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Dietary supplementation with sulforaphane attenuates liver damage and heme overload in a sickle cell disease murine model

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Sickle cell disease (SCD) is a recessively inherited blood disorder caused by abnormal β-globin production. The β-globin mutation changes erythrocyte morphology into a sickle shape and increases erythrocyte vulnerability to hemolysis. Oxidative stress and concomitant inflammation eventually result in damage to multiple organs. Nrf2 is a master regulator of the oxidative stress response, homeostasis, and metabolism. Keap1 modulates Nrf2 protein levels; Nrf2 inducers alter nuclear Nrf2 levels by interacting with Keap1. Genetic modification of Keap1 helps to reduce inflammation and tissue damage in SCD model mice through Nrf2 induction. Here, we investigated the benefits of a mild and safe Nrf2 agonist, sulforaphane (SFN), in ameliorating SCD pathology in a murine model. SFN is a phytochemical and is found in cruciferous vegetables as its inert precursor, glucoraphanin. We found that dietary SFN administration for 14 days or 2 months increased the expression of Nrf2-dependent cytoprotective genes, but SFN uptake did not have deleterious effects on the food consumption and growth of SCD model mice. SFN ameliorated the liver damage of SCD mice, which could be validated by the rescue of liver function and the significantly reduced liver necrotic area. SFN administration also helped to eliminate heme released from lysed sickle cells. These results indicate that dietary supplementation with SFN relieves SCD symptoms by inducing Nrf2 and support our contention that SFN is a potential drug for the long-term treatment of children with SCD. **© 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license. (http://creativecommons.org/licenses/by-nc-nd/4.0/)**

Sickle cell disease (SCD) is a recessive Mendelian hemoglobinopathy affecting millions of people worldwide and remains one of the major causes of morbidity and mortality globally. SCD results from a single amino acid substitution at the sixth residue of β-globin [1]. Hence, the mutant hemoglobin produced is called hemoglobin S (HbS). When deoxygenated, HbS molecules polymerize and form the characteristic sickled red blood cells (RBCs) [2]. Recurrent sickling of RBCs alters their membrane structure, making them highly adhesive to the vascular wall and other inflammatory cells, which facilitates intermittent vaso-occlusion; these changes eventually lead to ischemia–reperfusion injury and oxidative tissue damage [3]. Additionally, in the microvasculature, free heme and iron derived from hemolysis also exhibit pro-oxidative and pro-inflammatory properties and further increase the risk of vaso-occlusion [4,5].

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Endothelial cells activated as an inflammatory response recruit leukocytes, platelets, monocytes, and other cells to sustain an inflammatory state. As a result, homozygous SCD patients have severe multiple organ damage.

Currently, there are two drugs, hydroxyurea (HU) and L-glutamine, approved by the US Food and Drug Administration (FDA) for the treatment of SCD. HU has been found to increase fetal hemoglobin (HbF) and consequently improve the pathological effects of SCD. However, HU treatment generates adequate levels of HbF in approximately half of patients to alleviate the symptoms [6]. This therapeutic limitation highlights the need for alternate pharmacological interventions for SCD.

Transcription factor Nrf2 (nuclear factor erythroid 2-like 2) is a master regulator of the cellular response against oxidative stress and plays a vital role in cytoprotection against insults [7,8]. Keap1 (Kelch-like ECH-associated protein 1) is an adaptor protein for Cullin3-based ubiquitin E3 ligase. Keap1 acts as a stress sensor for electrophilic and oxidative insults and transmits the signals to Nrf2 [8,9]. Under unstressed conditions, Nrf2 is ubiquitinated by the Keap1 −Cullin3 ubiquitin E3 ligase complex in the cytoplasm and constitutively degraded via the proteasome [8]. However, upon exposure to reactive oxygen species (ROS) or electrophiles, the cysteine residues of Keap1 are modified, leading to disturbances in Nrf2 degradation. As a consequence, Nrf2 rapidly accumulates in the nucleus and forms a heterodimer with small Maf (sMaf) family proteins. The Nrf2 −sMaf heterodimer binds to cis-regulatory elements referred to as antioxidant-responsive elements [10], or electrophile-responsive elements [11], or collectively named CNC−sMaf binding elements (CsMBE) [12].

The Nrf2−sMaf heterodimer is responsible for the expression of various cytoprotective genes, such as genes encoding detoxification enzymes, including NADPH:quinone oxidoreductase 1 (encoded by *Nqo1*) and glutamate cysteine ligase catalytic and modifier subunits (*Gclc* and *Gclm*), and ROS elimination antioxidant enzymes, including heme oxygenase 1 (*Hmox1*) and peroxyredoxin 1 (*Prdx1*) [13−17]. Notably, both genetic Nrf2 activation and chemical Nrf2 induction lead to the amelioration of inflammation and tissue damage in sickle cell model mice [18]. In addition, the role of *Hmox1* in relieving inflammation and vaso-occlusion in SCD murine model mice has been well documented [19]. However, our previous approach used a genetic mouse model and highly efficacious drugs, which provides information about the mechanism but may not be a realistic treatment option in SCD patients. To this end, we examined the efficacy of mild Nrf2-inducing drugs in relieving SCD symptoms for future clinical use.

Sulforaphane (SFN) is an intriguing candidate in this category. SFN is an isothiocyanate found naturally in cruciferous vegetables as its inert precursor, glucoraphanin, which is hydrolyzed to SFN, a bioactive molecule, in the presence of myrosinase. Myrosinase can be found endogenously in the cruciferous vegetables themselves or is produced by the microflora in the colon [20]. SFN has been reported to induce Nrf2 signaling, as SFN binds directly to the thiol group of the cysteine 151 residue of Keap1 [21], leading to the inactivation of Keap1 ubiquitin ligase activity [22,23]. SFN is a safe drug that exhibits minimal adverse effects. However, SFN has been assumed to be a mild Nrf2 inducer and used as a health supplement. The efficacy of SFN for overt and severe disorders such as SCD has not been well examined or verified.

SCD is characterized by chronic inflammation and recurrence of acute attacks of oxidative tissue damage. In this study, we hypothesized that children with SCD can be protected from chronic tissue damage and/or acute oxidative tissue damage with SFN. Therefore, we examined whether oral administration of SFN ameliorates the symptoms of SCD model mice through Nrf2 activation. We found that dietary intake of glucoraphanin for 2 weeks relieved induced liver damage in the humanized SCD model mice. SFN also enhanced heme elimination in the liver. These results thus indicate that dietary intake of the mild Nrf2 inducer SFN improves liver damage in SCD model mice, supporting our contention that SFN is a potential drug for treatment of children with SCD.

Methods

Mice

We used both male and female homozygous SCD (hα/hα, βS/βS) and wild-type (WT) homozygous (hα/hα, βA/βA) mice generated by Townes and colleagues [24]. All animal experiments were approved by the Animal Care Committee at Tohoku University.

Broccoli sprout powder preparation

Broccoli sprout extract powder containing the SFN precursor glucoraphanin was industrially produced by Kagome Company, Ltd. (Chūō, Tokyo, Japan). Broccoli sprouts were grown from specially selected seeds for 1 day after germination. One-day-old broccoli sprouts were then boiled at 95°C for 30 min, and sprout residues were removed by filtration. The boiling water extract was mixed with a waxy cornstarch dextrin and then spray dried to yield the broccoli sprout extract powder [25].

Glucoraphanin administration

Four-week-old mice were fed broccoli sprout extract powder (obtained from Kagome Co., Ltd.) + MF (4.4% w/w) or MF (Oriental Yeast Co., Ltd., Tokyo). After continuous feeding for 2 weeks or 2 months, mice were subjected to analysis. Food intake and weight were recorded every day.

RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted with Sepazol RNA I Super G reagent (Nacalai Tesque) and reverse-transcribed with a ReverTra Ace

qPCR RT Kit (Toyobo) according to the manufacturer's instructions. The resulting cDNA was used as a template for quantitative reverse transcription polymerase chain reaction (RT-qPCR) using TaqMan with a QuantStudio 6 Real-Time PCR Analyser (Thermo Fisher Scientific). The primers used are described in the Supplementary Data (online only, available at www.exphem.org). The abundance of mRNA levels was normalized to *Hprt* abundance.

Plasma biochemistry

Plasma levels of alanine transferase (ALT), aspartate aminotransferase (AST), total bilirubin, and direct bilirubin were measured with a FUJI DRI-CHEM 7000 biochemical auto analyzer (Fujifilm). Indirect bilirubin was calculated by subtracting direct bilirubin from total bilirubin. Total plasma heme levels were measured calorimetrically at 400 nm using a Quanti-Chrome Heme Assay Kit (BioAssay Systems).

Reticulocyte count

The reticulocyte count was determined by flow cytometry according to the protocol previously described [26]. Samples were analyzed using a FACS Canto II (BD Biosciences).

Urine iron measurement

Urine iron content was measured using an iron assay kit according to the manufacturer's instructions (Metallo assay, Metallogenics, Chiba, Japan). Briefly, R-A buffer was added to the urine samples, and iron levels were determined based on the ferrozine method by measuring the absorbance twice (before and after adding R-R Chelate color) at 570 nm using a microplate reader FlexStation 3.

Hematological analysis

Hematological indices were measured using an automatic blood cell analyzer (Nihon Kohden).

Histological analysis

Livers were fixed in Mildform 10N (Wako Pure Chemical Industries) and processed into paraffin-embedded tissue sections. The liver sections were stained with Masson trichrome. The sections were analyzed using a Leica LD2500 microscope. Liver sections were stained with Prussian blue to detect iron and with anti-8-hydroxydeoxyguanosine (8-OHdG) antibody to detect oxidized nucleoside. Necrotic areas were measured for each mouse using BZ Analyzer software (KEYENCE) and are expressed as percentages of the total area.

Results

Glucoraphanin has no effect on food intake and growth in treated mice

To assess the therapeutic effect of SFN on SCD, we used the SCD homozygous ($h\beta^{S/S}$) and normal control ($h\beta^{A/A}$) murine models previously established by Townes and colleagues [24,27]. The $h\beta^{S/S}$ and $h\beta^{A/A}$ mice carry knocked-in human mutated β^S or normal β^A globin genes in place of mouse β-globin genes, respectively. These mice also carry knocked-in human α globin gene in place of mouse α genes. Because many of the SCD patients in Africa and India are infants, we hypothesized that SFN may be a well-tolerated, nontoxic alternative to currently available medications, such as HU, for use in children; hence, we used 4-week-old mice right after weaning for these experiments. The $h\beta^{A/A}$ and $h\beta^{S/S}$ mice were provided with either MF feed containing 4.4% broccoli sprout powder (SFN) or MF feed (vehicle) for 14 days *ad libitum*. Broccoli sprout extract powder was prepared from 1-day-old broccoli sprouts. We recorded the food intake and body weight every day and found that there were no apparent treatment-related effects on food consumption in any of the four groups (Figure 1A). The SFN-treated $h\beta^{S/S}$ mice on average ingested 3.64 ± 0.39 g of glucoraphanin-enriched MF-feed diet (containing 0.160 ± 0.02 g of glucoraphanin) per day, and vehicle-treated $h\beta^{S/S}$ mice ingested 3.85 ± 0.35 g of MF-feed diet per day. Similarly, the SFN- and vehicle-treated $h\beta^{A/A}$ mice ate 3.73 ± 0.26 g of glucoraphanin-enriched feed diet (containing 0.163 ± 0.01 g of glucoraphanin) and 3.87 ± 0.10 g MF-feed diet per day, respectively. Glucoraphanin is known to be biotransformed to SFN in the intestine of mice [28]. The glucoraphanin (SFN)-treated $h\beta^{A/A}$ and $h\beta^{S/S}$ model mice also had a growth curve similar to that of vehicle-treated $h\beta^{A/A}$ and $h\beta^{S/S}$ mice (Figure 1B), indicating that glucoraphanin did not have deleterious effects on food consumption and growth of SCD model mice.

Dietary intake of glucoraphanin induces mild Nrf2 activation

To determine whether SFN treatment increases Nrf2 activity, we first examined expression levels of Nrf2 target genes *Nqo1* and *Gstm1* in the liver of mice treated with glucoraphanin. We found that the mRNA levels of *Nqo1* and *Gstm1* in the liver had also increased 1.9 and 2.5 times, respectively, in RT-qPCR analyses of SFN-treated $h\beta^{S/S}$ mouse livers (Figure 1C). The $h\beta^{A/A}$ mice had less induction of Nrf2 target genes than did the $h\beta^{S/S}$ mice. Thus, dietary SFN treatment resulted in mild but significant induction of Nrf2 activity and its target gene levels that reinforced the antioxidant defense of the liver tissue. These effects of SFN treatment are consistent with those of expected Nrf2 inducers that will be used in long-term therapy of SFN children.

Two-week SFN treatment ameliorates liver damage in SCD mice

Because SFN increased the Nrf2-dependent cytoprotective genes in SCD mice, we next investigated whether glucoraphanin treatment helps to ameliorate or reverse liver damage, as liver damage has been reported to be a characteristic feature of SCD mice [27]. Therefore, we assessed whether liver damage was relieved by SFN treatment. Notably, we found that the necrotic area observed in vehicle-treated $h\beta^{S/S}$ mice (*yellow arrowheads*) was

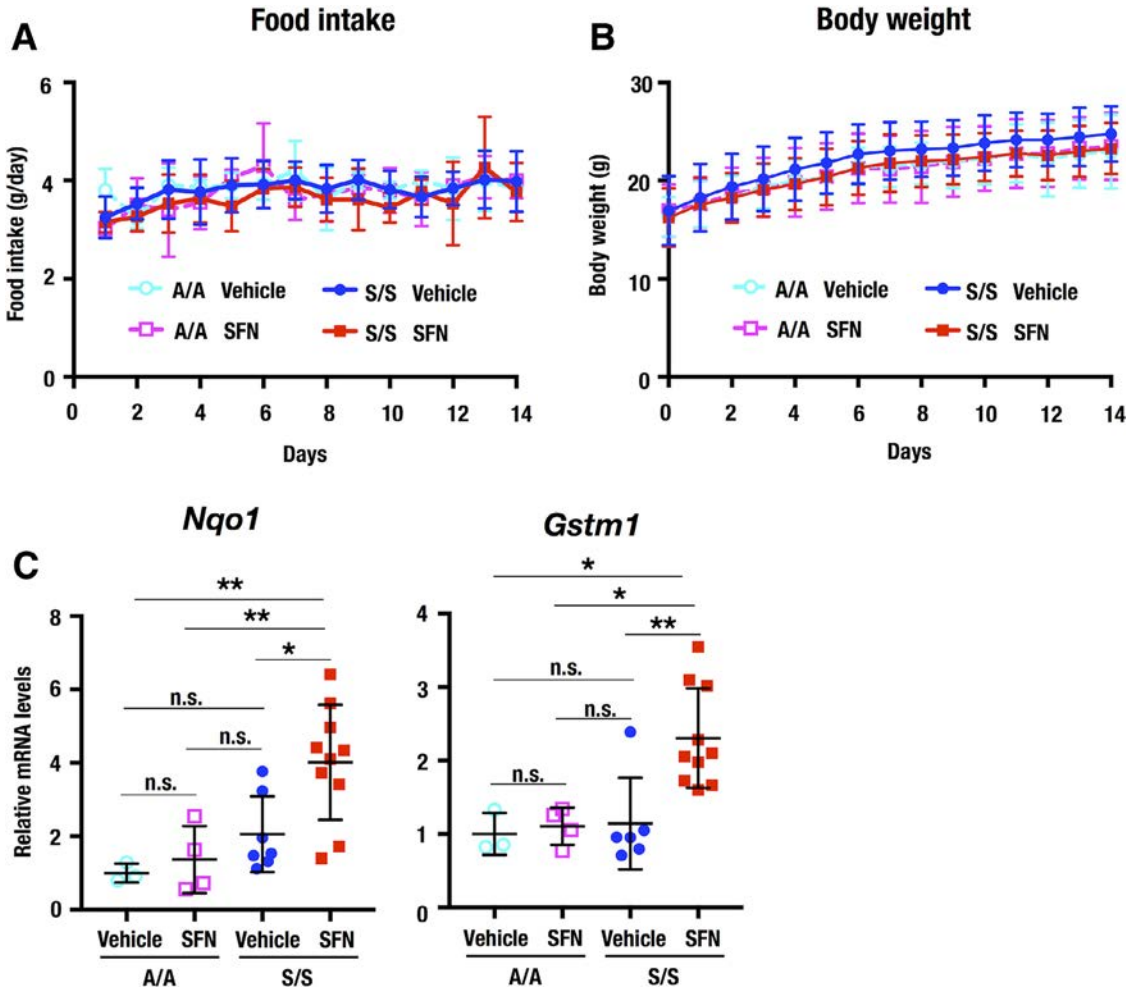

Figure 1. Dietary intake of glucoraphanin induces mild Nrf2 activation and has no effect on food intake or growth in treated mice. **(A)** Food intake of the vehicle-treated h$\beta^{A/A}$ ($n = 3$), SFN-treated h$\beta^{A/A}$ ($n = 4$), vehicle-treated h$\beta^{S/S}$ ($n = 6$), and SFN-treated h$\beta^{S/S}$ ($n = 6$) mice. Food intake was recorded every day. **(B)** Growth curves for the vehicle-treated h$\beta^{A/A}$ ($n = 3$), SFN-treated h$\beta^{A/A}$ ($n = 4$), vehicle-treated h$\beta^{S/S}$ ($n = 6$), and SFN-treated ($n = 6$) h$\beta^{S/S}$ mice. A steady time-dependent weight gain was observed in all groups. **(C)** Relative mRNA levels of Nrf2 target genes *Nqo1* (NADPH:quinone oxidoreductase 1) and *Gstm1* (glutathione *S*-transferase Mu1) in the vehicle-treated h$\beta^{A/A}$, SFN-treated h$\beta^{A/A}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ livers. Values are expressed as the mean ± standard deviation (SD). *$p < 0.05$; ** $p < 0.01$; n.s. = not significant (one-way analysis of variance).

diminished in SFN-treated h$\beta^{S/S}$ mice (Figure 2A). Histological analysis revealed that focal areas of necrosis (*yellow arrowheads*) in the livers were significantly decreased in SFN-treated h$\beta^{S/S}$ mice compared with vehicle-treated h$\beta^{S/S}$ mice and, as expected, were completely absent in the vehicle- and SFN-treated h$\beta^{A/A}$ mice (Figure 2B, C). Additionally, the plasma levels of ALT and AST, which are important markers of liver damage, were both significantly relieved in SFN-treated mice (Figure 2D). These results thus indicate that SFN treatment for 2 weeks effectively ameliorated liver damage in SCD mice.

SFN treatment is insufficient for reduction of hemolysis in SCD mice

To examine whether the 2-week SFN treatment improves anemia in SCD mice, we also analyzed red blood cell numbers and hemoglobin levels in the peripheral blood. We found that there were no significant differences in these red cell parameters with SFN treatment (Figure 3A, B). To analyze red cell production, which reflects hemolysis and compensatory stress erythropoiesis status, we examined the reticulocyte levels in the peripheral blood and spleen weights. The h$\beta^{S/S}$ mice had higher reticulocyte levels and spleen weight compared with h$\beta^{A/A}$ mice, reflecting that hemolysis and compensatory stress erythropoiesis occurred in the h$\beta^{S/S}$ mice (Figure 3C, D). We also found that reticulocyte counts and spleen weights were not decreased in the SFN-treated h$\beta^{S/S}$ mice compared with vehicle-treated h$\beta^{S/S}$ mice. These results indicated that hemolysis cannot be improved and that the 2-week SFN treatment is not sufficient to reduce hemolysis in SCD mice.

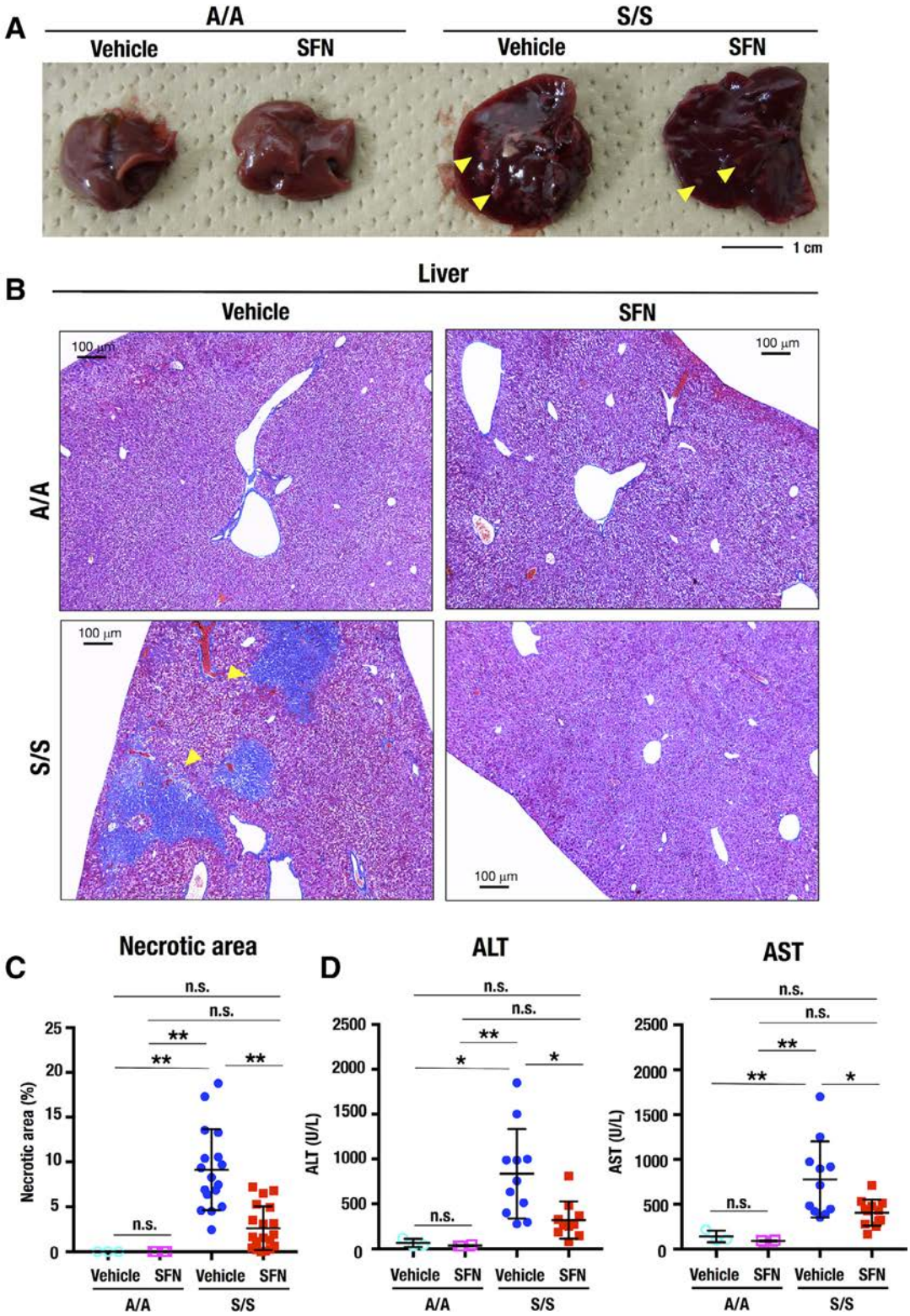

Figure 2. Two-week SFN treatment ameliorates liver damage in SCD mice. **(A)** Gross specimens of the livers of vehicle-treated h$\beta^{A/A}$, SFN-treated WT h$\beta^{A/A}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ mice. The *yellow arrowheads* indicate necrosis. **(B)** Liver pathology of vehicle-treated h$\beta^{A/A}$, SFN-treated WT h$\beta^{A/A}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ mice. The liver cross sections were stained with Masson trichrome. *Yellow arrowheads* indicate necrosis. **(C)** Quantitative analysis of the necrotic area in the liver. Necrotic areas were sized for each mouse using BZ Analyzer software (KEYENCE) and are expressed as percentages of the total area. **(D)** ALT and AST levels in the plasma. Values are expressed as the mean ± standard deviation. *$p < 0.05$; **$p < 0.01$; n.s. = not significant (one-way analysis of variance).

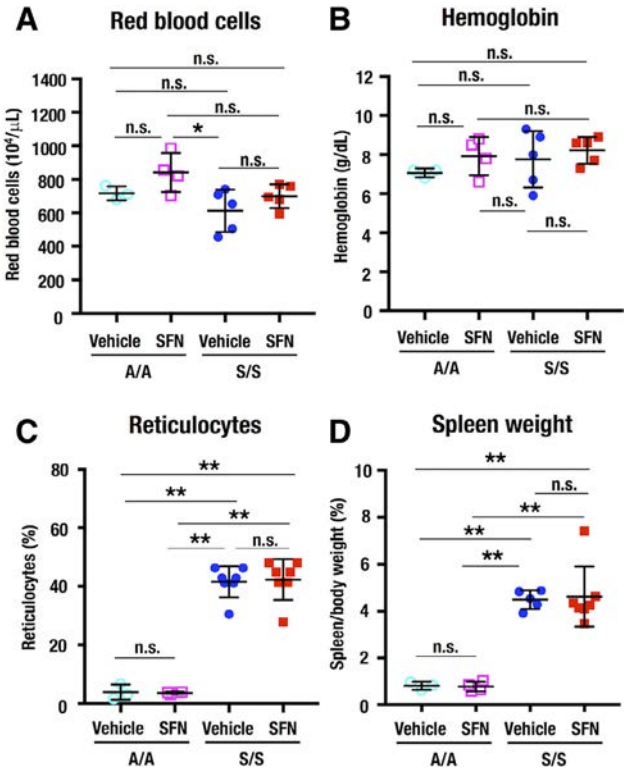

Figure 3. SFN treatment is insufficient for improvement of hemolysis in SCD mice. **(A–D)** Red blood cell numbers **(A)**, hemoglobin levels **(B)**, reticulocyte counts in the peripheral blood **(C),** and spleen weights **(D)** of the vehicle-treated $h\beta^{A/A}$, SFN-treated $h\beta^{A/A}$, vehicle-treated $h\beta^{S/S}$, and SFN-treated $h\beta^{S/S}$ mice. Values are expressed as the mean ± standard deviation. *$p < 0.05$; **$p < 0.01$; n.s. = not significant (one-way analysis of variance).

SFN treatment enhances heme detoxification in the liver of SCD mice

Hemolysis of sickled RBCs results in the release of high levels of heme into the bloodstream, and the increase in heme plays a role in activating endothelial cells and inducing ischemia–reperfusion injuries [29]. To determine whether SFN affects heme levels in plasma, we measured plasma heme levels along with the expression of one heme catabolism-related enzyme gene. We found that heme levels were increased in SFN-treated $h\beta^{S/S}$ mice compared with wild-type mice and heterozygotes ($h\beta^{A/S}$). Of note, the heme level was lower in SFN-treated $h\beta^{S/S}$ mice than in their vehicle-treated $h\beta^{S/S}$ counterparts, suggesting that enhanced heme detoxification induced by SFN treatment plays, at least partially, a role in ameliorating the liver damage of SCD mice (Figure 4A).

Heme is degraded into biliverdin, iron, and carbon monoxide by heme oxygenase 1 (HO-1, encoded by *Hmox1*) activity [29]. Biliverdin is further converted into bilirubin [30]. So, we next examined indirect and direct bilirubin levels in plasma. We found that both bilirubin levels were increased in $h\beta^{S/S}$ mice, but there was no difference in these parameters between the vehicle- and SFN-treated $h\beta^{S/S}$ mice (Figure 4B, C).

To assess iron deposition, we performed Prussian blue staining of the liver and found that iron deposition was markedly reduced in SFN-treated $h\beta^{S/S}$ mice compared with vehicle-treated $h\beta^{S/S}$ mice (Figure 4D, upper panels). Moreover, 8-OHdG staining in the liver sections of SFN-treated $h\beta^{S/S}$ mice also revealed reduced oxidized nucleosides (i.e., nuclear damage) compared with those of the vehicle-treated $h\beta^{S/S}$ mice (Figure 4D, lower panels). These results support our contention that heme detoxification by SFN treatment has a role in ameliorating liver pathology.

To determine the mechanism underlying relief of iron deposition, we quantified iron levels in the urine samples of all mice. We detected high iron levels in vehicle-treated $h\beta^{S/S}$ mice compared with $h\beta^{A/A}$ mice, but the iron levels were decreased in the SFN-treated $h\beta^{S/S}$ mice (Figure 4E). In fact, the iron levels in SFN-treated $h\beta^{S/S}$ mice were comparable to those of $h\beta^{A/A}$ mice. These results indicate that on SFN treatment, iron released is better recycled and not deposited in the liver, leading to the reduction in liver damage.

It is known that Nrf2 activates the expression of the *Hmox1* gene encoding HO-1, a rate-limiting enzyme of heme degradation [19,31]. The *Hmox1* mRNA levels of $h\beta^{S/S}$ mouse livers were much higher than those of $h\beta^{A/A}$ mouse livers, indicating the presence of substrate-mediated induction of *Hmox1* as a result of the abundant heme influx into liver from plasma (Figure 4F). SFN treatment did not change much the *Hmox1* level in SCD mouse livers, suggesting that substrate induction by the imported heme overpasses the *Hmox1* induction mediated by the SFN–Nrf2 pathway. The significant decrease in heme in SFN-treated SCD mice seems to reflect the induction of *Hmox1* in tissues other than the liver. We surmise that the decrease in heme in plasma and mild decrease in iron in urine of SFN-treated mice support the notion that iron is highly utilized in the bone marrow for stress erythropoiesis in SCD mice.

Two-month SFN treatment relieves liver damages

As a short-term (2-week) treatment resulted in improvement of liver damage and reduced the heme overload in the SFN-treated SCD mice, we next wanted to check what effect SFN has on SCD mice in long-term treatment. Hence, we treated 4-week-old SCD mice for 2 months (i.e., 8 weeks) until they were 12 weeks old. After the 2-month treatment, Nrf2 target gene *Nqo1* mRNA levels were upregulated in SFN-treated $h\beta^{S/S}$ mice compared with vehicle-treated $h\beta^{S/S}$ mice, suggesting that continuous Nrf2 activation is observed on long-term SFN treatment (Figure 5A). As was the case for the 2-week treatment, after 2 months of treatment we found SFN intake relieved

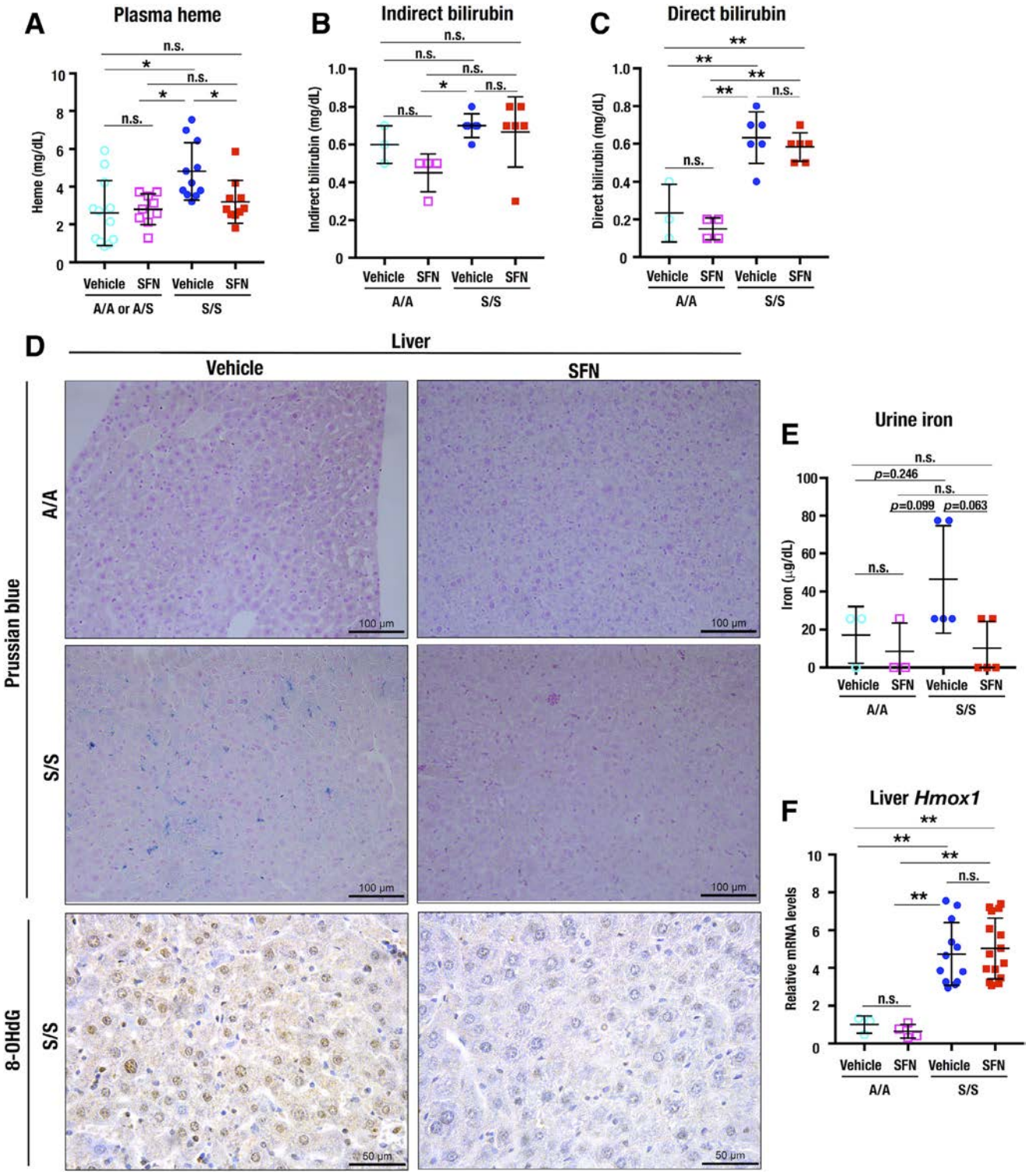

Figure 4. SFN treatment enhances heme detoxification in the liver of SCD mice. **(A)** Plasma heme levels of the vehicle-treated h$\beta^{A/A}$ and h$\beta^{A/S}$, SFN-treated h$\beta^{A/A}$ and h$\beta^{A/S}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ mice. **(B, C)** Indirect bilirubin **(B)** and direct bilirubin **(C)** levels of the vehicle-treated h$\beta^{A/A}$, SFN-treated h$\beta^{A/A}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ mice. **(D)** Prussian blue (upper panels) and 8-OHdG (lower panels) staining in cross sections of the liver indicating iron deposition and oxidized nucleoside (nuclear damage), respectively. **(E)** Iron content in urine of the vehicle-treated h$\beta^{A/A}$, SFN-treated h$\beta^{A/A}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ mice. **(F)** Relative *Hmox1* mRNA levels of the vehicle-treated h$\beta^{A/A}$, SFN-treated h$\beta^{A/A}$, vehicle-treated h$\beta^{S/S}$, and SFN-treated h$\beta^{S/S}$ livers. Values are expressed as the mean ± standard deviation. *$p < 0.05$; **$p < 0.01$; n.s. = not significant (one-way analysis of variance).

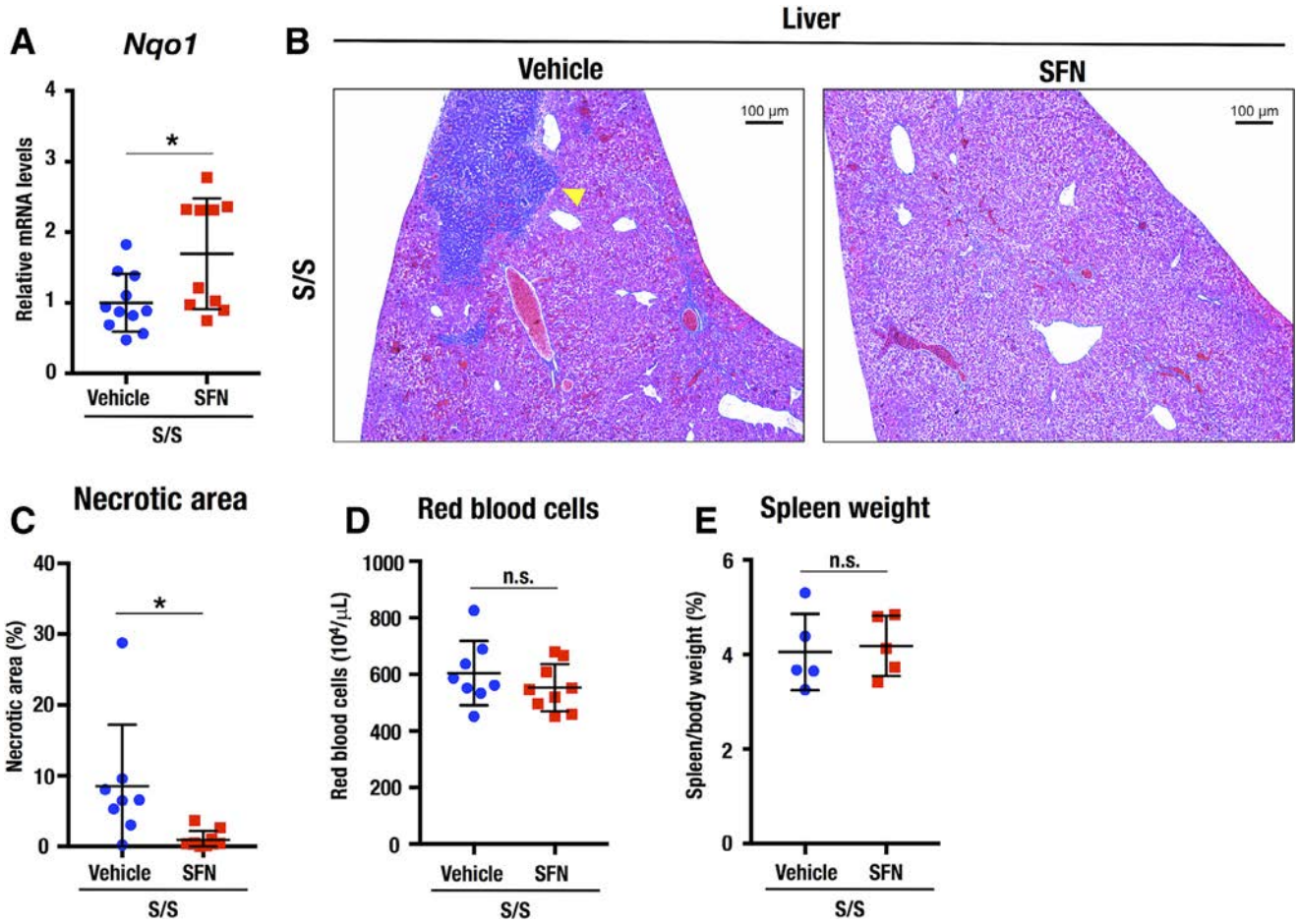

Figure 5. The liver-protective effect is maintained by continuous SFN treatment. **(A)** Relative mRNA levels of Nrf2 target gene *Nqo1* of 2-month vehicle- and SFN-treated h$\beta^{S/S}$ liver. **(B)** Liver pathology of 2-month vehicle- and SFN-treated h$\beta^{S/S}$ mice. The liver cross sections were stained with Masson trichrome staining. A *yellow arrowhead* indicates necrosis. **(C)** Quantitative analysis of the necrotic area in the liver of 2-month vehicle- and SFN-treated h$\beta^{S/S}$ mice. Necrotic areas were sized for each mouse using BZ Analyzer software (KEYENCE) and are expressed as percentages of the total area. **(D, E)** Red blood cell numbers **(D)** and spleen weights **(E)** of 2-month vehicle- and SFN-treated h$\beta^{S/S}$ mice. Values are expressed as the mean ± standard deviation. $^{**}p < 0.05$; n.s. = not significant (unpaired *t* test).

the liver damage, which could be confirmed by the decrease in liver necrosis (Figure 5B, C). Red blood cell numbers and spleen weights reflecting red cell production were not altered substantially by the 2-month SFN treatment (Figure 5D, E). These results thus indicate that the liver-protective effect of SFN is maintained during continuous SFN treatment of h$\beta^{S/S}$ mice up to 2 months.

Discussion

More than 100 years have passed since SCD was first described in the literature [32]. Over the years, many important studies have been carried out to elucidate the molecular and pathophysiological basis of the disease. However, translation of the knowledge obtained in academia to an effective treatment has been slow [33]. In this study, we provide preclinical lines of evidence related to the use of a natural Nrf2 inducer, SFN, to treat the complications associated with SCD, especially liver damage. As summarized in Figure 6A, we found that SFN treatment enhances heme and iron elimination, which relieves liver damage by eliminating oxidative stress. Given that SFN is a phytochemical that is isolated from edible cruciferous vegetables and hence can be delivered at presumably low toxicity and low cost, it will be an efficacious, tolerable, and practical alternative treatment, especially in children with SCD.

Although broccoli sprout powder contains other bioactive molecules such as folate, folate is inactivated on heating during the process of preparation of the supplement. The folate-to-glucoraphanin ratio is 1:15,000 (0.002 mg folate and 30 mg glucoraphanin/0.78 g of powder) in the broccoli sprout powder. Therefore, we assume that the folate level in broccoli sprout powder is very low and the amelioration of liver damage and reduction of heme levels are due primarily to the Nrf2 induction by SFN.

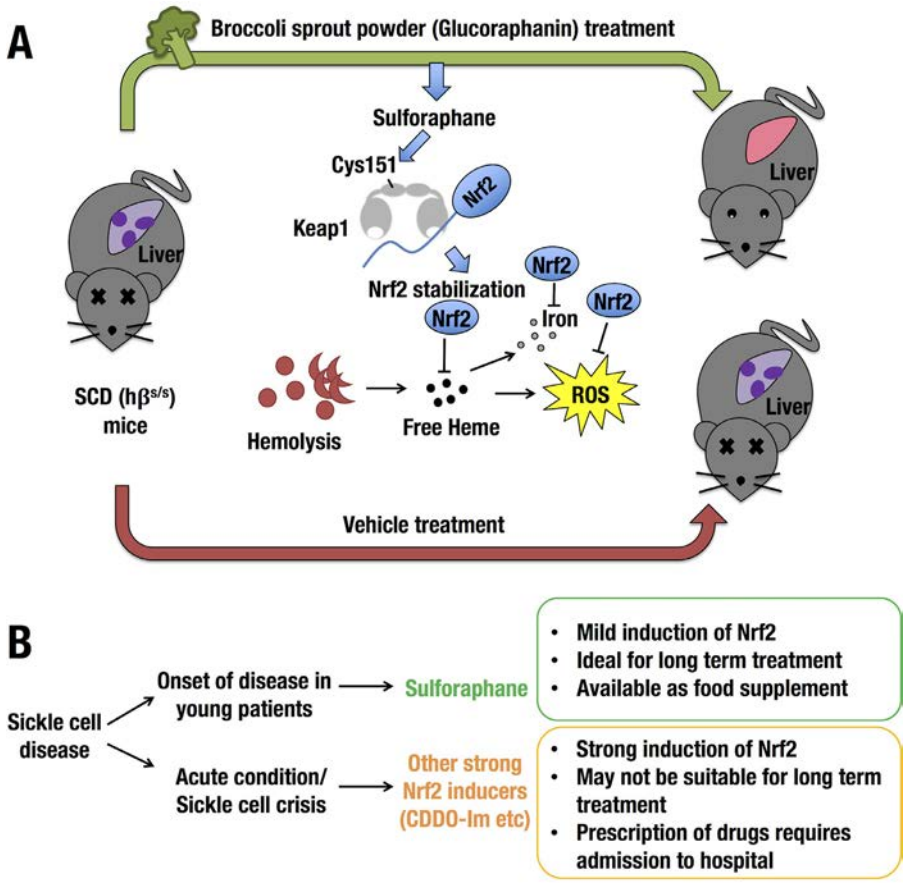

Figure 6. Therapeutic potential of SFN for SCD. **(A)** SFN binds to the cysteine (Cys) 151 residue of Keap1 protein, resulting in its structural modification. This, in turn, stabilizes the Nrf2 protein, which results in translation of a battery of cytoprotective genes. In the SCD murine model, SFN ameliorates organ damage by reducing oxidative stress, enhancing heme detoxification, and better recycling iron through enhanced induction of Nrf2 protein. **(B)** SFN is a phytochemical that is found in abundance in cruciferous vegetables. By virtue of its mild Nrf2 activation activity, it can be administered for a long duration without any associated toxicity. Hence, it is an ideal drug for young sickle cell patients during the onset of disease. Under severe conditions, such as those observed during sickle cell crises, a much stronger synthetic Nrf2 inducer can be prescribed for a short period to avoid severe oxidative injury to the tissues.

Previous reports have described the relationship between Nrf2 activation and cancer risk as a U-shaped curve. Although too high or too low Nrf2 activation can be detrimental and increase the risk of cancer, a mild and transient activation within the biologically effective dose (BED) and maximum tolerated dose (MTD) can confer cancer prevention [34,35]. As summarized in Figure 6B, this is the rationale behind the use of SFN in the present project instead of other stronger Nrf2 inducers. SFN would be an ideal chemical for the long-term treatment of children with SCD under unstressed conditions, because it exhibits no obvious toxicity. However, in acute conditions such as SCD crises, much stronger Nrf2 inducers than SFN should be administered without any delay to avoid severe development of oxidative tissue injury.

Conclusions

Our results indicate that Nrf2 activation by SFN treatment improves SCD symptoms by promoting the elimination of heme released by hemolysis and reversing liver damage by transcriptional activation of cytoprotective enzymes. These diverse effects of SFN suggest its future clinical use as a viable treatment for SCD.

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References

1. Ingram VM. Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell haemoglobin. Nature. 1957;180:326–328.
2. Bunn HF. Pathogenesis and treatment of sickle cell disease. N Engl J Med. 1997;337:762–769.
3. Li C, Wright MM, Jackson RM. Reactive species mediated injury of human lung epithelial cells after hypoxia–reoxygenation. Exp Lung Res. 2002;28:373–389.
4. Camus SM, De Moraes JA, Bonnin P, et al. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. Blood. 2015;125:3805–3814.
5. Repka T, Hebbel RP. Hydroxyl radical formation by sickle erythrocyte membranes: role of pathologic iron deposits and cytoplasmic reducing agents. Blood. 1991;78:2753–2758.
6. Steinberg MH, Lu ZH, Barton FB, Terrin ML, Charache S, Dover GJ. Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Multicenter study of hydroxyurea. Blood. 1997;89:1078–1088.
7. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997;236:313–322.
8. Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. 1999;13:76–86.
9. Kobayashi A, Kang MI, Okawa H, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol. 2004;24:7130–7139.
10. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem. 1991;266:11632–11639.
11. Friling RS, Bensimon A, Tichauer Y, Daniel V. Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. Proc Natl Acad Sci USA. 1990;87:6258–6262.
12. Otsuki A, Suzuki M, Katsuoka F, et al. Unique cistrome defined as CsMBE is strictly required for Nrf2-sMaf heterodimer function in cytoprotection. Free Radic Biol Med. 2016;91:45–57.
13. Malhotra D, Portales-Casamar E, Singh A, et al. Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. Nucleic Acids Res. 2010;38:5718–5734.
14. Chorley BN, Campbell MR, Wang X, et al. Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha. Nucleic Acids Res. 2012;40:7416–7429.
15. Hirotsu Y, Katsuoka F, Funayama R, et al. Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. Nucleic Acids Res. 2012;40:10228–10239.
16. Suzuki T, Motohashi H, Yamamoto M. Toward clinical application of the Keap1-Nrf2 pathway. Trends Pharmacol Sci. 2013;34:340–346.
17. Mitsuishi Y, Taguchi K, Kawatani Y, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. Cancer Cell. 2012;22:66–79.
18. Keleku-Lukwete N, Suzuki M, Otsuki A, et al. Amelioration of inflammation and tissue damage in sickle cell model mice by Nrf2 activation. Proc Natl Acad Sci USA. 2015;112:12169–12174.
19. Belcher JD, Mahaseth H, Welch TE, Otterbein LE, Hebbel RP, Vercellotti GM. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. J Clin Invest. 2006;116:808–816.
20. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. Cancer Epidemiol Biomarkers Prev. 1998;7:1091–1100.
21. Saito R, Suzuki T, Hiramoto K, et al. Characterizations of three major cysteine sensors of Keap1 in stress response. Mol Cell Biol. 2016;36:271–284.
22. Dinkova-Kostova AT, Talalay P. Direct and indirect antioxidant properties of inducers of cytoprotective proteins. Mol Nutr Food Res. 2008;52(Suppl 1):S128–S138.
23. Suzuki T, Yamamoto M. Stress-sensing mechanisms and the physiological roles of the Keap1-Nrf2 system during cellular stress. J Biol Chem. 2017;292:16817–16824.
24. Ryan TM, Ciavatta DJ, Townes TM. Knockout-transgenic mouse model of sickle cell disease. Science. 1997;278:873–876.
25. Yao W, Zhang JC, Ishima T, et al. Role of Keap1-Nrf2 signaling in depression and dietary intake of glucoraphanin confers stress resilience in mice. Sci Rep. 2016;6:30659.
26. Nobes PR, Carter AB. Reticulocyte counting using flow cytometry. J Clin Pathol. 1990;43:675–678.
27. Wu LC, Sun CW, Ryan TM, Pawlik KM, Ren J, Townes TM. Correction of sickle cell disease by homologous recombination in embryonic stem cells. Blood. 2006;108:1183–1188.
28. Fahey JW, Holtzclaw WD, Wehage SL, Wade KL, Stephenson KK, Talalay P. Sulforaphane bioavailability from glucoraphanin-rich broccoli: control by active endogenous myrosinase. PLoS One. 2015;10:e0140963.
29. Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, Jacob HS. Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species. Lab Invest. 1991;64:648–655.
30. Singleton JW, Laster L. Biliverdin reductase of guinea pig liver. J Biol Chem. 1965;240:4780–4789.
31. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol. 2003;24:449–455.
32. Herrick JB. Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. Originally published: Arch Intern Med (Chic). 1910;6:517–521. Re-published: Yale J Biol Med. 2001;74:179–184.
33. Suzuki M, Yamamoto M, Engel JD. Fetal globin gene repressors as drug targets for molecular therapies to treat the β-globinopathies. Mol Cell Biol. 2014;34:3560–3569.
34. Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? Carcinogenesis. 2010;31:90–99.
35. Yamamoto M, Kensler TW, Motohashi H. The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. Physiol Rev. 2018;98:1169–1203.

Supplementary Table 1. Primers and Probes for RT-qPCR.

Gene	Forward Primer	Reverse Primer	Probe
Hprt	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGG	ATCCAACAAAGTCTGGCCTGTATCCAAC
Nqo1	AGCTGGAAGCTGCAGACCTG	CCTTTCAGAATGGCTGGCA	ATTTCAGTTCCCATTGCAGTGGTTTGGG
Hmox1	CCA GCA ACA AAG TGC AAG ATTC	TCACATGGCATAAAGCCCTACAG	TCTCCGATGGGTCCTTACACTCAGCTTTCT
Gstm1	CCTATGATACTGGGATACTGGAACG	GGAGCGTCACCCATGGTG	CGCGGACTGACACACCCGATCC