Original Research *Feature article*

Safety and feasibility of the gene transfer of hemopexin for conditions with increased free heme

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Impact Statement

Toxicity of free extracellular heme to cells and tissues has been extensively demonstrated, so that this molecule is regarded as a key element in the pathogenesis of hemolytic diseases. Hemopexin (HPX), which is a liver-produced protein with high affinity for free heme, is responsible for keeping free heme away from cells and tissues, by shuttling heme to cells so that it is metabolized. In fact, HPX is capable of reversing the deleterious effects of free heme in models of sickle cell disease. Accordingly, increasing HPX expression is an attractive strategy to mitigate heme-mediated toxicity in hemolytic disorders. Here, we report that obtaining a sustained increase of HPX levels using a viral vector is a feasible strategy, with no evident safety concerns in animal models. Our study paves the way for exploring similar strategies in animal models of sickle cell disease and other hemolytic conditions.

Abstract

Heme is a fundamental molecule for several biological processes, but when released in the extracellular space such as in hemolytic diseases, it can be toxic to cells and tissues. Hemopexin (HPX) is a circulating protein responsible for removing free heme from the circulation, whose levels can be severely depleted in conditions such as sickle cell diseases. Accordingly, increasing HPX levels represents an attractive strategy to mitigate the deleterious effects of heme in these conditions. Gene transfer of liver-produced proteins with adeno-associated virus (AAV) has been shown to be an effective and safety strategy in animal and human studies mainly in hemophilia. Here, we report the feasibility of increasing HPX levels using an AAV8 vector expressing human HPX (hHPX). C57Bl mice were injected with escalating doses of our vector, and expression was assessed by enzyme immunoassay (ELISA), Western blot, and quantitative polymerase chain reaction (qPCR). In addition, the biological activity of transgenic hHPX was confirmed using two different models of heme challenge consisting of serial heme injections or phenylhydrazine-induced hemolysis. Sustained expression of hHPX was confirmed for up to 26 weeks in plasma. Expression was dose-dependent and not associated with clinical signs of toxicity. hHPX levels were significantly reduced by heme infusions and phenylhydrazine-induced hemolysis. No clinical toxicity or laboratory signs of liver damage were observed in preliminary short-term heme challenge studies. Our results confirm that long-term expression of hHPX is feasible and safe in mice, even in the presence of heme overload. Additional studies are needed to explore the effect of transgenic HPX protein in animal models of chronic hemolysis.

Keywords: Hemopexin, heme, inflammation, hemolysis, viral vector, gene transfer

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Introduction

Chronic intravascular hemolysis is recognized as one of the basic mechanisms of sustained inflammation in diseases such as sickle cell anemia (SCA), mainly due to the release of free hemoglobin and free extracellular heme.[1,2] Although heme is an essential molecule for most living beings,[3] free extracellular heme is toxic to cell membranes due to its amphipathic nature and its capacity to generate reactive oxygen species.[4] Moreover, several studies carried out in the last two decades have shown that free heme can also activate multiple components of innate immunity such as leukocyte recruitment,[5] neutrophil activation,[6] TLR-4-dependent inflammation,[7] complement activation,[8] neutrophil extracellular trap (NET) release,[9] inflammasome activation,[10] and tissue factor-dependent coagulation activation,[11] placing heme in a central position in the pathogenesis of SCA and other hemolytic diseases.[12–14]

Hemopexin (HPX) is a circulating protein responsible for binding free heme, thus preventing its toxicity to cells and tissues. However, in conditions associated with sustained release of free heme, such as SCA, HPX levels are consumed and free heme levels increase.[15] In animal models of SCA in which heme was able to trigger acute lung injury (STA)[16] or

vaso-occlusive crisis,[17] intravenous administration of HPX was able to reverse these conditions. Although the therapeutic use of HPX protein infusion for the treatment of acute lung injury is an attractive proposal, the need for repeated infusions of this protein may be a limiting factor.

Gene therapy using adeno-associated virus (AAV) has gained ground in recent years and shown a great potential for the treatment of diseases associated with reduced or absent levels of circulating proteins such as hemophilia.[18] Herein, we explored the feasibility and safety of using an AAV-vector capable of expressing human HPX (hHPX) in animal models of conditions associated with increased free heme release.

Materials and methods

Reagents

Heme (CAS: 16009-13-5, Frontier Scientific) was diluted in 1M NaOH, adjusted to pH of 7.4 with 3M HCl, filtered through a 0.22 um filter and immediately used. Phenylhydrazine (PHZ) (CAS 370-86-5, Sigma) was diluted in water for injection to a concentration of 50 mg/kg and immediately used. HPX plasma levels were measured by enzyme immunoassay (ELISA) using commercials kit from Abcam (Cambridge, UK), specific for the human (cat# ab108860) or murine (cat# ab157716) form of the protein. A specific anti-HPX monoclonal antibody (1:10.000) for hHPX (Abcam, ab133523) was used in Western blot analyses. Primers used for mRNA expression quantification are shown in Supplementary Table 1.

Animal models

All experiments with mice were approved by the Ethics Committee on the Use of Animals at Unicamp (CEUA) and obtained from the Centro Vivarium at Unicamp (CEMIB, Campinas, SP, Brazil). Male and female C57BL/6J mice were used between 6 and 10 weeks, kept in microisolators at a temperature of 20°C–22°C, in a light–dark 12 h, and fed with commercial chow and autoclaved water. Two models of heme challenge were used, adapted from previous studies, and based on (1) serial heme infusions or (2) PHZ-induced hemolysis.[19] Heme infusion consisted of a daily regimen of 35 or 70 uMol/kg heme for 7 days, intraperitoneally (i.p.). PHZ hemolysis was induced using a single dose of 75 mg/kg i.p.

Gene construct and vectors

Our vector was built on an AAV serotype 8 due to its liver tropism, which is the main site of physiological HPX production.[20] The cDNA of the hHPX gene was submitted to a codon optimization process (Genscript; Piscataway, NJ, USA) aiming to increase the efficiency of hHPX expression in mice. A specific liver promoter ApoE enhancer/human α1-antitrypsin (ApoE/hAAT) was also used, being composed of the gene of human α1-antitrypsin (hAAT) with four copies of the enhancer (enhancer) of the Human ApoE and a synthetic intron.[21] As a control, we used the same construction, lacking the hHPX gene sequence. The rAAV8 vectors with the cDNA of the hHPX optimized (hAAV8-hHPX) and the empty rAAV8 (AAV8-Empty) were produced by the company Virovek (Hayward, California, USA).

Gene transfer regimens and sample collection

Vector was injected under isoflurane anesthesia in animals aged between 8 and 10 weeks, retro-orbitally, in saline solutions. Doses ranged from 2×10^{12} to 2×10^{14} vg/kg. To monitor hHPX protein expression, blood was collected before (baseline) and 2 weeks after vector infusion, retro-orbitally in tubes containing 10% EDTA under isoflurane anesthesia. For long-term expression and safety monitoring, additional samples were obtained every 2–4 weeks. Heme challenge experiments were performed 2–4 weeks after vector infusion, after the confirmation of hHPX expression. For these experiments, blood was obtained before heme or PHZ infusion and thereafter, at specific timepoints detailed in the "Results" section. For terminal experiments, blood was obtained from the inferior vena cava in tubes containing 10% EDTA or 3.8% sodium citrate (for plasma separation), or in non-anticoagulated tubes (for serum separation). After plasma or serum separation by centrifugation at 2500 g or 1000 g for 15 min, respectively, aliquots were stored at −20°C until analyses. In addition, fresh liver samples were collected for real-time polymerase chain reaction (RT-PCR) and Western blot and processed. For RT-PCR experiments, samples were stored in a RNA stabilizer buffer. For Western blot, samples were stored in 1.5 mL tubes and immediately placed on dry ice. Both samples were processed with the TissueLyser II (Qiagen, Germany). For histological analysis, liver and right kidney were collected in histological cassettes and stored in 10% formaldehyde.

Confirmation of transgenic hHPX expression and of hHPX biological activity

Feasibility of hHPX gene transfer in mice was confirmed by short- and long-term measurement of mRNA and protein expression. mRNA expression was evaluated by RT-PCR using primers specific for the sequence of optimized hHPX. As control genes, we used Calr and Hsp90b1, both obtained from a database of housekeeping genes https://housekeeping.unicamp.br/?homePageGlobal.[22] Protein expression was measured by ELISA, using commercial kits specific for hHPX. Protein expression was also confirmed by Western blot of liver samples. The biological activity of transgenic hHPX was assessed by the consumption of hHPX upon infusion of heme or PHZ-induced hemolysis.

Safety parameters

Weight gain was monitored biweekly and expressed as a ratio to baseline weight (Δ weight). Mortality was monitored daily in the long-term follow-up experiments, and as detailed in the Kaplan–Meyer survival curves for the heme challenge models. Liver toxicity was assessed by measurements of liver enzymes (aspartate transaminase – AST and alanine transaminase – ALT), and nephrotoxicity by measurement of creatinine, both in serum samples. Hematological toxicity was assessed by complete blood counts in automated analyzers. Since increased delivery of iron to liver cells could result in cell toxicity, liver toxicity was also analyzed in hematoxylin and eosin (H&E) and evaluated for the presence of inflammation, fibrosis, and necrosis, by two independent observers who were blinded

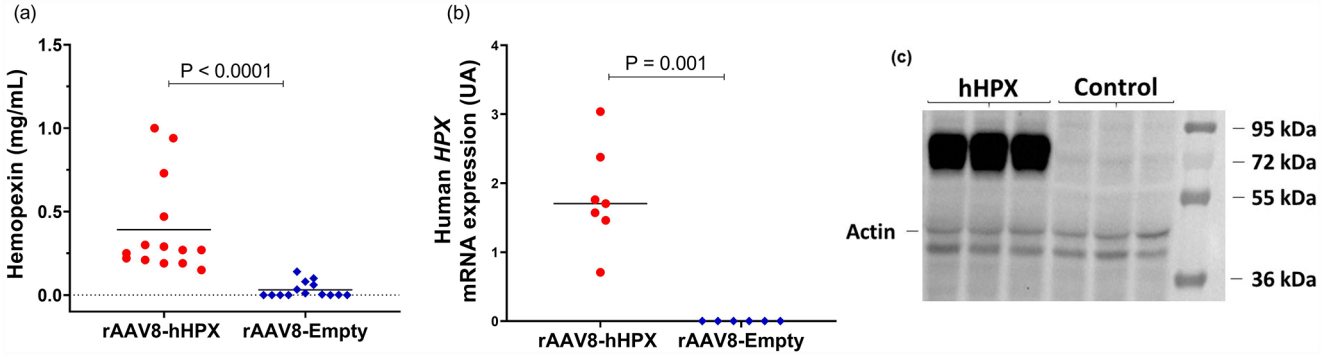


Figure 1. (A) hHPX levels in mouse plasma 2 weeks after transduction with 2×10^{13} vg/kg of rAAV8-hHPX vector ($n = 14$ per group). (B) mRNA expression of HPX in liver samples of mice transduced with 2×10^{13} of rAAV8-hHPX vector ($n = 7$) or with the control vector ($n = 6$). Results shown as median, and *P* values are from Mann–Whitney test. (C) hHPX by Western blot in liver samples of mice transduced with 2×1013 vg/kg of rAAV8-hHPX vector ($n = 3$) or with the control vector ($n = 3$).

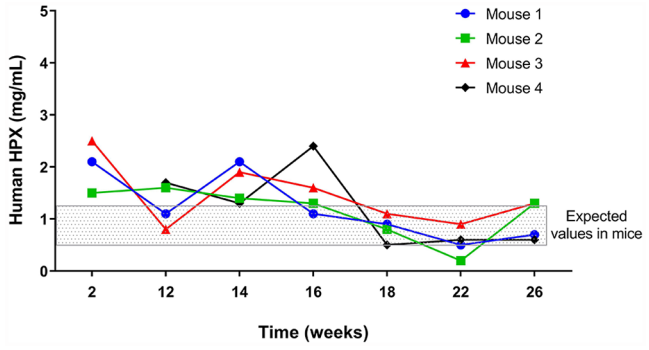


Figure 2. Human HPX levels in C57BL/6J mice transduced with 2×10^{13} vg/kg of rAAV8-hHPX vector evaluated for up to 26 weeks. Levels of hHPX were measured by ELISA. Dashed lines indicate reference range for murine HPX (0.55–1.25 mg/mL).

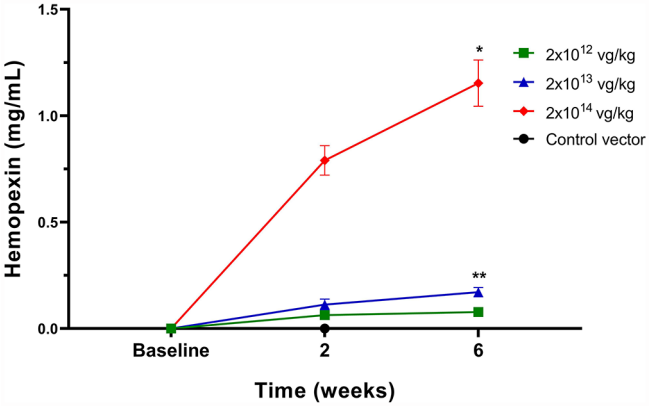


Figure 3. hHPX expression (mean and standard deviation) according to vector dose. C57BL/6J mice were transduced with three doses of the rAAV8-hHPX vector: 2×10^{12}, 2×10^{13}, and 2×10^{14} vg/kg. hHPX levels were measured by ELISA. *P* values refer to comparisons between Control Vector using ANOVA with mean and SEM ($n = 4$–5 per group). *$P = 0.0003$; **$P = 0.04$.

for the experimental group of each section. The presence of necrosis and fibrosis were grades using two previously described scores.[23,24]

Statistical analysis

Data were expressed as median and interquartile range. Comparison between groups was performed using the Mann–Whitney or Wilcoxon test. All statistical analyses were performed using the GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). A *P* value < 0.05 was considered as statistically significant.

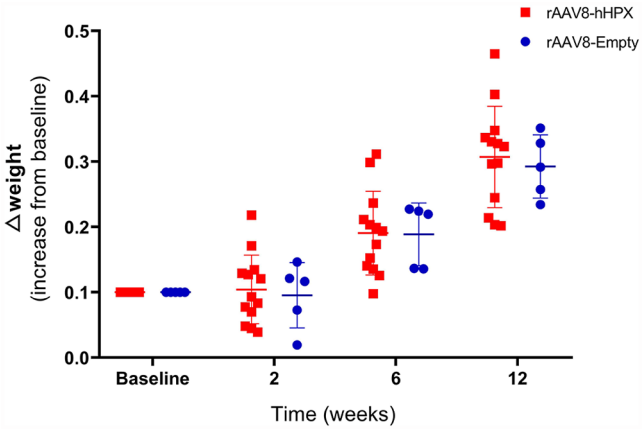


Figure 4. Weight gain variation of mice expressing hHPX ($n = 13$) compared to mice treated with the control (empty) vector ($n = 5$). Animals were transduced with three doses of the rAAV8-hHPX vector: 2×10^{12}, 2×10^{13}, and 2×10^{14} vg/kg. Results are expressed as weight gain (g)/baseline weight. All comparisons yielded non-significant *P* values (Mann–Whitney test).

Results

AAV8-hHPX induces the expression of hHPX in mice

Expression of hHPX was demonstrated by ELISA in whole blood of rAAV8-hHPX-treated animals, by the consistent detection of hHPX compared to animals treated with the control vector. The absence of hHPX expression in the control group confirmed the specificity of the ELISA used for hHPX (Figure 1(A)). Expression was also confirmed in liver samples of treated animals by RT-PCR (Figure 1(B)) and Western blot (Figure 1(C)).

We followed vector-transduced animals and observed that hHPX expression is sustained for at least 26 weeks (Figure 2) and that it is dose-dependent (Figure 3).

Transgenic expression of hHPX is not associated with significant clinical and laboratory toxicity

One of the main objectives of this study was to evaluate whether the expression of hHPX could be harmful to mice. As shown in Figure 4, no differences were observed in

Table 1. Hematological parameters of mice transduced with the vector or control.

	2×10^{12} vg/kg	2×10^{13} vg/kg	2×10^{14} vg/kg	rAAV-empty	P^a
Baseline					
Leukocytes (10^3/μL)	2.9 (2.3–42)	3.4 (3.2–3.6)	5.3 (4.0–5.4)	3.4 (3.1–4.0)	>0.9
Hemoglobin (g/dL)	12.7 (12.5–13.4)	13.2 (11.9–14.2)	12.0 (11.7–13.3)	12.0 (10.2–12.7)	0.3
Platelets (10^3/μL)	1085 (1029–1156)	1093 (981–1110)	1120 (920–1193)	1115 (1050–1268)	0.7
2nd week					
Leukocytes (10^3/μl)	4.4 (2.4–5.4)	2.5 (1.9–5.7)	2.5 (2.1–3.7)	4.8 (4.2–5.0)	0.3
Hemoglobin (g/dL)	12.5 (11.6–13.0)	9.5 (9.0–12.5)	13.0 (12.0–13.2)	12.5 (11.7–13.2)	0.3
Platelets (10^3/μl)	1108 (991–1164)	860 (745–1175)	1160 (987–1183)	1290 (1153–1388)	0.1
12th week					
Leukocytes (10^3/μL)	2.1 (1.9–5.8)	3.8 (2.0–3.9)	2.5 (2.1–4.0)	1.6 (1.5–4.2)	0.4
Hemoglobin (g/dL)	12.7 (12.1–13.0)	12.5 (12.0–12.5)	13.0 (12.0–13.0)	11.5 (9.0–12.5)	0.2
Platelets (10^3/μL)	1175 (993–1383)	1190 (1185–1290)	1295 (980–1340)	1085 (857–1500)	>0.9

Results shown as median and interquartile range (IQR); $n = 4$–5 animals per dose.
[a]Kruskal–Wallis test.

Table 2. Biochemical parameters of mice transduced with the vector rAAV8-hHPX or rAAV8-empty.

	rAAV8-hHPX ($n = 6$)	rAAV-empty ($n = 3$)	P^a
ALT (U/L)	40.0 (27.0–69.0)	28.0 (20.0–58.0)	0.59
Creatinine (U/L)	0.17 (0.14–0.18)	0.16 (0.12–0.16)	0.27

Results shown as median and interquartile range (IQR).
hHPX: human hemopexin; ALT: alanine transaminase.
[a]Mann–Whitney test.

weight gain of animals expressing hHPX when compared to the group that received the control vector (Figure 4).

No signs of hematological or liver toxicity were observed in animals expressing hHPX (Tables 1 and 2).

No evidence of inflammation, fibrosis, or necrosis were observed in liver sections of mice transduced with the rAAV8-hHPX or with the control (Empty) vector (Figure 5).

Increase of extracellular heme (heme challenge) is associated with hHPX consumption and no safety concerns

After confirming that the vector is capable of expressing hHPX, we next assessed the biological activity of hHPX and its safety in models associated with the infusion/release of extracellular heme. For this, we assessed the "consumption" of hHPX in models of serial heme infusions and PHZ-induced hemolysis (Figure 6(A)). Expression of hHPX was confirmed prior to both experiments (Figure 6(B) and (C)), and hHPX consumption was confirmed after heme challenge (Figure 6(D) and (E)), using ELISA.

As safety parameters in the context of heme challenge, we analyzed leukocyte, platelet, and hemoglobin counts (Table 3); liver enzymes (ALT/AST); and creatinine (Table 4). No significant differences were observed in hematological parameters. A mild, yet significant increase in ALT levels was observed in mice that received the control vector when compared to rAAV8-hHPX treated mice. In addition, a trend toward lower erythrocyte count was observed in mice treated with the control vector.

Finally, no signs of liver toxicity or increased iron deposition were observed in liver sections obtained from mice 24 h after infusion of 75 mg/kg of PHZ (Figure 7). Iron deposition was evaluated in sections stained with Perls (data not shown).

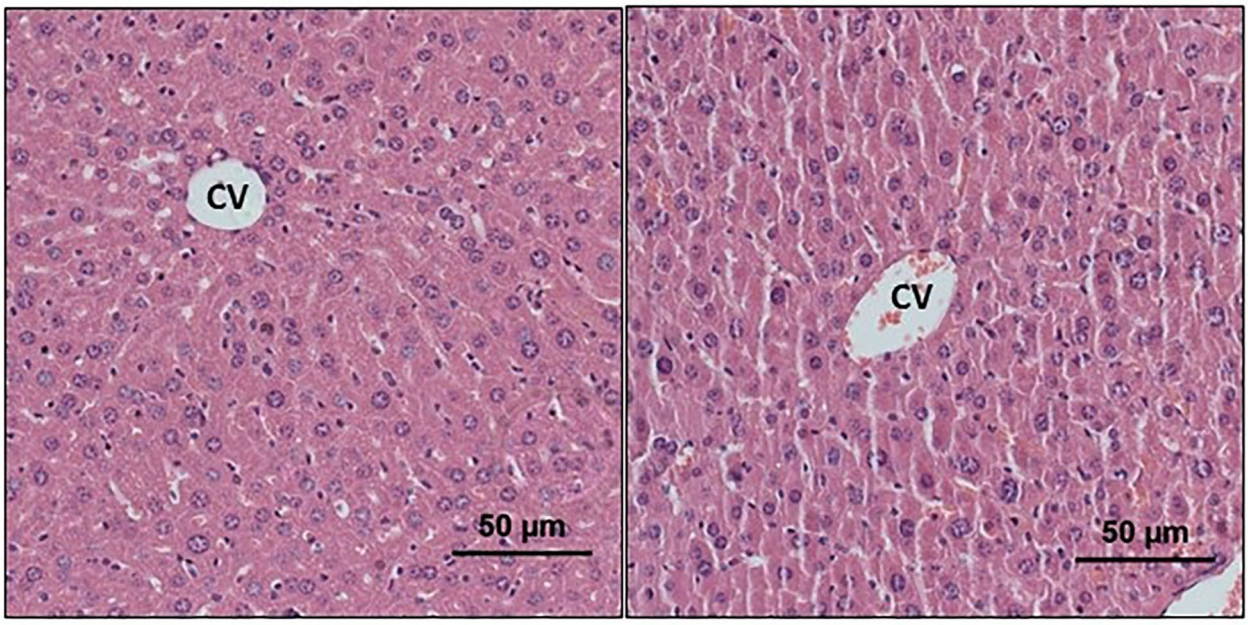
Discussion

Release of extracellular heme is an important element of the pathogenesis of several hemolytic disorders such as SCA, malaria, and sepsis, in which endogenous mechanisms that evolved to protect cells and tissues from heme toxicity are consumed. The main contribution of our study was to demonstrate the feasibility and preliminary safety of a gene therapy–based strategy designed to promote the sustained delivery of HPX.

HPX is a circulating protein produced mainly in the liver, with a high affinity of free heme. HPX: heme complexes are targeted to hepatocytes via the receptor CD91, thereby precluding the contact of free extracellular heme with cells and tissues.[20] In the intracellular milieu, heme is released from HPX and metabolized into carbon monoxide, biliverdin, and iron by heme oxygenase 1 (HO-1), while HPX returns to the circulation and is capable of binding other heme molecules.[25] Despite this recycling mechanism, it has been demonstrated that upregulation of endogenous HPX is not sufficient to provide enough levels to cope with massive amounts of heme released in conditions associated with intravascular hemolysis, in which HPX levels are markedly decreased.[26–29] Given the deleterious effects of heme in these conditions, demonstrated mainly in animal models,[16,17] but also in human studies,[30] a strategy capable of enhancing the efficiency of endogenous heme-sequestering mechanisms could be attractive, particularly for patients with congenital conditions.

Accordingly, several studies demonstrated that HPX infused as a purified protein can reduce the proinflammatory effects of heme in these conditions. The most robust evidences were obtained in animal models of sickle cell disease, in which the infusion of hHPX was capable of reducing heme-induced vaso-occlusion measured by intravital

(a)



(b)

	rAAV8-hHPX				rAAV8-Empty		
	M1	M2	M3	M4	M1	M2	M3
Inflammatory infiltrate	None	None	Single-cell	Single-cell	None	Single-cell	Single-cell
Necrosis	None	None	None	None	None	None	None
Fibrosis	None	None	None	None	None	None	None

Figure 5. (A) Representative images of H&E-stained liver sections from mice treated with rAAV8-hHPX or control (rAAV8-empty) vectors. No evidence of inflammation, fibrosis, or necrosis were observed. Original magnification: 200×. (B) Results from individual mice (M). *n* = 3–5 per group. Analysis performed by two blinded observers. Single-cell necrosis denotes sparse and rare events. CV: centrilobular vein.

microscopy in dorsal skin flaps[17,31] and heme-induced acute lung injury, rescuing most mice from death.[16] In fact, an elegant proof of concept study supporting the benefit of the gene transfer of HPX in animal models of sickle cell disease (SCD) has been previously published in which HPX expression was driven by a non-viral vector.[32] However, given the transient expression of transgene proteins when non-viral vectors are used, our study aimed to test the feasibility of a strategy capable of promoting long-term HPX expression.

We were able to demonstrate sustained and dose-dependent hHPX expression in mice transduced with our vector, which was designed to optimize liver transduction by using an AAV8 backbone[33] and liver-specific promoters.[21] The rationale for using hHPX as opposed to murine HPX was to facilitate the characterization of transgenic HPX expression with species-specific antibodies and primers. Moreover, most studies that demonstrated that HPX infusion can improve inflammation in animal models of hemolysis used preparations of hHPX, thus confirming cross-species biological activity. A codon optimization step was included so as to facilitate expression. We also explored the biological activity of transgenic hHPX in mice using two independent models of heme challenge, with the objective to demonstrate whether hHPX was consumed upon heme release. We were able to show a marked decrease of HPX levels after direct infusions of heme, or by the induction of intravascular hemolysis by PHZ, thus supporting the biological activity of hHPX. The demonstration that HPX expression is sustained for at least 26 weeks supports the feasibility of a therapeutic strategy based on the concept of enhancing endogenous mechanisms to cope with increased release of free extracellular heme. Unfortunately, methods that measure circulating heme are

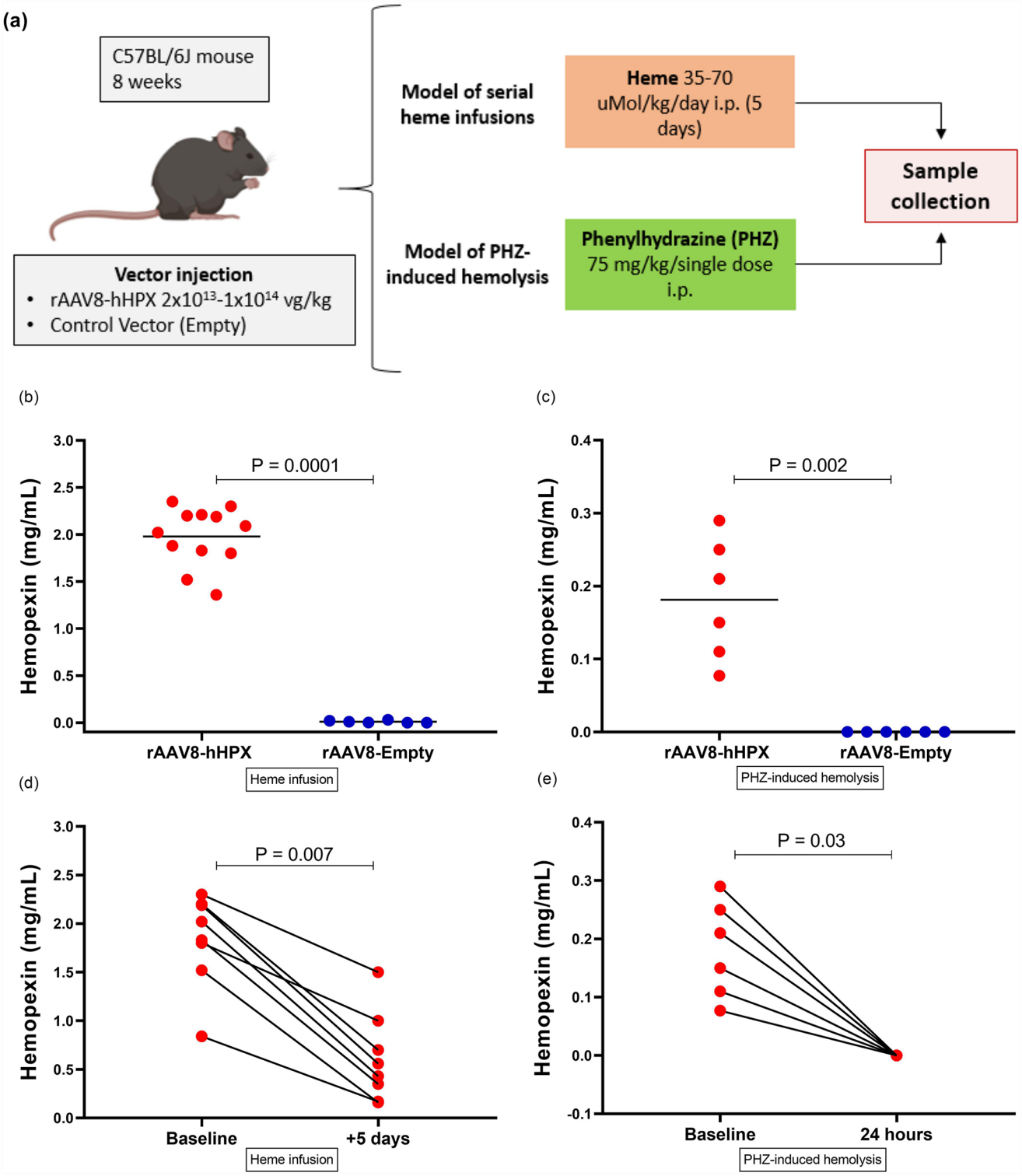


Figure 6. (A) Experimental design: 8-week-old mice were transduced with 2 × 1013–1 × 1014 vg/kg rAAV8-hHPX or control vector (rAAV-empty) and separated into two experiments. Part ($n = 18$) received serial infusions of heme 35–70 µmol/kg i.p. daily for five days, and were euthanized on the fifth day. Part of the animals ($n = 6$) received a single infusion of phenylhydrazine 75 mg/kg i.p. and were euthanized after 24 h or assessed for survival. (B and C) Confirmation of hHPX expression restricted to rAAV8-hHPX-treated animals, 24 h before the experiments; horizontal bar represents median, and *P* values are from the Mann–Whitney test. (D) hHPX levels before and after (day + 5) serial heme infusions showing hHPX consumption in all mice. (E) hHPX levels before and after (+ 24 h) PHZ-induced hemolysis showing hHPX consumption; horizontal bar represents median, and *P* values are from the Wilcoxon test. Levels of hHPX were measured by ELISA.

not capable of discriminating free heme from protein-bound heme, so that we are not able to measure the effectiveness of our strategy by measuring heme. Accordingly, in the study that explored HPX gene transfer using non-viral vectors, levels of circulating heme were not significantly reduced,[32] which could be related to these methodological limitations.

Safety is a critical issue in gene transfer studies, particularly when long-term effects are expected. Although

Table 3. Hematological parameters after heme challenges or PHZ-induced hemolysis.

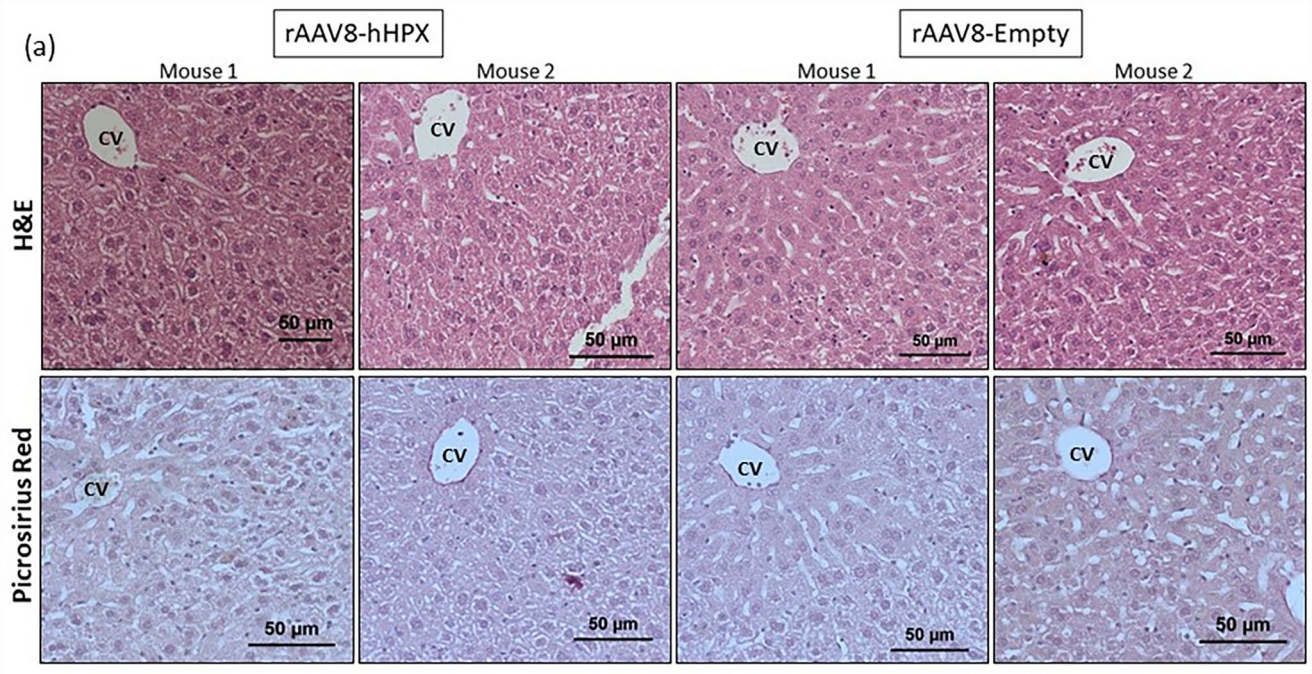
	rAAV8-hHPX	rAAV8-empty	P^a
Heme infusion			
Leukocytes (10^3/μL)	3.7 (2.1–5.4)	2.6 (1.9–5.6)	0.85
Erythrocytes (10^6/μL)	8.4 (7.4–10.2)	6.6 (5.4–8.1)	0.06
Platelets (10^3/μL)	1108 (654–1260)	963 (871–1074)	0.18
PHZ infusion			
Leukocytes (10^3/μL)	106.0 (63.4–120.1)	113.1 (35.7–137.2)	0.95
Erythrocytes (10^6/μL)	5.4 (4.8–5.7)	5.3 (4.9–5.9)	0.71
Platelets (10^3/μL)	715 (354–834)	549 (527–786)	0.77

Results shown as median and interquartile range (IQR); $n = 6–9$.
hHPX: human hemopexin; PHZ: phenylhydrazine.
[a]Mann–Whitney test.

Table 4. Biochemical parameters of mice after serial heme infusion induction or hemolysis with PHZ.

	rAAV8-hHPX	rAAV8-empty	P^a
Heme infusion			
ALT (U/L)	17.5 (12.0–20.7)	20.0 (15.0–23.0)	0.62
AST (U/L)	38.5 (33.2–52.7)	44.0 (34.0–52.0)	0.62
PHZ-induced hemolysis			
ALT (U/L)	25.5 (21.2–27.7)	30.0 (27.0–40.0)	0.02
Creatinine (U/L)	0.19 (0.14–0.24)	0.16 (0.15–0.18)	0.20

Results shown as median and interquartile range (IQR); $n = 6–8$ per group.
PHZ: phenylhydrazine; hHPX: human hemopexin; ALT: alanine transaminase; AST: aspartate transaminase.
[a]Mann–Whitney test.



(b)

	rAAV8-hHPX					rAAV8-Empty				
	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
Inflammatory infiltrate	None	None	None	None	None	None	None	None	None	None
Necrosis	Single-cell	Single-cell	None	None	Single-cell	Single-cell	Single-cell	Single-cell	Single-cell	Single-cell
Fibrosis	None	None	None	None	None	None	None	None	None	None

Figure 7. (A) Representative images of liver sections stained with H&E or Picrosirius Red from mice treated with rAAV8-hHPX or control (rAAV8-Empty) vectors, 24 h after infusion of PHZ 75 mg/kg. No evidence of inflammation, fibrosis, or necrosis were observed. Original magnification: 200×. (B) Results from individual mice (M). $n = 5$ per group. Analysis performed by two blinded observers. Single-cell necrosis denotes sparse and rare events. CV: centrilobular vein.

AAV-based strategies are considered safe in regard to the risk of insertional mutagenesis and innate-immunity-induced inflammation, we also explored safety parameters that could illustrate systemic adverse events such as hematological parameters and weight gain, with no signs of toxicity, even in models of heme challenge. In addition, transgene-related toxicity was also explored by assessing biochemical and histological parameters of liver toxicity. We hypothesized that increased delivery of heme-HPX complexes to hepatocytes could result in liver toxicity mediated either by iron or by changes in the cellular homeostasis of liver cells due to increased HPX production and of heme metabolism. Again, no signs of toxicity were observed in two independent models of heme challenge.

Our study has limitations that need to be acknowledged. First, assessment of the effect of our strategy on free heme levels, which would be a very intuitive efficacy parameter, is not feasible using classical methods used in the literature,[34] so that we had to rely on the consumption of HPX upon heme challenge. Second, we did not explore the benefits of our strategy in a model of a condition associated with chronic heme-mediated inflammation, which represents the most attractive target for our therapeutic strategy. In fact, the focus of our study was to demonstrate the feasibility and the safety of long-term HPX expression. Ongoing studies are exploring the effect of our vector in classical models of SCD.

Conclusions

In conclusion, we demonstrate that gene therapy with an AAV8-based vector is capable of promoting the long-term expression of HPX, which is consumed by challenges with heme. Long-term transgenic expression of HPX was not associated with signs of systemic or liver toxicity. Further studies are warranted to explore the benefit and safety of this strategy in animal models of conditions associated with sustained free heme release such as SCD.

AUTHORS' CONTRIBUTIONS

FDL contributed to study design; obtained and processed samples from experimental animals; performed biochemical/hematological assays, ELISA tests, real-time PCR, and Western blot and histology; and analyzed data and drafted the manuscript. BWH contributed with Western blot and real-time PCR assays and statistical analyses. CRPDM and ITB-J contributed to obtain samples from experimental animals. FFC oversaw and provided resources and infra-structure for all the tests. EVDP designed the study, oversaw and provided resources and infra-structure for all the tests, contributed to data analysis, and drafted the manuscript. All collaborators revised and approved the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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