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A TRANSGENIC MOUSE MODEL EXPRESSING EXCLUSIVELY HUMAN HEMOGLOBIN E: INDICATIONS OF A MILD OXIDATIVE STRESS

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Abstract

Hemoglobin (Hb) E (β26 Glu→ Lys) is the most common abnormal hemoglobin (Hb) variant in the world. Homozygotes for HbE are mildly thalassemic as a result of the alternate splice mutation and present with a benign clinical picture (microcytic and mildly anemic) with rare clinical symptoms. Given that the human red blood cell (RBC) contains both HbE and excess α-chains along with minor hemoglobins, the consequence of HbE alone on RBC pathophysiology has not been elucidated. This becomes critical for the highly morbid β^E-thalassemia disease. We have generated transgenic mice exclusively expressing human HbE (HbEKO) that exhibit the known aberrant splicing of β^E globin mRNA, but are essentially non-thalassemic as demonstrated by RBC α/β (human) globin chain synthesis. These mice exhibit hematological characteristics similar to presentations in human EE individuals: microcytic RBC with low MCV and MCH but normal MCHC; target RBC; mild anemia with low Hb, HCT and mildly elevated reticulocyte levels and decreased osmotic fragility, indicating altered RBC surface area to volume ratio. These alterations are correlated with a mild RBC oxidative stress indicated by enhanced membrane lipid peroxidation, elevated zinc protoporphyrin levels, and by small but significant changes in cardiac function. The C57 (background) mouse and full KO mouse models expressing HbE with the presence of HbS or HbA are used as controls. In select cases, the HbA full KO mouse model is compared but found to be limited due to its RBC thalassemic characteristics. Since the HbEKO mouse RBC lacks an abundance of excess α-chains that would approximate a mouse thalassemia

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(or a human thalassemia), the results indicate that the observed in vivo RBC mild oxidative stress arises, at least in part, from the molecular consequences of the HbE mutation.

Keywords

Hemoglobin E; transgenic mouse model; β-thalassemia; red blood cells; oxidative stress; cardiac function

INTRODUCTION

Hemoglobin E [HbE, β26 (Glu→Lys)] is the most common Hb variant worldwide, with the greatest frequency in Southeast Asia. Given the wave of migration to North America, the HbE carrier frequency is almost equal to that of the HbS gene carrier [1; 2]. The β^E mutation, located at exon 1 of the β globin gene close to the intervening sequence of the normal mRNA splicing site, results in an alternative β-globin mRNA splice. The consequence is a reduction of β^E mRNA that eventually leads to less β^E globin synthesis [3; 4] and consequently excess α-globin chains. Hence, the HbE mutation results in both a mild thalassemia and a Hb structural change; and also confers the adaptive advantage of resistance to malaria [5; 6; 7; 8; 9].

HbE is structurally unstable as shown in vitro [10; 11; 12]. The instability is usually explained by the location of the single amino acid substitution [β26 (Glu→Lys)] at the α 1β1 interface. Recent high resolution crystallographic data shows the β26 Lys substitution disrupts the α1β1 interface hydrogen bond and imparts a charge repulsion [13; 14; 15; 16]. This would explain in vitro observations of increased HbE dissociation to dimers and monomers, with a heightened propensity to form hemichromes that could explain the reported oxidative damage to the red blood cell (RBC) membrane and membrane components [12; 17; 18; 19; 20; 21; 22].

Yet, to date, there is no evidence that the characteristic *in vitro* instability of HbE gives rise to *in vivo* red cell consequences or pathophysiology. A study of HbE/β thal human RBC reported that the thalassemic nature of both HbE and β thal is the basis for the RBC alterations; but HbE instability is indicated with mild oxidative stress, globin chain synthesis at higher than body temperature (39°C and 41°C, and possibly in HbE patients with fevers [23]. Since it is established that β-thalassemic RBC with excess α-chains give rise to damaging oxidative species (e.g, [24; 25; 26]), it becomes experimentally challenging to determine the origin of reactive species in a RBC with both excess α-chains in the presence of HbE.

The question of the potential role of oxidative damage by HbE becomes further complicated when considering the varying clinical presentations of HbE diseases [9; 27; 28]. Homozygous HbE disease is benign with a mild hypochromic microcytosis, target cells, and minimal anemia with reduced RBC survival [29; 30]. HbE/β^0-thalassemia presents a panoply of phenotypes from very severe to a mild $\beta^{/+}$ thalassemia [31]. This is surprising since either one allele for HbE (e.g., the trait) or one allele for β^0-thal trait results in benign clinical conditions. Controversy remains as to whether the altered RBC indices in HbE related disorders are due to unstable HbE or to the excess α-chain oxidative stress within the RBC, or both [12; 23]. Only recently have transgenic mice expressing HbE been generated in attempts to probe this issue [32; 33]. Using an extraction technique, RBC membranes derived from transgenic mice containing human HbE or HbS or HbC in the presence of Hb Mouse single (HbM^{single}) exhibit enhanced membrane lipid peroxidation correlated with enhanced binding of HbE, HbS, and HbC to the mouse RBC membrane [22]. These results

are consistent with the known enhanced mutant hemoglobin binding to the human RBC membrane in the order HbE>HbC>HbS>HbA and correlations of oxidative stress [17; 34; 35; 36]. Thus, the enhanced binding of HbE to the RBC membrane and its observed consequences points to the relevance and potential role of RBC oxidative stress in the pathophysiology of HbE diseases.

In the present study, transgenic mice expressing *exclusively* human HbE [human $\alpha_2\beta^E{}_2$] [i.e., full mouse globin knockout, (KO)] have been generated with a closely balanced α:β globin chain synthesis ratio. Of note in this model is that the RBC is without mouse globins and without human HbF or HbA_2 or other minor human hemoglobins. Thus, this HbEKO transgenic mouse model, uniquely lacking a significant excess of α-chains, provides a unique opportunity to assess RBC oxidative stress and membrane damage concomitant with the sole presence of HbE. Hematological analyses (RBC smears, density gradient, RBC index measurements) show RBC parameters that approach the human HbE disease counterparts. A mild oxidative stress is indicated in these HbEKO mouse RBC. The C57 (background) mouse and full KO mice models expressing HbE and HbS or HbA are used as controls. In selective cases, the HbA full KO mouse model is compared but found to be limited due to its RBC thalassemic characteristics [37] [38].

To the best of our knowledge, this is the first transgenic HbE mouse model with RBC expressing solely human HbE *without a significant* excess of α-chains and without mouse globins. This mouse model provides the opportunity to directly assess RBC alterations correlated with the sole presence of HbE. The data indicate that the HbE mutation gives rise to a mild oxidative stress with minimal cardiac dysfunction. In addition, this mouse model provides the further advantage of a HbE RBC without human minor hemoglobins or mouse globin chains that permits the isolation of pure HbE needed for in vitro studies without contamination by HbA_2 (a hemoglobin that co-migrates with HbE in cation and anion exchange chromatographic separations) studies [13; 14; 15; 39].

MATERIALS AND METHODS

Full KO HbE, HbAE, HbSE transgenic mice were generated by further breeding the founder and partial KO mice [32]. All of the mice described express cointegrated human α and human β^E and/or β^A or β^S transgenes [32; 37; 40]. Founder mice were backcrossed onto a C57BL/6 (C57) background, 8 or more times. HbAE and HbSE full KO mice were generated by respectively breeding HbE full KO with mice expressing HbA ([37]) or HbS [40]; also known as NY1KO) as partial KOs. The offspring were then bred to HbE full KO mice to obtain the heterozygous KOs (e.g., HbSE full KO and HbAE full KO). Although 3 lines of HbE mice were generated [32], only one line was used to generate HbEKO as well as the HbAE and HbSE mice described in this manuscript. The HbAE full KO express ~58% of HbA and ~42% HbE, while the HbSE full KO mouse express ~63% HbS and 37% HbE. All mice are hemizygous for all transgenes. Knockout status (absence of mouse globins) was determined by both HPLC and IEF. Thal mice, homozygous for the mouse globin β-major deletion [41], and C57BL/6 mice are used as controls. Adult mice used in the experiments ranged in age from 5 months to 16 months.

Mouse blood was drawn from the tail vein into heparinized capillary tubes using a protocol approved by the Animal Study Committee of the Albert Einstein College of Medicine. Whole blood smears were prepared as previously described [32].

RBC indices were obtained on the ADVIA 120 Multispecies Hematology System (Siemens Diagnostics, Deerfield, IL).

Isoelectric focusing (IEF) (Isolab, Akron OH) and high performance liquid chromatography (HPLC) (Shimadzu, Columbia MD) were used to identify the Hb RBC content and quantify the globin chains obtained from RBC hemolysates.

Reticulocyte α:β globin chain synthesis

Globin chain synthesis, an established technique in our laboratory [42; 43] was measured using H^3-leucine incubated with washed RBC according to a modification of the method of Weinberg et al. [44] and as described by Fabry et al. [43]. Briefly, 50 microL of whole blood were incubated as described by Weinberg et al. [44] and the globin chains were separated by reverse phase HPLC as described by [43]. Only two peaks were detected in the HPLC and the counts vs fraction number illustrated in Figure 1B. The HPLC technique used is a potentially more accurate technique than previously employed urea gels due to better resolution.

Reticulocytes and spleen β^E-globin mRNA analysis

Sacrifice and dissection of HbE full KO mice were performed according to the AECOM Animal Institute guidelines. mRNA extraction and RT-PCR analysis were performed using erythrocytes (reticulocytes) and spleen according to methods described in Qiu et al. [45]. β^E-globin mRNA splicing was assessed by methods described by Jamsai et al. [33], Winichagoon et al. [4], and Traeger et al. [3].

RBC osmotic fragility

RBC osmotic fragility was measured by modified protocols described by Lew et al. [46]. A pair of 96-well plates was used: a U-bottom plate for spinning down the unlysed cells and a flat-bottom plate for optical density measurements. Each column of the U-bottom plate contained 250μl solutions per well (a total of 12 wells), with each solution having different osmolarities ranging from 100 to 0 relative tonicity units (lysis media). These were prepared by mixing appropriate volumes of 2 solutions: 2mM Hepes-Na (pH 7.5, 100% lysis) and 10mM Hepes buffer pH 7.5 + NaCl (330mOsm, 0% lysis).

A 12-channel pipette was used to deliver 5μl of RBC suspensions (5% HCT) to the corresponding row in the U-bottom plate. Each row generates a full hemolysis curve. The plate was centrifuged for 5 min at 1100 RPM and 150μL of supernatant was transferred with a 12-channel pipette to the corresponding row in the flat bottom plate. Hb concentration was measured by its absorption at the Soret band (using 414nm), and used to calculate the percent lysis of RBC.

RBC density gradient

Percoll-Stractan density gradients were prepared as described by Fabry et al. [47].

Isopropanol precipitation test of the transgenic mouse hemolysates

The isopropanol precipitation of the hemolysate was performed as described by [48]. The hemolysates (0.62mM heme) were incubated at 37°C in 17% isopropanol (pH 7.35 0.1M Tris-HCl buffer). At 30min intervals, the hemolysates were centrifuged and the absorption of the supernatant at 578nm was measured.

RBC membrane lipid peroxidation

A Becton Dickinson FACS Scan was used to measure RBC membrane peroxidation by peroxy radicals and hydroperoxy species as described by Maulik et al., [49; 50]. Washed RBCs (~ 10^6 counts) were incubated with Fluorescein-dihexadecanoyl-phosphatidylethanolamine (Fluor-DHPE, Molecular Probes, Eugene, OR) at a final

concentration of 50μM at 37°C for 1hour. After further washing to remove any free probe, RBCs with or without 2mM H_2O_2 were then incubated at room temperature for 1–2 hr before flow cytometric measurements.

Phosphatidylserine (PS) exposure using flow cytometry

RBC membrane PS exposure was measured using fluorescein labeled annexin V as described by [51].

Reactive oxygen species (ROS) Levels

Intracellular RBC ROS were detected by flow cytometry using a membrane permeable fluorescein derivative, 2,7 dichlorodihydrofluorescein diacetate (DCDHF, Cayman Chemicals, Ann Arbor, MI) as described by [52; 53; 54]. After deacetalyation and membrane transport, the probe is oxidized by the intracellular ROS to fluorescent DCF. Briefly, washed RBCs (~ 10^6 counts) were incubated with DCDHF (final concentration 0.4mM) at 37°C for 15 min. After further washing to remove any free probe, RBC with or without 0.5mM H_2O_2 were then incubated at room temperature for 1–2 hours followed by flow cytometry.

Fluorescence determination of hemolysate zinc protoporphyrin levels

Hemolysate ZPP, PPIX, and FHDP levels were measured using the one-step spectroscopic assay described by [55]. A Perkin-Elmer 650-10S spectrofluorometer with right angle optics was used to record the fluorescence spectra of FHDP (ex. 321nm, em. 466nm), PPIX (ex. 365nm, em.626nm) and ZPP (ex 365nm, em. 594nm) in hemolysates diluted and matched at 50μM heme using pH 7.35 potassium phosphate buffer. A 10nm excitation and emission slit width, sensitivity gain 10 was used.

RBC membrane associated free iron

Membrane free iron was determined by its reactivity with ferrozine in the presence of a membrane solubilizing agent sodium dodecyl sulfate (SDS) and reducing agents such as ascorbic acid and sodium metabisulfite according to the established methods [56]. Briefly, washed RBCs were lysed by a large volume (40:1 buffer: packed RBC) of ice-cold 5mM phosphate buffer (pH 8.0,) and washed by the same cold buffer until white ghosts were obtained. Solubilized membranes (in 1% SDS) were incubated with 0.02% ascorbic acid for 5min followed by addition of ferrozine and neocuproin (to eliminate the contribution of copper interference) to initiate the colorimetric reaction (λmax=562nm). The absorption of colored iron-ferrozine complex was measured by a Perkin-Elmer Lamda 20 spectrophotometer.

RBC antioxidant enzyme activities and glutathione levels

RBC catalase activity was determined based on the decomposition of H_2O_2 by hemolysate catalase [57]. The enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were determined by SOD and GSH-PX assay kits (Cayman Chemicals, Ann Arbor, MI).

Organ weights and tissues

Organs were washed in isotonic saline, dried, and weighed. Tissues were preserved in 10% buffered formalin. 2–3 thin histological sections were stained with hematoxylin and eosin. Organ weight comparisons were determined by percent total body weight.

Mouse echocardiograms

The mouse echocardiograms were recorded by high resolution ultransography (echo) units and high-speed high fidelity digital data acquisition systems (Vevo 770, Visual Sonics, Toronto, Canada). These systems provide noninvasive, real-time visualization of cardiac structures at a spatial resolution of approximately 30 μm. These systems are equipped with a 40 MHx ultrasonic scan head and accessorized with a rail and platform system, which allows for stable positioning of the mouse with respect to the scan head, an anesthesia system designed to deliver regulated concentrations of isoflurane (1.0%), a thermostatically controlled heating pad, and a means to simultaneously record the electrocardiogram as well as monitor heart rate and body temperature. The anesthesized mouse was placed in the supine position on the rail system and superficial hair on the chest was removed. Echo gel was added to the bare skin. The echocardiograms were performed on adult mice, ages 5–8 months. These protocols are approved by the AECOM Animal Institute.

Quantification of left ventricular wall motion and determination of wall motion abnormalities were performed using both B-mode and M-mode imaging. The echocardiography system is also capable of acquiring Doppler images and the pulsed-wave Doppler modality can be used to determine the velocity and direction of blood flow within a region of interest. *In vivo* transthoracic echocardiography of the left ventricle using a 30 MHz 700-Series scanhead (RMV-707B) capturing 140 frames per second interfaced with a Vevo 770 were performed as described previously [58]. Ventricular parameters were measured using the leading-edge technique. M-mode (sweep speed = 200 mm/sec) echocardiograms were captured from parasternal, short- and long-axis 2D views of the left ventricle at the mid-papillary level. Left ventricular end diastolic diameters (LVEDD), left ventricular end systolic diameters (LVESD), aortic diameter (AoD), aortic velocity time integral (AoVTI), and heart rate (HR) were measured at baseline and following myocardial reperfusion. LV percent fractional shortening (FS) were calculated according to the following equation: LV%FS = ((LVEDD-LVESD)/LVEDD) × 100. Stroke volume (SV) were calculated from the product of the aortic cross-sectional area $((AoD/2)^2 \times \pi)$ and the AoVTI. Cardiac output (CO) will be calculated from the product of the stroke volume and heart rate. The cardiac output values were corrected for the animals' weights (in μL/min/g). All data were calculated from 10 cardiac cycles per experiment.

RESULTS

A non-thalassemic transgenic HbEKO mouse model has been generated on a C57 mouse background. It should be pointed out that the difference between normal hemoglobin genetics and transgene genetics is that transgenes are incorporated into a single site, and, in this HbE model, the site has a random mix of LCRhumanα and LCRhumanβ^E with an unknown ratio of α:β^E genes. Reticulocyte and spleen extractions of β^E-globin mRNA exhibited the expected 16 base pair (bp) difference in RNA splices of 98bp and 82bp (alternative splice) (Figure 1A) that arise in the human mutation and are similar to that described in [33]. Despite the presence of the alternate splice, non-thalassemic ratios of human α:β^E chains are found in the HbEKO RBC as determined by globin chain synthesis in reticulocytes (Table 1, Figure 1A). Identification and quantification of globin chains and the absence of mouse hemoglobins were determined and verified by high performance liquid chromatography (HPLC) (Figure 1B) and isoelectric focusing (IEF) (Figure 1C). Hence, the HbE mouse model is demonstrated to be a full knockout with RBC expression of only human HbE [$\alpha_2\beta^E{}_2$].

Hematological parameters and RBC properties of HbE, HbAE, HbSE, HbA full KO transgenic mouse models and C57 mice

The HbEKO mouse model RBC indices reveal increased RBC RDW and reticulocytes, low MCV and MCH, normal MCHC, increased RBC counts, low HCT and HGB compared to C57, indicative of a mild anemia (Table 2). Interestingly, mice expressing HbS/HbE and HbA/HbE have near normal Hb, HCT, and RBC counts; hence, the presence of HbA or HbS with HbE results in substantial corrections as seen in the red cell indices (Table 2). In contrast to HbSE and HbAE transgenic mouse RBC that exhibit a somewhat decreased MCV compared to C57 ($P<0.008$ and $P< 0.0009$, respectively), the HbEKO mouse RBC exhibit a large reduction in MCV.

Blood smears obtained from HbEKO mice exhibit RBC hypochromia, target cells, stress reticulocytes, and other abnormalities in the order: HbE>HbSE>HbAE>HbA>C57 (Fig 2A). C57 mice serve as controls since the HbEKO transgenic mice are on a C57 mouse background. Although the ideal control might be a HbAKO mouse model, the mildly thalassemic RBC characteristics of the HbAKO RBC and generation by a different transgene exclude it from serving as a proper control. The mildly thalassemic nature of the HbAKO mouse results from the nature of the construct LCRhumanα humanβ^{A}. In this case, unlike the HbEKO mouse construct, the ratio of α :β is 1:1 but since the α gene is closer to the LCR than the β gene, we anticipate that the α-globin chain synthesis will be favored, as indeed is the case. Considering the aforementioned characteristics of the HbAKO mouse model, the C57 background mouse is used as a standard control for the comparisons, with some selective comparisons made with the thal, HbAEKO, HbSEKO, and HbAKO mouse models. Hematological characteristics of the thal mice are reported in [38].

Density gradients of RBC obtained from HbE and HbSE mice show broader distributions compared to the HbAE, HbA, and C57 mice (Figure 2B). Notably, HbAE RBC densities are similar to those of C57 mice, whereas thal mice [38; 41; 59] exhibit the broadest distribution. HbAKO mice exhibit the least dense RBC distribution. Mice expressing HbC show increased RBC density [47]. Since HbC has the same charge and a nearly identical isoelectric point as HbE, the HbE density differences are not a consequence of charge.

The density gradients (Figure 2) show a pattern for HbEKO that does not look like the broad density distribution seen in the mouse model for beta-thalassemia (Figure 2B). We speculate that this is because (1) there is less chain imbalance in the HbEKO mouse, leading to fewer unpaired, disruptive α-chains and a higher final hemoglobin tetramer level and (2) there is more disruption of cation transport in the thal mouse than in the HbEKO mouse (leading to both low and high MCHC cells) because chain synthesis is better balanced in the latter case.

RBC osmotic fragility assesses the resistance of RBC to lyse as a function of decreasing salt (NaCl) concentration (ie., increasing hypotonic environments). This parameter is reflective of the erythrocyte surface area/volume ratio. Clinically, it has been used as an indicator of specific hemoglobinopathies, in particular, to screen for β-thalassemia and HbE Disease [60; 61; 62]. It is therefore, not surprising to find RBC of HbE, HbSE, HbAE full knockout transgenic mice and thal mice exhibiting significantly decreased osmotic fragility (i.e., enhanced osmotic resistance) compared to C57 in the order thal>HbE>HbSE>HbAE transgenic mice > C57 mice (Figure 3B). The relative tonicity, shown in the osmotic fragility curves at the point of 50% lysis [i.e., the median corpuscular fragility (MCF)], shifts to lower tonicities in AE>SE>HbE mouse RBC, indicating an enhanced resistance to osmotic lysis in RBC from mice expressing exclusively (100%) HbE. Furthermore, thal mice exhibit a modestly increased resistance to hypotonic lysis compared to HbE mice. The observed decreased osmotic fragilities in HbE, HbAE and HbSE transgenic mice and/or in

thal mice RBC are consistent with those reported in human counterparts (sickle cell anemia, HbC, iron-deficency anemia, β-thal, and HbE patients, e.g., [62; 63; 64; 65; 66; 67; 68; 69].

Human HbE is an oxidatively unstable Hb variant with an unusual susceptibility to isopropanol precipitation [10]. In order to test that HbE expressed by transgenic mice exhibits structural instability, hemolysates from HbE, HbAE, HbSE mice were incubated in 17% isopropanol (0.1M Tris-HCl, pH 7.35) at 37°C, compared to HbC and C57 mice. As anticipated from that known about the stability of human Hb mutants, hemolysates from transgenic mice exclusively expressing HbE exhibit the greatest precipitation propensity, with HbE>HbSE>HbAE>HbC>C57 (Figure 3B). HbE is more vulnerable to isopropanol precipitation than HbS, although HbS is known to exhibit enhanced surface denaturation and mechanical precipitation. HbC, having the same positive charge as HbE, is significantly more stable than HbE as assessed by the isopropanol precipitation assay.

ZPP levels are elevated about 2-fold in transgenic hemoglobin KO mouse models expressing, respectively, HbE, HbSE, HbAE, and thal mice compared to C57 mice ($P \ll 0.05$) and HbA mice compared to HbE mice ($P<0.02$) (Figure 4A). PPIX levels or free heme degradation products (FHDP) were not significantly different when comparing HbEKO and HbAKO mice (data not shown).

Indications of RBC mild oxidative stress in the HbEKO mouse model

Enhanced RBC membrane associated iron and membrane lipid peroxidation— RBC are highly susceptible to oxidative damage due to the presence of both high concentrations of membrane unsaturated fatty acids and the oxygen carrying Hb, a potent promoter of oxidation [70]. It is likely that the observed elevated levels of ZPP (Figure 4A) and membrane-associated iron (Figure 4B) in the HbE mouse RBC may promote membrane peroxidation. RBC membrane-associated free iron in the HbE mouse RBC is elevated ~ 2-fold (Figure 4B). Free iron refers to non-heme and non-ferritin iron that react within 2-minutes in the ferrozine assay. Multiple forms of iron are found to be associated with the RBC membrane in hemoglobinopathies (e.g., [24; 71]).

A novel fluoresceinated phosphoethanolamine (Fluor-DHPE)-based flow-cytometric method [50; 72] is used to detect membrane lipid peroxidation in the HbE mouse RBC compared to C57. Fluor-DHPE is lipophilic and primarily observed on the outer membrane leaflet. Upon interaction with membrane peroxyl radicals ($ROO^{\bullet}$) or hydroperoxide (ROOH), Fluor-DHPE fluorescence is quenched. Figure 6 shows RBCs labeled with Fluor-DHPE before and after H_2O_2 treatment. The HbEKO mouse microcytic cells exhibit a smaller Fluor-DHPE fluorescence intensity and a lower forward scatter than C57 at both basal levels and with H_2O_2 exposure, indicating greater quenched fluorescein fluorescence. The mean fluorescein fluorescence [analysed by Cytomation Summit software (V. 3.1), CO, USA] of HbE mouse RBC show a decrease in the basal level, and a further decrease in H_2O_2 stimulated RBC when compared to C57 and the HbAKO mouse model (Figure 4C), indicating enhanced membrane lipid peroxidation. Enhanced lipid peroxidation of the RBC membrane is correlated to enhanced HbE binding to the RBC membrane [55].

PS exposure—PS exposure in the circulation triggers rapid removal of RBC that have lost their phospholipid asymmetry as a result of increased RBC oxidative stress and metabolism [73; 74; 75]. Significant PS exposure has been reported in human sickle cell disease, human β-thalassemia and in transgenic sickle cell mice and thal mice [73; 76; 77; 78]. PS exposure did not appear to be significantly different in the HbEKO transgenic model when compared to the HbAKO transgenic model but was significantly different when compared to the C57 mouse (data not shown).

Cytosolic ROS levels—HbEKO transgenic mice exhibited a relatively increased basal cytosolic ROS levels when compared to the HbAKO mice and C57 mice (Figure 4D).

Altered H_2O_2-related antioxidant enzyme activity in RBC from HbE transgenic mice—An upregulation of the activity of antioxidant enzymes could account for the above indications of a mild oxidative stress. Superoxide dismutase catalyzes the dissociation of superoxide to oxygen and hydrogen peroxide. Catalase and glutathione peroxidase (GSH-PX) are two antioxidant enzymes that protect RBC against peroxide in a compartmentalized manner. Catalase has been shown to play a predominant role in scavenging exogenously added H_2O_2 away from the plasma membrane, while GSH-PX is more effective in scavenging membrane peroxides and hydroperoxides and in directly preventing the formation of heme degradation products in RBC [48; 79; 80]. Therefore, RBC catalase, superoxide dismutase (SOD), and GSH-PX activities were compared (Figure 5).

Decomposition of exogenously added H_2O_2 to HbEKO mouse hemolysates follows an exponential decay (Figure 5A inset) with a 4-fold increase in rate compared to C57 (Figure 5A), indicating a significantly enhanced catalase activity in full KO HbE mouse hemolysate. Superoxide dismutase activity is reduced in the HbEKO mouse (Figure 5C). GSH-PX activity, as a function of NADPH oxidation rates, is based on the glutathione redox cycle [composed of GSH-PX, glutathione reductase (GSSG-RX) and the cosubstrate GSH and NADPH]. GSH-PX activity in the HbEKO mouse exhibits a 50% decrease compared to the C57 mouse (Figure 5B). This is consistent with elevated membrane lipid peroxidation since GSH-PX scavenges lipid hydroperoxides close to the membrane region.

Do these small changes in indicators of mild oxidative stress correlate with organ damage?

Since most HbE/β-thal patients die of cardiac disease [81], we assessed cardiac function by echocardiograms on the HbEKO mouse model compared to the C57 background mouse. Left ventricular (LV) echocardiography was performed under baseline conditions in the C57 mouse (n = 5) and in the HbEKO mutant mouse model (n = 5) and LV percent (%) ejection fraction (EF) and LV fractional shortening were assessed (Figure 6). LVEF was significantly ($p < 0.02$ vs. wild type) depressed in the HbEKO mouse (Figure 6A) and LV fractional shortening was significantly ($p < 0.02$ vs. wild type) attenuated in the HbEKO mouse (Figure 6B). These data clearly demonstrate that baseline LV contractile function is significantly decreased in the HbEKO mouse.

The echocardiogram findings were reproduced on the same mice 3 months later (Figure 6, “Post”) and the observations were the same, indicating that the left ventricular (LV) dysfunction observed in the HbE full KO mice are significant and reproducible, and validating and the use of a smaller mouse population (n=5) for statistically significant echocardiograms. Histological preparations of the heart stained with hematoxylin and eosin showed no muscle pathology and there was no difference in heart weight as a function of body mass.

In addition to the heart, organ weight as a function of percent body mass and histological preparations of the kidney, spleen, and liver were obtained for the HbEKO mouse compared to the C57 mouse. No marked significant differences were observed in the kidney, spleen, or liver.

DISCUSSION

RBC are highly susceptible to oxidative damage due to the presence of both high concentrations of membrane unsaturated fatty acids and an unstable Hb, a potent promoter

of oxidation [70]. It is well established that HbE is highly unstable in vitro. However, instability of human HbE in RBC has not been demonstrated because HbE and β-thalassemia are usually present concomitantly. Thus, the presence of free α-globin chains, that are highly unstable, may confound the origin or contribution of HbE to the pathophysiology.

We now report that a HbEKO transgenic mouse model has been generated that produces RBC with exclusively HbE (human α- and β^E-globins), a closely balanced human α:β globin chain synthesis, no human minor hemoglobins, and no mouse globin chains. Like transgenic knockout mouse models for other hemoglobinopathies including sickle cell disease, our HbE mouse model may not perfectly mimic the homozygous human condition of HbE. Nevertheless, we find our model to be consistent with the hypochromic, microcytic RBC and a number of human HbE RBC parameters reported for human HbE homozygotes and the overall mild effects of human homozygous HbE [9]. Hence, this HbEKO transgenic mouse model presents an opportunity to assess the RBC alterations and pathophysiology attributed to the presence of human HbE without a significant excess of human α-globin chains, in contrast to the β^E-globin transgenic mouse model generated by [33] that does not produce human α-globin but rather the equivalent mouse globin. Whether mouse α-globin will stabilize or destabilize the hemoglobin α1β1 interface is not clear; however, given the number of amino acid differences between mouse and human α-chains, it is unlikely that there will be no effect.

The transgenic HbEKO mouse model exhibits altered RBC parameters that appear similar to the HbE homozygous human counterpart, and indicative of an overall mild oxidative stress associated with an elevation in RBC membrane lipid peroxidation, cytosolic ROS, and ZPP levels. We must consider two possible explanations for the microcytic nature of HbEKO cells: (1) it does have something to do with β^E chain synthesis, although possibly something more complex and interesting than simply creating a thalassemic condition. This possibility may provide new insight into the perplexing nature of HbE by suggesting additional contributing pathological factors beyond those already established. Or, (2) we cannot completely rule out that it may, more trivially, simply be due to the failure of the transgene to produce sufficient tetramer due to insufficient copy number of both α and β constructs, a possibility that is not present in human disease. Regardless, the microcytic nature of HbEKO mouse cells mimics the HbE human counterpart.

Enhanced reactive species seen in the HbEKO mouse RBC may give rise to the observed mild oxidative stress. As noted earlier, unstable globins (e.g., HbS and free α-chains arising in β-thalassemia) yield hemichromes, metHb, free heme, and iron that bind to the RBC membrane result in membrane oxidative damage, including lipid peroxidation [82; 83; 84]. With the enhanced binding of HbE to the mouse RBC [22] (as well as the human RBC membrane [34]), not only is a rationale provided to investigate oxidative stress in RBC, but also a possible mechanistic explanation for the observed mild oxidative stress.

We highlight the term *mild* oxidative stress since PS exposure did not appear to be significantly different in the HbEKO transgenic model when compared to the HbAKO transgenic model. PS exposure is an integral part of ineffective erythropoiesis or increased apoptosis resulting in premature removal during RBC formation. In cases of high oxidative stress, compensation by removal of these cells cannot fully occur, and, as a result, PS exposed cells would be observed in the circulation. The lack of a significantly increased PS exposure in this HbEKO transgenic model is consistent with a mild or minimized oxidative stress wherein PS exposing cells, if they are formed, are rapidly removed and cannot be identified in peripheral blood. Interestingly, this finding is consistent with that reported on a β-thal mouse rescued with β^E-globin [85].

Some premature RBC removal in the HbEKO mouse is indicated by the mildly elevated reticulocyte count, indicating a higher RBC turnover (consistent with a mild or minimized oxidative stress) along with enhanced membrane lipid peroxidation, elevated cytosolic ROS, increased catalase activity, and a significant decrease in GSH-PX and SOD activity. The lack of a significantly enlarged spleen is also consistent with a benign, mild anemia.

The elevated ZPP levels may also be a consequence of the mild oxidative stress. ZPP levels are also markers for in vivo events of Hb synthesis and ineffective erythropoiesis. Increased porphyrin levels have been detected in the blood of HbE patients, sickle cell disease patients, even in the *absence* of lead poisoning and iron deficiency [86; 87; 88]. Thal mice, known to exhibit ineffective erythropoiesis due to globin chain imbalance [79], show greatly elevated levels of ZPP compared to the C57 and HbE mice (data not shown). This is consistent with elevated ZPP reported in human β-thal patients [18; 89].

Elevated ZPP/heme ratios have been used to diagnose pre-anemic iron deficiency [90]. It is noteworthy that elevated levels of ZPP have been shown to interfere with RBC malaria parasite propagation by binding to heme crystals to inhibit the process of hemozoin formation in *Plasmodium falciparum* [91]. Most human hemoglobinopathies have the greatest gene frequencies in malaria-endemic area, and these hemoglobinopathies are associated with elevated ZPP, such as in β-thal, HbC diseases, sickle cell disease and HbE disease. Elevated ZPP may be one mechanism of several contributing to resistance to malaria.

Further evidence suggesting altered erythropoiesis in the HbEKO mouse is supported by the RBC morphology and RBC indices (in particular, microcytosis and target cells). The number of target cells is correlated with the percent expression of HbE. Consistently, HbE mice exhibit more target cells than HbSE and HbAE mice. The presence of target cells may be explained by increased RBC surface membrane area to volume ratio that may result from a larger RBC membrane surface area or a decrease in cellular Hb content. A significantly altered surface to volume ratio is indicated by low osmotic fragility and low MCV. Notably, the HbEKO mouse has a significant increase in reticulocytes compared to the HbAKO mouse and HbAEKO mouse. The 10% reticulocyte count in HbSE mice may result from a shorter RBC lifespan due to the combined presence of HbS and HbE.

The conclusion that the HbEKO transgenic mouse model displays a mild oxidative stress is further supported by small but significant changes in cardiac function. Emerging evidence shows that reactive oxidative species (ROS) such as, free heme and heme-free globins are inducers of endothelial and cardiac pathophysiology (e.g., [92; 93], and reactions of nitric oxide (NO), nitrite, and hemoglobin contribute to homeostasis of the endothelium and cardiac physiology [94]. Minimal reactive species may be generated during low levels of RBC hemolysis in the HbEKO mouse that could partially account for the mild oxidative stress. Regarding the apparent requirement of normal levels of NO for normal cardiovascular physiology, it is noteworthy, that HbE has recently been showed in vitro to be a less active nitrite reductase compared to HbA [39]. This introduces a novel hypothesis for the role of HbE in HbE pathophysiologies that will be the subject of future investigations.

CONCLUSIONS

The sole presence of HbE is correlated with elevated reactive oxygen species and mild oxidative stress accompanying alterations in hematological HbEKO transgenic mouse RBC indices (low HGB, HCT, MCH, normal MCHC, elevated RBC counts, elevated reticulocytes). These altered RBC indices observed in the HbEKO transgenic mouse are

consistent with those reported in human HbE homozygotes (HbE disease) [8; 9]. The minimal pathophysiology in both the human homozygote and this HbEKO transgenic mouse model may be explained by a non-overloaded and sufficient RBC redox systems. Remaining to be defined are (1) the specific primary molecular events intrinsic to the HbE tetramer that initiate the RBC mild oxidative stress and/or hemolysis, and (2) the specific molecular events arising in HbE/β-thalassemia RBC (containing both HbE and free α-chains) that lead to an unexpected severe morbidity. One hypothesis arising from this transgenic HbEKO mouse model characterization is that, in HbE/β-thalassemia disease, HbE instability in the RBC becomes further exacerbated by high levels of free α-chains; it is the coupling of HbE instability and α-chain instability that results in an overall heightened RBC oxidative stress that overrides the RBC redox capacity, leading to the release of free radicals with consequential severe pathophysiology.

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Abbreviations

Hb	hemoglobin(s)
HCT	hematocrit
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
MCHC	mean corpuscular hemoglobin concentration
RDW	red cell distribution width
HbEKO	a full mouse globin knockout expressing only human HbE
HbAKO	full mouse globin knockout expressing only human HbA
ROS	reactive oxygen species
FHDP	free heme degradation products
ZPP	zinc protoporphyrin IX
RBC	red blood cell(s)
thal	thalassemia
C57	C57Bl/6 mouse

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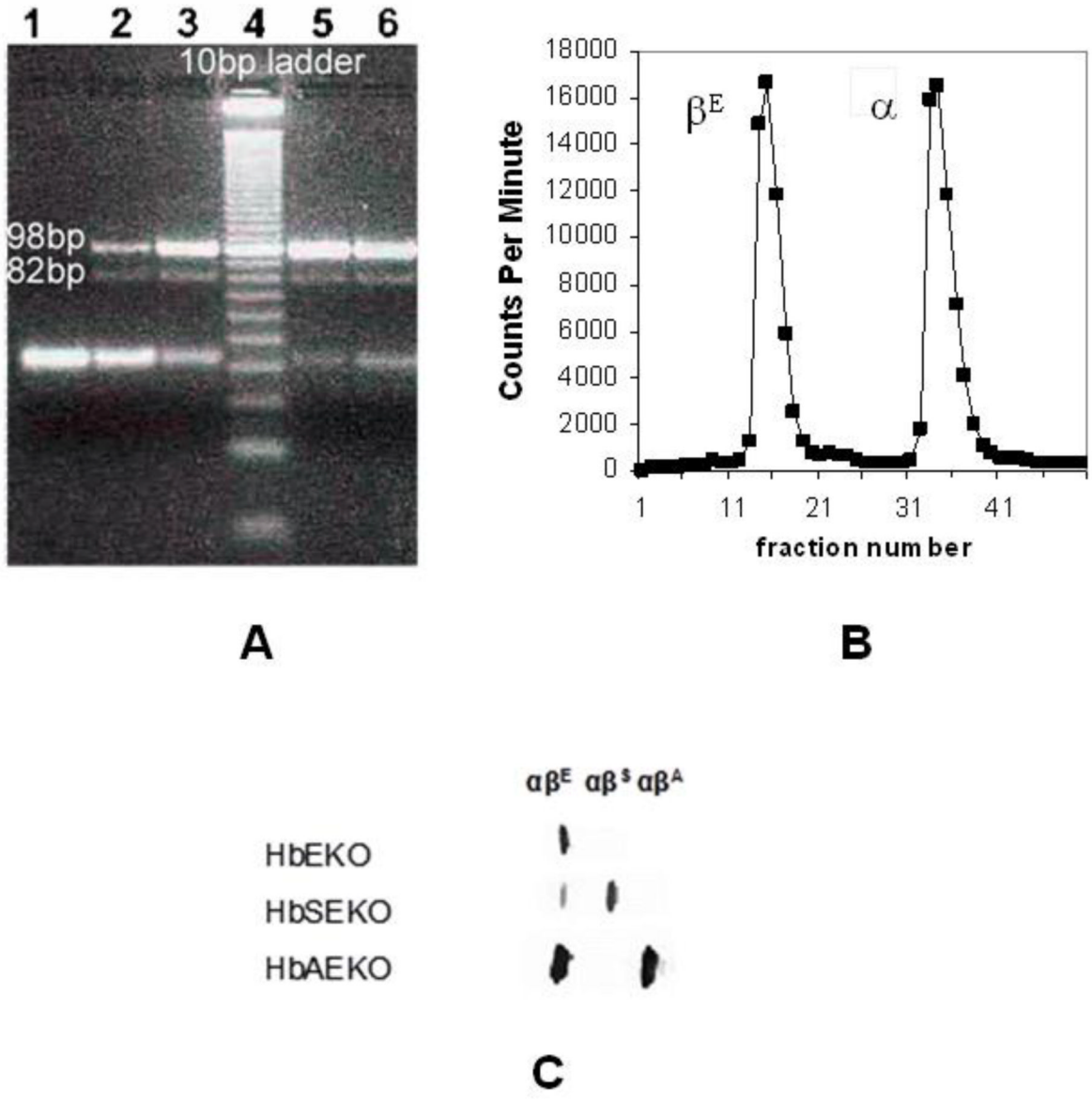


Figure 1.

The HbEKO mouse expresses 100% HbE without excess α-chains. (A) Globin mRNA analysis of peripheral blood reticulocytes demonstrates the alternate splice in founder (on FVB background) (n=2) and HbEKO mice (n=2). RT-PCR product: 5 μl (A&B) and 7 μl (C&D) loaded on 5% wide range agarose gel at 40–50 volts for 1–3 hours. Lane 1 is the no template control; lane 2 & 3, RNA extraction from 2 different founder mice; lanes 4, 10bp ladder and lanes 5 & 6, from 2 different HbEKO transgenic mice. (B) Globin chain synthesis in reticulocytes. See Materials & Methods for details. (C) Isoelectric focusing (IEF) of Hb obtained from RBC lysates of transgenic mouse models expressing only human α-globins (right peak) with human β-globins (left peak). $\alpha\beta^E$ denotes the Hb tetramer with the human α-chain and the specific human β^E mutant. The HbAEKO IEF was run on a separate gel and somewhat overloaded compared to the HbEKO and HbSEKO giving rise to the denser appearance of the bands. Importantly, all the HbE are aligned and no other globins (human or mouse) are present.

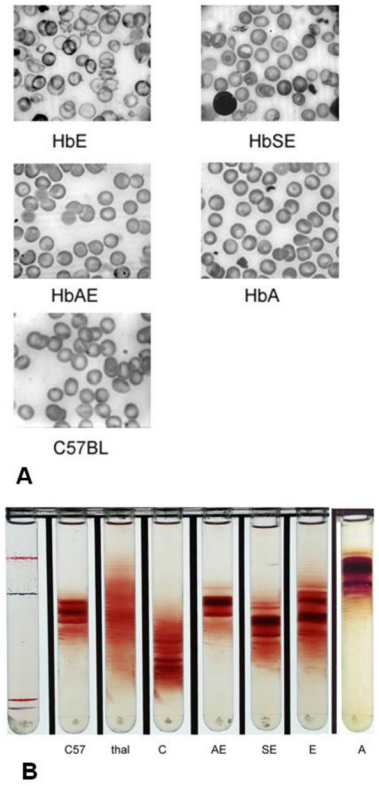
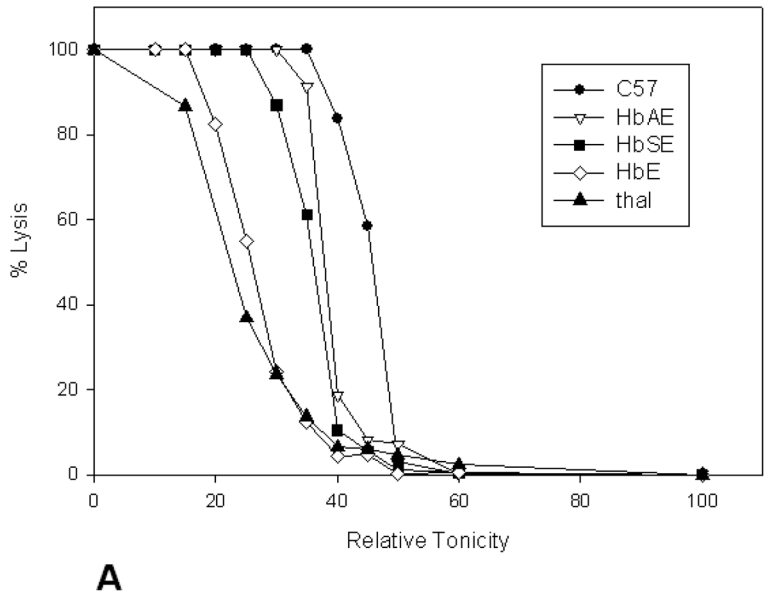


Figure 2.

(A) Blood smears (Wright's stain) from transgenic mice that exclusively express human HbE (HbE), HbS/HbE (HbSE), HbA/HbE (HbAE), and HbA compared to a blood smear from a C57 mouse. Blood smears and hematological parameters for β-thal mouse are found in [38]. (B) Percoll-Stractan density gradients of RBCs from control C57BL (C57), β-thal (thal) and transgenic mice expressing exclusively human HbC (C), HbA/HbE (AE), HbS/HbE (SE), HbE (E), and HbA (A).



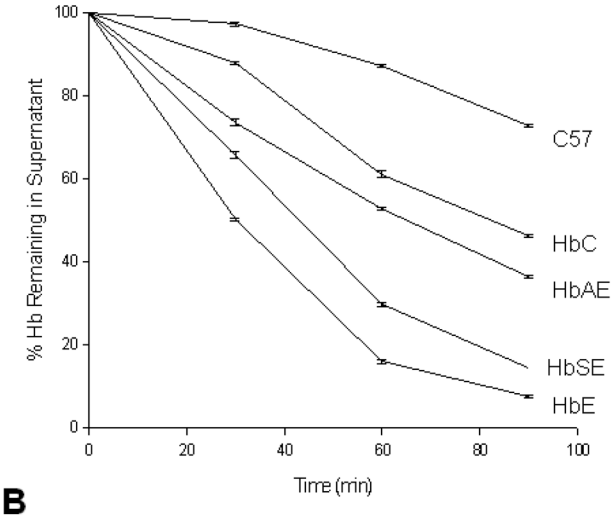


Figure 3.

(A) Osmotic fragility of HbE, HbAE, HbSE, and thal mouse RBC compared to C57. (B) Isopropanol precipitability of hemolysates obtained from the C57 background mouse and the following transgenic mouse models: HbCKO, HbAEKO, HbAEKO, HbSEKO, and the HbEKO mouse.

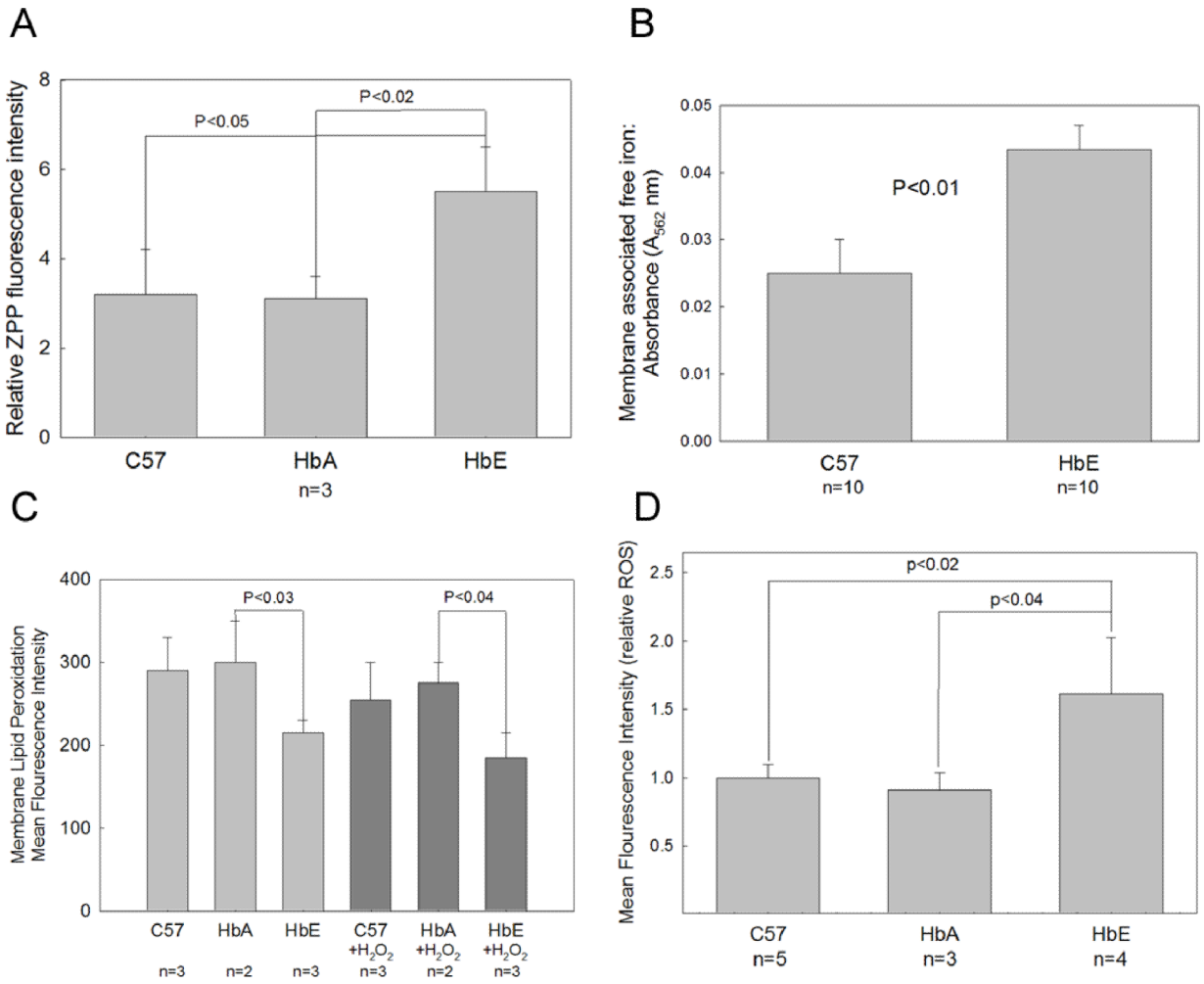


Figure 4.

(A) Relative ZPP levels are enhanced for the HbEKO transgenic mouse model compared to the HbAKO transgenic mouse model. (B) Relative membrane associated free iron is elevated in the HbEKO mouse compared to C57 mice. (C) Relative RBC membrane lipid peroxidation. Flow cytometry shows a decrease in the mean fluorescence intensity compared to the HbAKO and C57 mouse, signifying an enhanced RBC membrane lipid peroxidation for the HbEKO mouse before and after H_2O_2 stimulation. (D) Relative RBC ROS levels is elevated for the HbEKO mouse RBC compared to HbAKO RBC and C57 RBC.

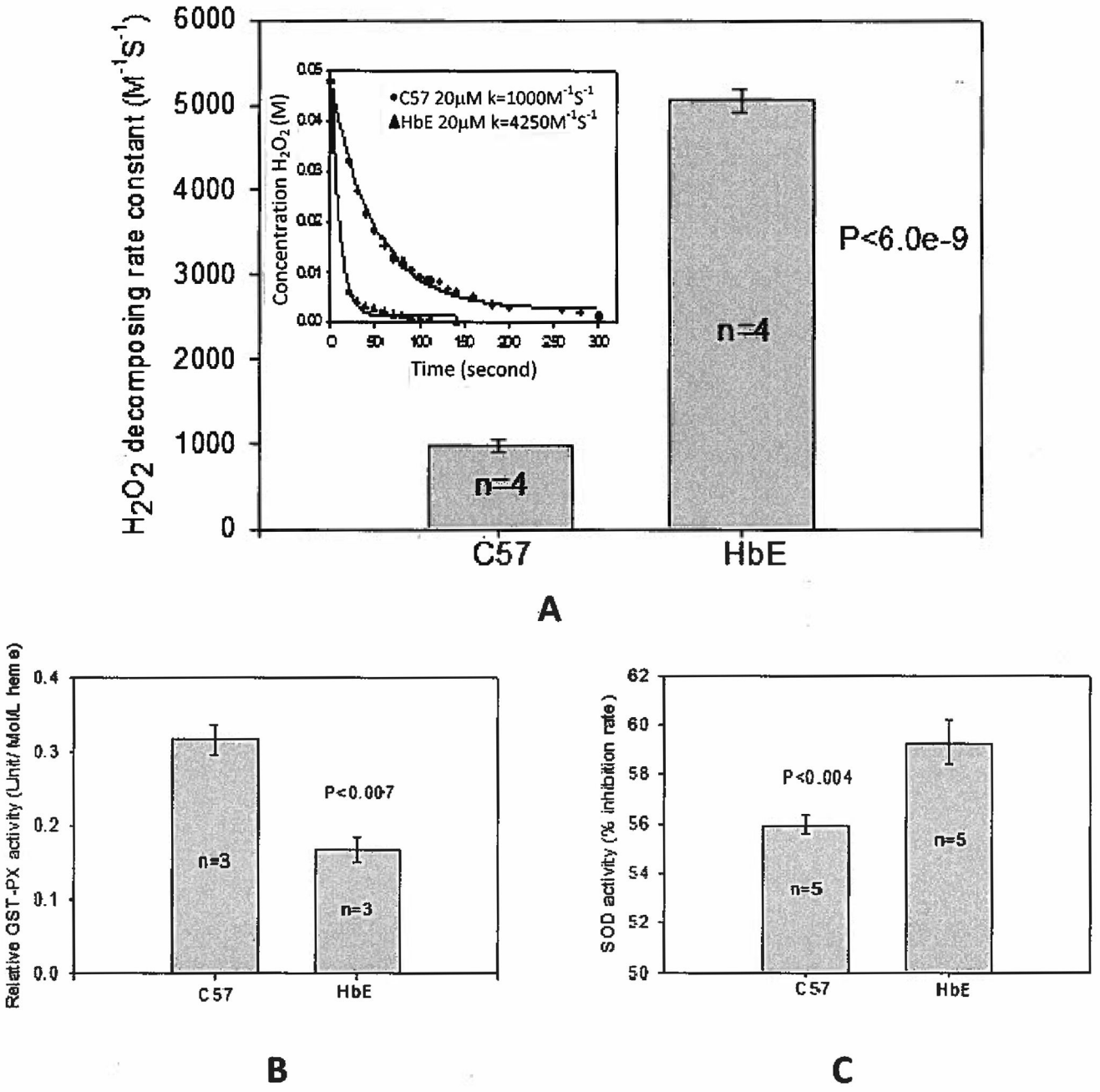


Figure 5.

Catalase (A) activity is enhanced while GSH-PX (B) and SOD (C) activities are reduced in hemolysates from HbE KO mouse compared to C57.

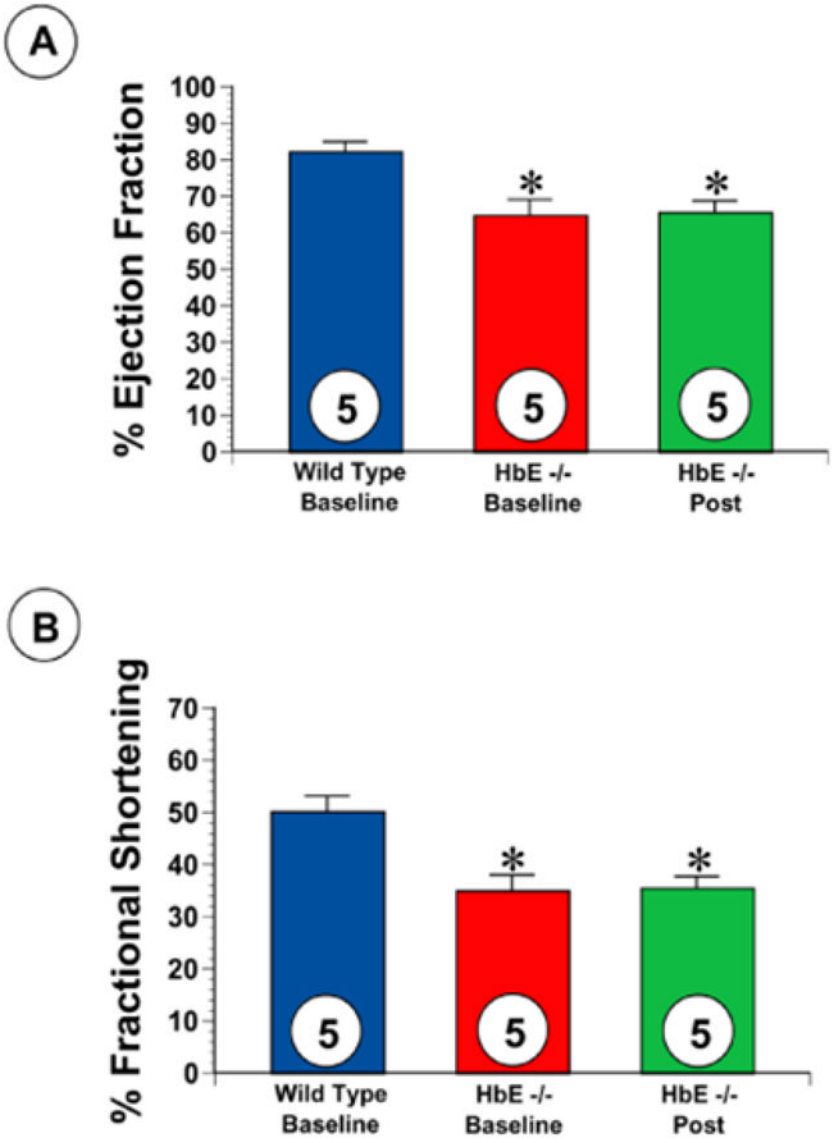


Figure 6.

Left ventricular (LV) echocardiography was performed under "baseline" conditions in the C57 mouse (n = 5) and in the HbEKO mutant mouse model (n = 5). (A) Left ventricular ejection fraction (LVEF) was significantly depressed in the HbEKO mouse [$p < 0.02$ vs. C57 (denoted as wild type)]. (B) LV fractional shortening was significantly ($p < 0.02$ vs. wild type) attenuated in the HbEKO mouse compared to C57. The same mice were re-evaluated three months later after recovery ("Post") and they exhibited the same pattern of decreased contractile function (A,B).

Table 1

Comparative Globin chain synthesis in transgenic mouse models.

Mouse	β/α chain ratio	n
C57*	1.05± 0.04	3
Thal	0.79	From: Fabry et al., 2001
HbAKO	0.92 ± 0.04	3
HbEKO	0.93 ± 0.04	5

*The C57 mouse RBC hemoglobin consists of mouse β-single chain and α-mouse globin.

Table 2

Hematological parameters for HbEKO, HbAEKO, HbSEKO, HbAKO, and C57 mice. The hematological values are shown as mean ± standard deviation of the mean.

Full KO transgenic mice/C57 BL	RBC ($\times 10^6$ cell/μl)	Reticulocytes (%)	HGB (g/dl)	HCT (%)	MCH (pg)	MCV (fl)	MCHC (g/dl)	RDW (%)
C57 (n=7)	10.9±1.1	3.1±0.5	16.1±1.3	50.2±5.2	14.8±0.6	46.5±2.2	31.2±1.5	13.7±0.4
HbA (n=3)	11.97±1.0	2.2±0.4	13.2±1.0	45.2±2.4	10.9±0.3	37.8±1.2	28.8±0.8	15.4±1.2
HbE (n=5)	13.5±0.9	7.2±2.5	13.2±0.8	42.8±3.1	9.8±0.10	31.6±1.5	32.0±2.0	24.4±3.9
HbSE (n=3)	11.9±0.7	10.4±1.5	15.5±1.3	49.2±2.8	13.1±0.9	41.6±1.1	31.5±3.1	24.0±2.6
HbAE (n=4)	11.6±0.7	3.7±0.7	15.3±1.1	46.8±3.6	13.0±0.8	40.0±1.3	31.1±0.7	16.6±0.6
P value (HbA vs C57)	*P<0.3*	*P<0.03*	*P<0.03*	*P<0.2*	*P<6.0e-5*	*P<0.0005*	*P<0.08*	P<0.02
P value (HbE vs. C57)	*P<0.003*	*P<0.006*	*P<0.006*	*P<0.03*	*P<2.0e-7*	*P<5.0e-7*	*P<0.5*	*P<8.0e-5*
P value (HbSE vs. C57)	*P<0.3*	*P<5.0e-6*	*P<0.6*	*P<0.7*	*P<0.02*	*P<0.008*	*P<0.9*	*P<7.0e-5*
P value (HbAE vs. C57)	P<0.3	P<0.08	P<0.4	P<0.3	P<0.02	P<0.0009	P<0.9	P<0.0003
P value (HbE vs HbA)	*P<0.06*	*P<0.03*	*P<1.0*	*P<0.4*	*P<0.002*	*P<0.0002*	*P<0.06*	*P<0.02*
P Value (HbE vs. HbAE)	P<0.02	*P<0.05*	*P<0.03*	*P<0.2*	*P<9.0e-5*	*P<0.0002*	*P<0.5*	P<0.02