribosomes appeared decreased in the treated β-thalassemia mice, compatible with a decrease in reticulocyte count. Histopathological examination of liver sections of control and treated mice revealed no discernible difference in the extent of hepatic extramedullary hematopoiesis or significant difference in either the degree or the distribution of iron staining (Küpffer cells vs. hepatocytes). There was no light microscopic evidence of toxic hepatocellular injury in the treated groups. Treated and untreated animals also had similar histological features in the spleen, including comparable amounts of erythropoiesis in the red pulp

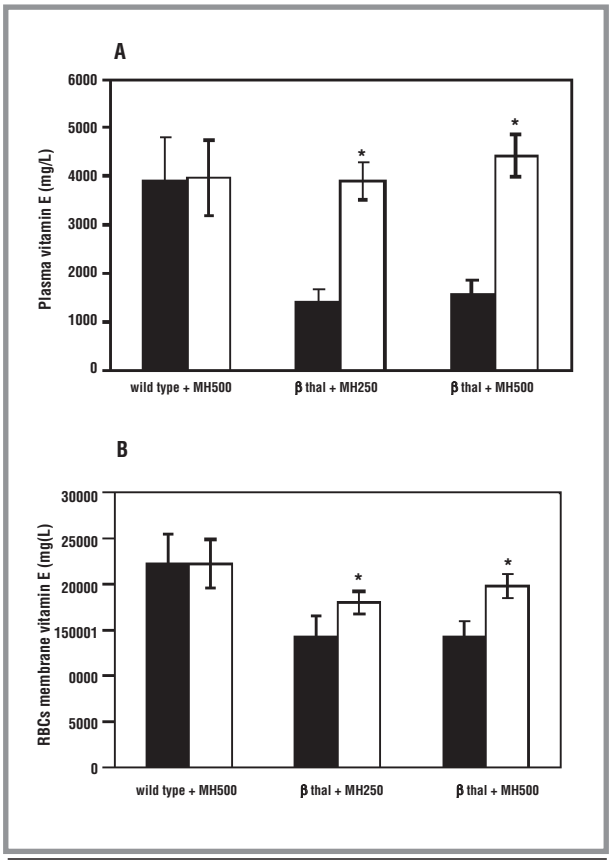


Figure 4. Effects of monoHER treatment on plasma and membrane vitamin E levels in wild type and β-thalassemia mouse strains. Plasma (A) and erythrocyte membrane (B) vitamin E levels at baseline and after 21 days of treatment in wild-type mice treated with monoHER 500 mg/kg once a day (n=6), in β-thalassemia mice treated with monoHER 500 mg/kg once a day (n=6) and in β-thalassemia mice treated with monoHER 250 mg/kg twice a day (n=6). Data are presented as mean ± SD of *n* experiments; **p*<0.05, β-thalassemia mice baseline vs. 21 days of treatment; *p*<0.05, β-thalassemia vs. wild-type at baseline.

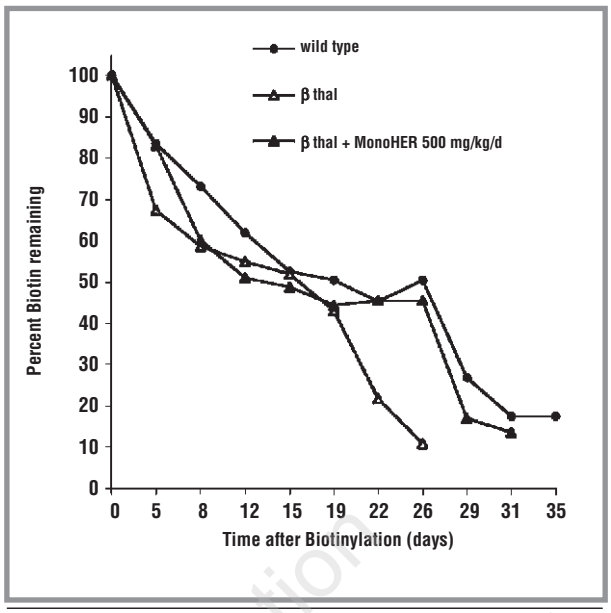


Figure 5. Red cell survival in wild-type, untreated β-thalassemia and monoHER-treated β-thalassemia mice. NHS-biotin was used for *in vivo* labeling of red cells, and streptavidin-PE was used for flow-cytometric quantification of circulating biotin-labeled cells. T_{50} was 23.3±2.5 days in wild-type mice (n=5), 13.2±2.8 days in β-thalassemia mice (n=4) and 13.6±3.9 days in β-thalassemia monoHER-treated mice (n=4; *p*< 0.05 β-thalassemia vs. wild-type mice). T_{20} was 22.7±2.9 days in β-thal mice (n=4) and 27.5±1.2 days in β-thalassemia monoHER treated mice (n=4, *p*<0.05). The percentage of biotinylation is shown for a representative mouse for each group. Essentially similar results were obtained in three additional mice for each group.

and macrophage iron staining. MonoHER treatment did not produce significant changes in tissue iron content: iron contents of liver and spleen (mean±SD) were 249±109 μg/g and 1,644±354 μg/g, respectively in untreated β-thalassemia mice. In β-thalassemia mice treated with 500 mg monoHER /kg/day, liver and spleen iron contents were 231±104 μg/g and 1,743±392 μg/g, respectively. In β-thalassemia mice treated with 250 mg monoHER /kg/twice daily, liver and spleen iron contents were 199±67 μg/g and 1,659±394 μg/g, respectively.

Effect of monoHER treatment on β-thalassemia mouse erythrocyte survival and erythropoiesis

As shown in Figure 5, red cells from β-thalassemia mice had a significantly ($p < 0.006$) shorter half-life (13.2±2.8 days) than those from normal controls (23.3±2.5 days). MonoHER (500 mg/kg/day i.p.) was administered to β-thalassemia mice and after 18 days of therapy, red cells were labeled with biotin. Administration of monoHER continued throughout for a total of 54 days. MonoHER treatment did not improve the β-thalassemia red cell half-life (Figure 5, red cell half-life 13.6±3.9 days vs. 13.2±2.8 days in untreated β-thalassemia mice). However, a significant improvement was seen in the survival of the remaining 20% of the cells, which lasted 27.5±1.2 days in monoHER-treated mice vs. 22.7±2.9 days in untreated β-thalassemia mice ($p < 0.05$). As in the previous experiments, monoHER administration caused a significant decrease in the reticulocyte count (baseline: 1.361±0.9 cells×10^6/mL, n=6 vs. monoHER, day 54 0.82±0.29 cells×10^6/mL, n=6, $p < 0.05$), in conjunction with small but significant reductions in HDW and in RDW (*data not shown*). The percentage of annexin-V positive erythrocytes significantly decreased in the β-thalassemia treated group compared to in untreated β-thalassemia mice (untreated: 10.1±4.2 %, n=15; monoHER, day 54 4.5±2.8%, n=6, $p < 0.0001$). No significant changes in Hct, Hb, MCV, MCHC, reticulocyte parameters such as MCVr, RDWr, HDWr, and spleen weights were detected with 54 days

of monoHER treatment (*data not shown*). Histological analysis of liver and spleen of these animals showed no significant decrease in the extent of splenic or hepatic extramedullary hematopoiesis. Several animals, however, had geographic subacute infarcts of the peripheral portions of the liver that could reasonably be attributed to the trauma of 54 days of chronic intraperitoneal injections. Staining of marrow preparations for the cell-surface erythroid-specific antigen TER-119, and for transferrin receptor (CD71), allows studies of erythroblasts maturation.[28] Based on CD71 positivity, different subpopulations of maturing erythroblasts can be identified, with R4 being the most immature and R6 the most mature (Figure 6). Control mice show that approximately 50% of the cells are in the immature R4 pool, and 20% are in the mature R6 pool. MonoHER treatment induced a significant increase in the R4 pools [from 73±6.4 (n=4) to 84±3.6(n=6), $p < 0.05$)] and significant decreases in the R5 [from 13.3±4.7 (n=4) to 4.0±0.92 (n=6), $p < 0.05$)] and R6 pool [from 8.8±2.6 (n=4) to 4.3±1.1 (n=6), $p < 0.05$)]. These changes could be interpreted as a reduction of inefficient erythropoiesis at the R5 and R6 stages. However, it is also possible that the predominance of the most immature erythroid precursors and the paucity of later forms result from increased premature death of maturing precursors.

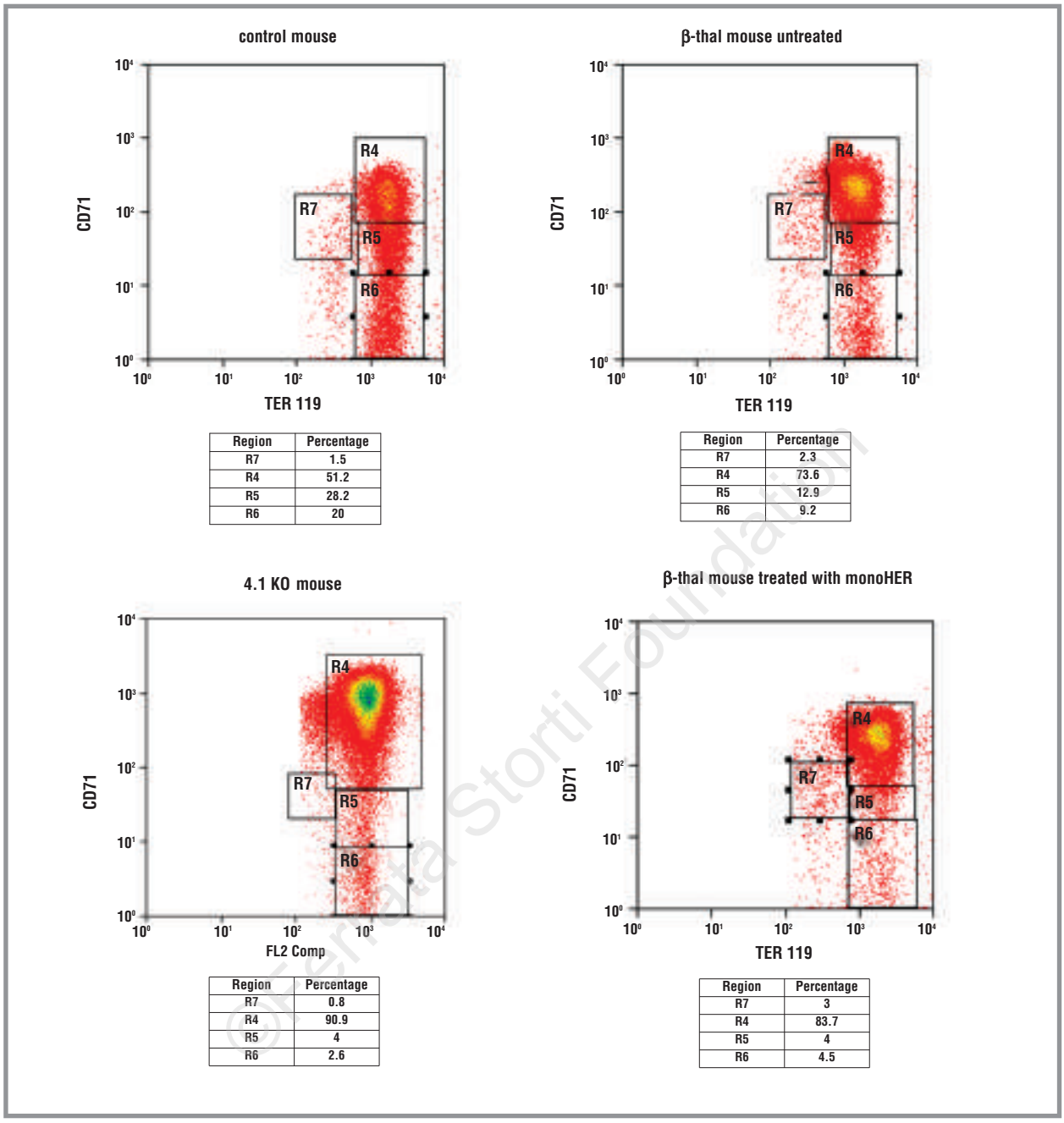
Discussion

We have characterized the red cell features of a mouse model for β-thalassemia, which closely resembles human β-thalassemia intermedia.[22] β-thalassemia mice had dehydrated erythrocytes, decreased erythrocyte K content and increased activity of K-Cl co-transport (Table 1, Figure 2). Activation of K-Cl cotransport was clearly present at baseline in isotonic conditions and became more evident with hypotonic challenge or pretreatment with the protein kinase inhibitor staurosporine (Figure 2). K-Cl co-transport activation by oxidative damage has been demonstrated *in vitro* with a variety of agents known to induce oxidative damage to the red cell membrane.[13,36-39] K-Cl co-transport activation due to oxidative membrane damage was also demonstrated in the anemia associated with ribavarin therapy.[40] The functional up-regulation of K-Cl co-transport seen in β-thalassemia mouse erythrocytes differs from that seen *in vitro* with human β-thalassemia erythrocytes, since there was little effect of *in vitro* DTT treatment of mouse erythrocytes on K-Cl co-transport activity, while a significant effect had been described in the human counterpart.[14] This may indicate an effect of reticulocytosis and cell age or irreversibility of some of these effects *in vitro*. β-thalassemia mice showed marked deficiency in both erythrocyte membrane and plasma levels of vitamin E (Figure 4). In the human counterpart, membrane and plasma lipid peroxidative damage results in marked depletion of lipid antioxidants, such as vitamin E and A.[16,41,42] Vitamin E depletion enhances the susceptibility of membrane and plasma lipids to oxidative damage.[42]

Significant structural alterations of β-thalassemia erythrocyte membranes, related to oxidative damage, were demonstrated by the marked annexin V positivity, a marker of loss of red cell membrane asymmetry (Figure 3). The annexin V positivity of erythrocytes in this mouse model differs from that of the human counterpart: in human β-thalassemia (and in sickle cell disease), annexin V positivity seems to be limited to a defined subpopulation of erythrocytes.[7,11] In this β-thalassemia mouse model, annexin V positivity was more uniformly distributed and appeared to reflect a shift of the entire red cell population (Figure 3). This difference may be related to the more homogeneous genetic background of this mouse model and/or to the fact that, contrary to most of the human patients, these mice were not splenectomized.

In human β-thalassemia, membrane clustering of hemichromes and band 3 induced by Hb denaturation results in deposition of IgG and removal by macrophages.[8,9,43,44] Similarly to the human counterpart, β-thalassemia mouse erythrocytes showed increased IgG membrane binding (Table 1). The presence of membrane oxidative damage in human β-thalassemia provides the rationale for testing anti-oxidant therapies.[2,45,46]

A pilot trial with large doses of oral vitamin E, prompted by the abnormally low levels of this vitamin in plasma of patients with β-thalassemia intermedia, showed a decrease in the levels of malonylaldehyde, but not in transfusion requirements.[16] Rutin, a quercetin rutoside member of the flavonoid family, prevented primaquine-induced methemoglobin formation *in vitro*, but could not restore normal hemoglobin from pre-formed methemoglobin.[17] The polyphenol curcumin caused a significant inhibition of lipid peroxidation in β-thalassemic red cell ghosts.[47] Tea polyphenols have shown antioxidant properties *in vitro*.[18] N-allylsecoboldine seemed to protect β-thalassemia erythrocytes from peroxyl, hydroxyl radicals and H_2O_2 induced damage, resulting in decreased hemolysis and lipid peroxidation *in vitro*.[48] Semi-synthetic flavonoids, such as monoHER and related compounds have been reported to be powerful *in vitro* antioxidants and to prevent doxorubicin cardiotoxicity *in vivo* in mice.[20,21,49-51] MonoHER and related compounds possess both iron chelating and radical scavenging properties, although iron chelation does not seem to play a role in their antioxidant activity, at least *in vitro*.[52] MonoHER treatment of β-thalassemia mice resulted in substantial improvements in several parameters related to membrane oxidative stress and lipid peroxi-



Region	Percentage
R7	1.5
R4	51.2
R5	28.2
R6	20

Region	Percentage
R7	2.3
R4	73.6
R5	12.9
R6	9.2

Region	Percentage
R7	0.8
R4	90.9
R5	4
R6	2.6

Region	Percentage
R7	3
R4	83.7
R5	4
R6	4.5

Figure 6. Changes in erythroblast maturity with monoHER treatment in β-thalassemic mice. Bone marrow cells were analyzed with flow cytometry for positivity for TER119 and CD71 labels, as detailed in ref. #28. The relative percentage of nucleated erythroid cells in each of the regions of the density plots is indicated below each plot for four representative animals. Upper left: control mouse; lower left: protein 4.1 deficient mouse;[61] upper right: untreated β-thalassemia mouse; lower right: β-thalassemia mouse treated with monoHER. A significant increase in the R4 pool [from 73±6.4 (n=4) to 84±3.6 (n=6), $p < 0.05$)] and significant decreases in the R5 [from 13.3±4.7 (n=4) to 4.0±0.92 (n=6), $p < 0.05$)] and R6 pools [from 8.8±2.6 (n=4) to 4.3±1.1 (n=6), $p < 0.05$)] were observed with monoHER treatment.

dation: (i) vitamin E levels in both plasma and erythrocyte membrane rose from abnormally low to values close to those of normal control mice (Figures 4A-B); (ii) the percentage of annexin V positive cells decreased markedly to levels close to those of normal controls (Figure 3); (iii) the abnormally high membrane binding of IgG diminished toward normal values (Table 1). Although we did not measure K-Cl co-transport activity in monoHER-treated β-thalassemia mice, the increased erythrocyte K content (Table 1) and improved cell hydration (Table 1) indicate that monoHER therapy affected ion transport and volume control of β-thalassemia erythro-

cytes. It has been elegantly shown in an *in vitro* system that the ability of flavonoids to reduce free radical-stimulated erythrocyte K loss is strictly associated with their ability to prevent membrane lipid peroxidation.[53] The fact that the average values for MCV and other red cell indices did not change with monoHER therapy can be explained by the simultaneous changes in dense cell and reticulocyte fractions (Table 1). A recent report in a different β-thalassemia mouse model suggested that ineffective erythropoiesis may play a dominant role in the pathogenesis of anemia.[54] Although there were laboratory signs of decreased hemolysis or reduced ineffective erythropoiesis (reduced serum bilirubin, LDH, and absolute reticulocyte counts), no significant changes in Hct or Hb were seen in β-thalassemic mice treated with monoHER (Tables 1 and 2). Red cell survival showed an improvement with monoHER treatment (Figure 5). However, this improvement was limited to a fraction of cells (approximately 20%), while the half-life of the entire population was unchanged. The duration of our treatment was limited by the fact that prolonged use of the intraperitoneal route is associated with local and systemic complications; longer treatment might have resulted in improvement of the anemia. In addition, we observed a discrepancy between the apparent reduction in ineffective erythropoiesis observed in the blood stream and the change toward a more immature erythroblast population in the bone marrow (Figure 6). This raises the possibility that some small negative effects of monoHER on erythroblasts may have limited the benefit associated with the increased survival of at least one fraction of cells.

It remains to be determined how the encouraging results obtained with monoHER in β-thalassemia mice can be applied to human β-thalassemia. Since the human disease exhibits a much more substantial reduction in red cell survival, it is possible that a reversal of the membrane abnormalities induced by oxidant damage could improve anemia. The reversal of lipid peroxidation and the reduction of PS positive red cells induced by monoHER may be clinically relevant for the hypercoagulable state recently described in human β-thalassemia.[55,56] The *in vivo* reversal of oxidant damage observed here may be relevant not only for thalassemias, but also for other anemias characterized by membrane oxidative damage, such as sickle cell anemia. The role of oxidation in the pathophysiology of sickle cell disease[57] and the evidence of reduced antioxidant defenses in patients with sickle cell disease[58-60] suggest that antioxidant therapy should be considered. Recently, studies with a different class of antioxidants which possess catalytic superoxide dismutase and catalase activities, have shown partial correction of anemia and hemolysis in a mouse model with absent mitochondrial superoxide dismutase and oxidative stress-induced hemolytic anemia.[33] The *in vivo* efficacy of monoHER in preventing oxidative stress and lipid peroxidation should prompt consideration of its use in several hematologic disorders characterized by oxidative damage to the erythrocyte.

LDF, CB: conception and design, analysis and interpretation of data, drafting the article; FT, MH, KA, AR, MDF, TL, FM: analysis and interpretation of the data; FAK, AB, WJFvdV: design, interpretation of data, drafting of the article. The authors reported no potential conflicts of interest.

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