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Pharmacokinetics and pharmacodynamics of single dose of inhaled nebulized sodium nitrite in healthy and hemoglobin E/β-thalassemia subjects

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ABSTRACT

Inhaled sodium nitrite has been reported to decrease pulmonary artery pressure in hemoglobin E/β-thalassemia (HbE/β-thal) patients with pulmonary hypertension. This study investigated the pharmacokinetics and pharmacodynamics of inhaled nebulized sodium nitrite in 10 healthy subjects and 8 HbE/β-thal patients with high estimated pulmonary artery pressure. Nitrite pharmacokinetics, fraction exhaled nitric oxide (FE_{NO}), estimated right ventricular systolic pressure (eRVSP) measured by echocardiography, and platelet activation were determined. Nebulized sodium nitrite at doses used in this study (37.5 and 75 mg for healthy subjects and 15 mg for HbE/β-thal patients) was well tolerated and did not cause changes in methemoglobin levels and systemic blood pressure. Absorption of inhaled nitrite was rapid with the absolute bioavailability of 18%. In whole blood, nitrite exhibited the dose-independent pharmacokinetics with clearance (CL) of 1.5 l/h/kg, volume of distribution (V_d) of 1.3 l/kg and half-life ($t_{1/2}$) of 0.6 h. CL and V_d of nitrite was higher in red blood cells (RBC) than whole blood and plasma. HbE/β-thal patients had lower nitrite CL and longer $t_{1/2}$ in RBC than healthy subjects. FE_{NO} increased immediately after inhalation. Following nitrite inhalation, eRVSP remained unchanged but platelet activation was suppressed as evidenced by inhibition of adenosine diphosphate (ADP)-induced P-selectin expression and increase in phosphorylated vasodilator-stimulated phosphoprotein ($P\text{-}VASP^{Ser239}$) in platelets. There were no changes in markers of oxidative and nitrosative stress after inhalation. Our results support further development of inhaled nebulized sodium nitrite for treatment of pulmonary hypertension in β-thalassemia.

1. Introduction

Recent data demonstrate increased oxidative stress and decreased nitric oxide (NO) in β-thalassemia, which are implicated in vascular complications including thrombosis, pulmonary hypertension and cardiac disorders [1,2]. Oxidative stress, chronic hypoxia and iron overload contribute to endothelial dysfunction in β-thalassemia, leading to NO deficiency as shown by decreased circulating nitrite [3–6]. In thalassemia, NO synthesis by NO synthase in endothelial cells is inefficient to maintain vasodilation, resting platelets, and vascular integrity [4,7]. Attempts to increase NO activity, either through phosphodiesterase-5 inhibition by sildenafil [8,9] or sodium nitrite inhalation [10], have been shown to decrease pulmonary artery pressure in β-thalassemia.

Nitrite anion (NO_2^-) is an endogenous NO derivative stored in blood and tissues. Under hypoxia and acidosis, nitrite is bioactivated to NO by heme-containing proteins resulting in hypoxic vasodilation [11] and platelet inhibition [12]. Nitrite is converted to NO selectively under hypoxia and acidosis as reported in exercising muscles [11] and lungs [13]. Nitrite inhalation has been shown to reduce pulmonary artery pressure in patients with Group 2 pulmonary hypertension associated

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with left heart failure with preserved ejection fraction [14,15]. Previously, our group demonstrated that nitrite inhalation transiently reduced pulmonary artery pressure without change in systemic blood pressure and methemoglobin levels in HbE/β-thal patients who had pulmonary hypertension [10]. Besides, inhaled nitrite suppressed platelet aggregation, P-selectin expression and platelet-leukocyte aggregates, and increased P-VASP^{Ser239} (a marker of cGMP/protein kinase G signaling) in platelets of healthy subjects [16].

The pharmacokinetics, safety and tolerability of nebulized sodium nitrite has been described in healthy subjects [17], but they were not known in thalassemia. Because most nitrite in blood reacts with hemoglobin in RBC, we hypothesized that the pharmacokinetics of nitrite would be altered in HbE/β-thal patients who had abnormal RBC and decreased nitrite reductase activity of hemoglobin E [18,19]. Here, the pharmacokinetics of inhaled sodium nitrite was investigated in healthy subjects and HbE/β-thal patients who had high estimated pulmonary artery pressure as a new study separated from our previous report [10]. The effects of nitrite inhalation on eRVSP measured by echocardiography and platelet activation were also assessed.

2. Materials and methods

2.1. Study setting and subjects

The study protocol was an open-label trial approved by the Ethical Clearance Committee on Human Rights Related Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (ID 03-56-27), and registered in Thai Clinical Trials Registry (TCTR20150518002). Written informed consent was obtained from each subject in compliance with the Declaration of Helsinki. Eighteen subjects, including 10 healthy subjects and 8 HbE/β-thal patients who had record of elevated eRVSP by echocardiography were enrolled in this study. Baseline blood test was determined at Pathological Laboratory of Faculty of Medicine Ramathibodi Hospital. eRVSP was measured at baseline and during inhalation by transthoracic echocardiography.

2.2. Sodium nitrite solution and administration

Sterile sodium nitrite solution (1,000 mg/20 ml) was prepared at Faculty of Pharmaceutical Sciences, Chulalongkorn University. As the reported maximum tolerated dose of inhaled sodium nitrite was 90 mg (1.2 mg/kg of body weight, mean body weight of 77 kg) [17], the healthy subjects received 37.5 and 75 mg of sodium nitrite inhalation in upright position (0.6 and 1.2 mg/kg of body weight, mean body weight of 60.7 kg). Because HbE/β-thal patients with pulmonary hypertension had limited exercise tolerance, anemia, high methemoglobin levels and low body weight, the dose for these patients was reduced to 15 mg (0.3 mg/kg of body weight, mean body weight of 48.1 kg) in order to minimize the risk of toxicity. The 15-mg dose has been shown to be effective to decrease pulmonary artery pressure [10]. Sodium nitrite was diluted with saline and placed in a nebulizer (Beurer IH25/1, Beurer Medical, Ulm, Germany). The inhalation time was 15 min at flow rate of 0.22 ml/min. Normal saline solution was used as control.

In a separate study, three healthy subjects who previously received sodium nitrite inhalation were administered intravenous sodium nitrite infusion at dose of 0.2 mg/min for 15 min.

2.3. Measurement of nitrite and nitrate

Venous blood samples were collected via catheter using heparin (143 units/10 ml) as anticoagulant at various time points including baseline and at time 0, 5, 10, 25, 40, 60, 90, and 120 min after the end of nebulization. Whole blood and RBC samples were mixed immediately with nitrite-stabilizing solution containing 0.8 M ferricyanide, 10 mM N-ethylmaleimide and 1% NP-40 in a 4:1 ratio (v/v, sample/stabilizing solution) [20]. Plasma and RBC were separated by centrifugation at 14,000 × *g* for 2 min. All blood samples were immediately frozen on dry ice and stored at −80 °C. Total urine excretion samples were collected at baseline and 120 min after the end of sodium nitrite administration to measure total volume. Then, 1 ml of urine at each time point was aliquoted and stored at −80 °C. Nitrite in whole blood, plasma, RBC, and urine was measured by the tri-iodide-based chemiluminescence-NO analyzer (CLD88; Eco Medics AG, Duernten, Switzerland) [21]. Plasma nitrate was measured by vanadium(III)-based chemiluminescence at 90 °C.

2.4. Measurement of FE_{NO}

Exhaled breath samples were collected at baseline and 0 and 10 min after the end of sodium nitrite administration using FE_{NO} offline collection kit collector attached to a reusable Mylar bag (ECO MEDICS AG, Duernten, Switzerland), which is impermeable and non-reactive to NO. FE_{NO} was measured by connecting a Mylar bag to a sample inlet system on a chemiluminescence-NO analyzer [22].

2.5. Safety

Blood pressure (systolic, diastolic and mean arterial) and heart rate were measured using a non-invasive automated blood pressure meter at various time points including baseline, every 5 min during inhalation and various time points after the end of inhalation. Methemoglobin levels and hematologic parameters were measured at baseline and 120 min after the end of inhalation.

2.6. Determination of pharmacokinetic parameters

Pharmacokinetic parameters of nitrite in whole blood, plasma and RBC were calculated by a non-compartmental model using PK-Solver [23]. Maximum concentration (C_{max}) and time to C_{max} (T_{max}) were directly obtained from the observed data. Area under the concentration-time curve from time zero to the last observation ($AUC_{0\text{-}120}$) was calculated using the linear trapezoidal rule. $AUC_{0\text{-}\infty}$ was calculated as the sum of $AUC_{0\text{-}120} + C_{last}/k_e$; where C_{last} is the last observed concentration and k_e is the terminal elimination rate constant based on linear regression fitted to last five data points. Elimination $t_{1/2}$ was calculated as $\ln 2/k_e$. Absolute bioavailability (F) was calculated from ratio of the dose-normalized area under the curve: $[AUC_{0\text{-}120}/dose]_{inhalation}/[AUC_{0\text{-}120}/dose]_{intravenous}$. The apparent total body CL and V_d were calculated as the following: $CL = dose.F/AUC_{0\text{-}\infty}$ and $V_d = dose.F/(AUC_{0\text{-}\infty}.k_e)$. Renal CL was calculated as UV/P; where U was the concentration of nitrite excreted in urine at time 120 min after the end of administration; V was the urine flow rate and P was the concentration of nitrite in plasma at time 120 min after the end of administration.

2.7. Echocardiography for measurement of eRVSP

The estimated pulmonary artery pressure was presented as eRVSP which was calculated as $eRVSP = 4(TRV_{max})^2 + RAP$ (mmHg). Right atrial pressure (RAP) was estimated from the size and collapsibility index of inferior vena cava [24]. Each subject received saline nebulization for 15 min and had rest for 15 min. Thereafter, sodium nitrite nebulization was administered for 15 min. The eRVSP was measured every 1 min during inhalation.

2.8. Measurement of platelet activation by flow cytometry

Venous blood samples were collected at baseline and after inhalation using 3.8% sodium citrate as anticoagulant. Ten-fold diluted whole blood was stained with PE-labeled CD62P (P-selectin) and PE-Cy5-labeled CD42b (platelet marker). Platelets were stimulated by 20 μM ADP or 20 μM U46619 (thromboxane A_2 receptor agonist). Blood samples were fixed with 1% paraformaldehyde and analyzed by Accuri C6 (BD Bioscience, San Jose, CA). The percentages of P-selectin expression were calculated from 10,000 CD42b positive events.

2.9. Western blot analysis of P-VASP^{Ser239} in platelets

Whole blood was centrifuged at 120 × *g* for 3 min to obtain platelet-rich plasma. Platelets were separated from platelet-rich plasma by centrifugation at 500 × *g* for 5 min. The platelet samples were added with lysis buffer (50 mM Tris, 0.5% NP-40 and 150 mM NaCl, pH 7.4) containing a proteinase inhibitor cocktail III (1:1000 Calbiochem, La Jolla, CA). Protein (10 μg protein) was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk overnight and then incubated for 1 h with anti-P-VASP^{Ser239} (Millipore, Billerica, MA), anti-VASP (Cell Signaling Technology, Danvers, MA) or GAPDH (Cell Signaling Technology, Danvers, MA). Secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) were incubated with membranes for 1 h. The bands were detected by enhanced chemiluminescence detection (Biorad, Hercules, CA) and quantified by Image J software (National Institutes of Health).

2.10. Measurement of oxidative and nitrosative stress markers

Plasma malondialdehyde and 8-isoprostane were measured as oxidative stress markers and plasma nitrotyrosine was measured as nitrosative stress marker. Malondialdehyde was measured using spectrofluorometric method based on the reaction with thiobarbituric acid [25]. Plasma 8-isoprostane was measured by commercial 8-isoprostane enzyme immunoassay kits (Cayman Chemical, Michigan, USA). Plasma nitrotyrosine was measured by competitive enzyme-linked immunosorbent assays (Abcam, Cambridge, UK).

2.11. Statistical analysis

Data are expressed as mean ± standard deviation (SD) or median (min, max), depending on normality. Group means of pharmacokinetic parameters of nitrite in whole blood, plasma and RBC were compared by ANOVA with Tukey's multiple comparison test. Paired *t*-test, unpaired *t*-test or Mann-Whitney *U* test were used to compare values between 2 groups. All statistical tests and graphical figures were performed using GraphPad Prism 5 (GraphPad software, LA Jolla, CA, U.S.A.). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Subjects' characteristics

Ten healthy subjects and 8 HbE/β-thal patients with elevated eRVSP were enrolled in this study. Means ± standard deviation (SD) of age were 29.6 ± 8.9 and 36.7 ± 9.3 years, respectively (Table 1). HbE/β-thal patients had lower weight and body mass index than healthy subjects. The hemoglobin levels were lower but white blood cell count, platelet count, methemoglobin, ferritin, and serum iron were higher in HbE/β-thal patients than healthy subjects. HbE/β-thal patients had increased levels of methemoglobin and direct bilirubin. HbE/β-thal patients had undergone splenectomy for mean duration of 23.7 years. There was no difference in blood nitrite and nitrate compared between healthy and HbE/β-thal subjects. Seven HbE/β-thal patients received iron chelation therapy with deferiprone, two patients received hydroxyurea, but none of them received medication for pulmonary hypertension.

3.2. Safety and tolerability

Nebulized sodium nitrite at doses used in our study was well tolerated as all subjects could complete the protocol. There was no orthostasis or hypotension with tachycardia in all subjects. No change in blood pressure and oxygen saturation was observed during inhalation and up to 120 min after inhalation (Table 2). Neither methemoglobin nor hemoglobin were changed in healthy subjects and thalassemia patients at time 120-min after inhalation of 37.5 and 15 mg sodium nitrite.

Table 1
Baseline characteristics of subjects.

Parameters, unit	Healthy	Thalassemia
Female/male	5/5	4/4
Age, years	29.6 ± 8.9	36.7 ± 9.3
Body weight, kg	60.7 ± 10.1	48.1 ± 5.4*
Body mass index, kg/m^2	22.2 ± 1.9	18.4 ± 2.3*
Hemoglobin, g/dl	14.0 ± 1.9	7.3 ± 1.1*
White blood cells, × 10^3 cells/μl	6.8 ± 2.2	12.0 ± 4.8*
Platelets, × 10^3 cells/μl	242 ± 54	595 ± 119*
Venous methemoglobin, %	0.5 (0.2, 0.7)	3.2 (2.3, 6.8)*
Serum ferritin, ng/ml	45 (10, 183)	1428 (180, 3298)*
Serum iron, μg/dl	101.5 ± 30.4	185.3 ± 15.0*
Direct bilirubin, mg/dl	0.20 ± 0.09	0.85 ± 0.3*
Aspartate aminotransferase, U/l	19 (13, 35)	44 (22, 119)*
Alanine aminotransferase, U/l	19 (8, 98)	31 (9, 91)
Creatinine, mg/dl	0.9 (0.5, 1.2)	0.4 (0.3, 0.7)*
Nitrite, nM		
Whole blood	182.7 ± 80.6	148.9 ± 87.4
Plasma	119.8 ± 65.8	92.6 ± 63.4
Red blood cells	213.4 ± 65.8	163.9 ± 84.6
Plasma nitrate, μM	22.3 ± 8.2	27.8 ± 18.1
Years after splenectomy	–	23.7 ± 8.8
Systolic blood pressure, mmHg	121.9 ± 14.8	120.9 ± 18.4
Diastolic blood pressure, mmHg	68.5 ± 9.2	62.7 ± 7.1
Mean arterial blood pressure, mmHg	86.1 ± 9.8	81.7 ± 7.4
Heart rate, beats per min	79.1 ± 13.0	77.1 ± 8.7
Right ventricular systolic pressure, mmHg	20.7 ± 4.7	64.3 ± 20.4*
Medications		
Deferiprone	–	7
Deferasirox	–	–
Hydroxyurea	–	2

Values are means ± SD or median (min, max) depending on normality, or number of subjects. *$P < 0.05$ (unpaired *t*-test or Mann-Whitney *U* test).

3.3. Pharmacokinetics of nebulized sodium nitrite

At baseline, the nitrite concentration was higher in RBC than plasma while the concentration was higher in plasma after inhalation. Inhaled nitrite was rapidly absorbed with the time to C_{max} occurring within 5 min after inhalation (Fig. 1). In healthy subjects, C_{max} and AUC calculated from nitrite concentrations in whole blood, plasma and RBC were proportional to dose (Table 3). C_{max} was proportional to dose while C_{max}/dose and AUC/dose ratios were not different between 37.5- and 75-mg dose. AUC was lower in RBC than whole blood and plasma. CL and V_d were higher in RBC than whole blood and plasma. The $t_{1/2}$ was longer in RBC than whole blood and plasma. CL, V_d and $t_{1/2}$ were independent of dose. The renal CL calculated from urine data was approximately 0.19 ml/min/kg (0.0114 l/h/kg) which accounted for 0.76% of the total plasma nitrite CL. In HbE/β-thal patients, C_{max}/dose, AUC/dose and renal CL were not significantly different from those of healthy subjects. Nitrite CL was lower and $t_{1/2}$ was longer in RBC of HbE/β-thal patients than those of healthy subjects. V_d values in RBC of HbE/β-thal patients was not different from those of heathy subjects. No change in plasma nitrate concentrations was found after nebulization in healthy subjects and HbE/β-thal patients (Fig. 2).

Following intravenous infusion of 3-mg sodium nitrite for 15 min in healthy subjects (n = 3), nitrite exhibited the first-order kinetic with CL, V_d and $t_{1/2}$ similar to those of inhalation (from whole blood data; CL 1.2 ± 0.3 l/h/kg, V_d 1.2 ± 0.3 l/kg, and $t_{1/2}$ 0.7 ± 0.2 h). C_{max} was achieved immediately after the end of intravenous infusion. The absolute bioavailability of nebulized sodium nitrite in our study was 18%, 19% and 14% in whole blood, plasma and RBC, respectively.

3.4. Pharmacodynamic effect on the estimated pulmonary artery pressure

FE_{NO} was measured as an indicator of pulmonary NO exposure arising from nitrite conversion to NO. FE_{NO} increased to the maximum immediately after the end of inhalation in healthy subjects and

Table 2
Laboratory values at baseline and 120 min after the end of sodium nitrite inhalation in healthy subjects and hemoglobin E/β-thalassemia patients.

Laboratory variables	Healthy (n = 10) 37.5-mg sodium nitrite		Thalassemia (n = 8), 15-mg sodium nitrite	
	Baseline	Time 120 min	Baseline	Time 120 min
Methemoglobin, %	0.5 (0.2, 0.7)	0.6 (0.3, 1.0)	3.2 (2.3, 6.8)	3.4 (2.5, 6.8)
Hemoglobin, g/dL	14.0 ± 1.9	13.8 ± 1.9	7.3 ± 1.1	7.3 ± 1.1
White blood cells, $x10^3/\mu L$	6.8 ± 2.2	8.4 ± 3.5	12.0 ± 4.8	12.9 ± 5.1
Platelets, $x10^3/\mu L$	242 ± 54	246 ± 63	595 ± 119	555 ± 105
Systolic blood pressure, mmHg	122 ± 16	111 ± 9	121 ± 17	112 ± 11
Diastolic blood pressure, mmHg	69 ± 9	71 ± 13	63 ± 6	64 ± 11
Heart rate, beats per min	79 ± 13	71 ± 9	77 ± 8	77 ± 17
Peripheral oxygen saturation, %	99.0 ± 0.0	98.0 ± 0.0	97.7 ± 1.6	97.1 ± 2.0

Values are means ± SD or median (min, max).

thalassemia patients (Fig. 3). Return of FE_{NO} to baseline values was observed at 10 min after inhalation. In healthy subjects, no change in the eRVSP was observed (Fig. 4A). In HbE/β-thal patients, although the reduction in eRVSP reached the statistical significance at certain time points during inhalation (using paired *t*-test for comparison between nitrite and saline), the changes were inconsistent (Fig. 4B). After

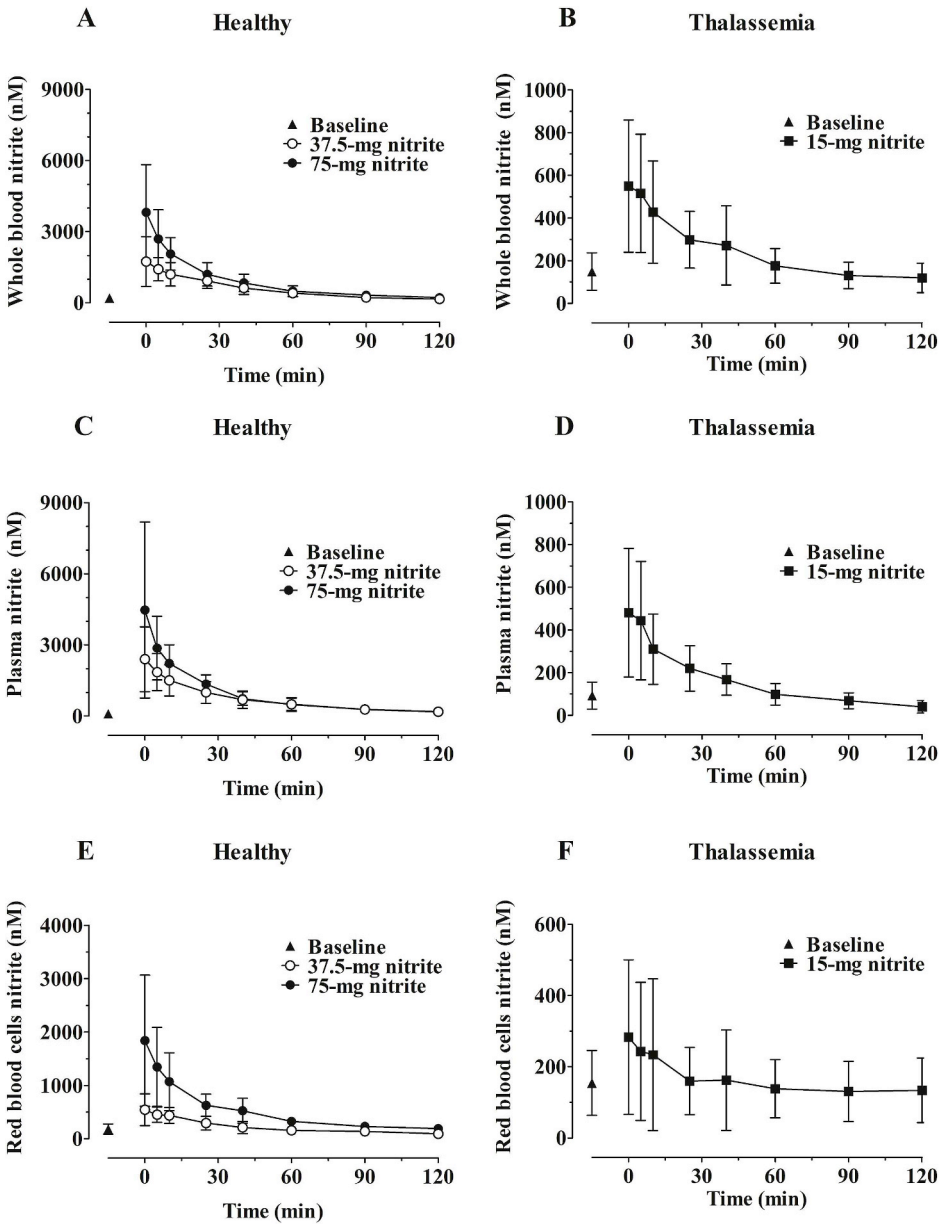

Fig. 1. Concentration-time profiles of whole blood nitrite (A and B), plasma nitrite (C and D), and red blood cells nitrite (E and F) in healthy subjects (n = 10) and hemoglobin E/β-thalassemia patients (n = 8). Each point represents mean ± SD.

Table 3
Pharmacokinetics of inhaled nitrite measured in whole blood, plasma and red blood cells.

	Dose (mg)	C_{max} (nM)	C_{max}/dose (nM/mg)	$AUC_{0-\infty}$ (nM.h)	$AUC_{0-\infty}$/dose (nM.h/mg)	CL (l/h/kg)	V_d (l/kg)	$t_{1/2}$ (h)	CL_{renal} (ml/min/kg)
Healthy (n = 10)	37.5								0.19 ± 0.2
Whole blood		1917.4 ± 941.4	51.1 ± 25.0	1284.1 ± 472.8	34.2 ± 12.6	1.5 ± 0.6	1.3 ± 0.6	0.6 ± 0.0	
Plasma		2554.8 ± 1293.1	68.1 ± 34.5	1524.9 ± 721.9	40.7 ± 19.3	1.4 ± 0.6	1.3 ± 0.6	0.7 ± 0.0	
Red blood cells		609.3 ± 251.4*	16.3 ± 6.6*	613.9 ± 310.9*	16.4 ± 25.9*	2.6 ± 1.3*	3.8 ± 1.3*	1.2 ± 0.6*	
Healthy (n = 10)	75								0.18 ± 0.1
Whole blood		3820.8 ± 2008.4	51.0 ± 26.9	1915.4 ± 725.7	25.5 ± 9.8	1.3 ± 0.3	1.3 ± 0.3	0.7 ± 0.0	
Plasma		4547.2 ± 3655.9	60.6 ± 48.7	1916.8 ± 552.8	25.6 ± 7.3	1.2 ± 0.3	1.1 ± 0.3	0.6 ± 0.3	
Red blood cells		1856.0 ± 1215.3*	24.7 ± 16.1*	1251.2 ± 359.6*	16.7 ± 4.7*	2.4 ± 0.9*	3.3 ± 1.3*	1.0 ± 0.3*	
Thalassemia (n = 8)	15								0.44 ± 0.4
Whole blood		563.7 ± 302.9	37.6 ± 20.1	638.1 ± 290.8	42.5 ± 19.5	1.3 ± 0.8	1.6 ± 0.6	1.1 ± 0.3#	
Plasma		490.7 ± 292.2	32.7 ± 19.5	380.6 ± 188.7	25.4 ± 12.7	2.1 ± 1.7	1.7 ± 0.8	0.6 ± 0.3	
Red blood cells		292.1 ± 210.2	19.5 ± 13.9	641.4 ± 418.3	42.8 ± 28.0	1.4 ± 0.6#	3.7 ± 1.4*	1.9 ± 0.6*#	

Data are means ± SD.
Abbreviations: $AUC_{0-\infty}$, area under the concentration-time curve from time zero to infinity; C_{max}, maximum concentration; CL, total clearance; CL_{renal}, renal clearance; $t_{1/2}$, half-life; V_d, volume of distribution.
*$P < 0.05$ compared with whole blood and plasma data (ANOVA with Tukey's multiple comparisons test).
#$P < 0.05$ compared with the values of healthy subjects at 37.5 mg dose (unpaired *t*-test).

inhalation, the eRVSP remained unchanged. In HbE/β-thal patients, the tricuspid annular plane systolic excursion (TAPSE) and right ventricular (RV) fractional area change before and after inhalation were not different (TAPSE, 2.23 ± 0.47 vs 2.24 ± 0.34 cm; RV fractional area change, 46.7 ± 14.7 vs 45.9 ± 17.2%). The left ventricular ejection fraction before and after inhalation was not different (65.2 ± 7.8 vs 65.4 ± 6.8%). There was no difference in systemic blood pressure during and 120 min after nitrite inhalation in healthy subjects and thalassemia patients (Fig. 4C and D).

3.5. Pharmacodynamic effect on platelet activation

Platelet activity in HbE/β-thal patients was determined by P-selectin expression on platelets. Immediately after nitrite inhalation, P-selectin expression in response to stimulation with ADP (20 μM) and U46619 (20 μM) decreased (Fig. 5A and B). P-$VASP^{Ser239}$ and P-$VASP^{Ser239}$/total P-VASP ratio increased immediately after inhalation (Fig. 5C and D).

3.6. Effect on oxidative and nitrosative stress

In both healthy subjects and HbE/β-thal patients, there were no significant differences in plasma malondialdehyde, 8-isoprostane and nitrotyrosine between baseline and after inhalation (Table 4).

4. Discussion

The main findings of our study are (1) nebulized sodium nitrite at the doses used in this study are safe and well tolerated; (2) absorption of nebulized nitrite into systemic circulation is low with absolute bioavailability of 18%; (3) nitrite exhibits dose-independent kinetics with similar CL and V_d in whole blood and plasma; (4) CL and V_d of nitrite are higher in RBC than whole blood and plasma; (5) non-renal clearance, presumably by RBC, plays major role in nitrite elimination as the renal CL is only 0.76% and $t_{1/2}$ of nitrite is longer in RBC than whole blood and plasma due to distribution of nitrite into RBC (higher V_d in RBC); (6) in HbE/β-thal patients, CL of nitrite decreases in RBC, leading to longer $t_{1/2}$ of nitrite in whole blood and RBC; (7) increased FE_{NO} after nitrite inhalation indicates conversion of nitrite to NO in lungs despite no change in eRVSP; and (8) inhaled nitrite can decrease platelet activity in thalassemia patients.

Significant decrease in blood pressure was not observed in our study even at 75-mg sodium nitrite dose that caused plasma C_{max} of 4.5 μM. Decreases in blood pressure correlates with blood nitrite concentrations. The relation of blood nitrite and blood pressure has been reported in healthy subjects who received nitrite and nitrate via different routes. For instance, oral ingestion of beetroot juice lowered blood pressure in healthy subjects [26]. Decrease in systolic and diastolic blood pressure by 8–10 mmHg was observed 3–4 h after ingestion of beetroot juice with C_{max} of 0.6 μM. In a study with healthy volunteers, inhalation of 176-mg sodium nitrite increased plasma nitrite to 20–30 μM immediately post-dose and led to 33-mmHg decrease in mean arterial pressure and 40-beasts/min increase in heart rate [17]. The maximum tolerated dose of inhaled sodium nitrite was reported to be 90 mg which caused C_{max} of 10–20 μM. By intra-arterial infusion of nitrite, C_{max} of 16 μM resulted in a decrease in mean arterial pressure of 7 mmHg [11], and C_{max} of 30 μM also caused 10-mmHg decrease in mean arterial pressure [27]. By intravenous infusion, 10–15 mmHg drop in mean arterial pressure was seen when plasma nitrite was 2 μM [28]. Thus, nitrite at 15-mg dose with plasma C_{max} of 0.5 μM in our study, which was previously reported to reduce RVSP in HbE/β-thal patients with pulmonary hypertension [10], is unlikely to cause hypotension.

Thalassemia patients have increased baseline methemoglobin levels, which is associated with oxidative stress and antioxidant depletion including ascorbate [29]. Following nitrite inhalation, there were no changes in methemoglobin levels in both healthy and HbE/β-thal subjects. Therefore, methemoglobinemia does not occur following nitrite

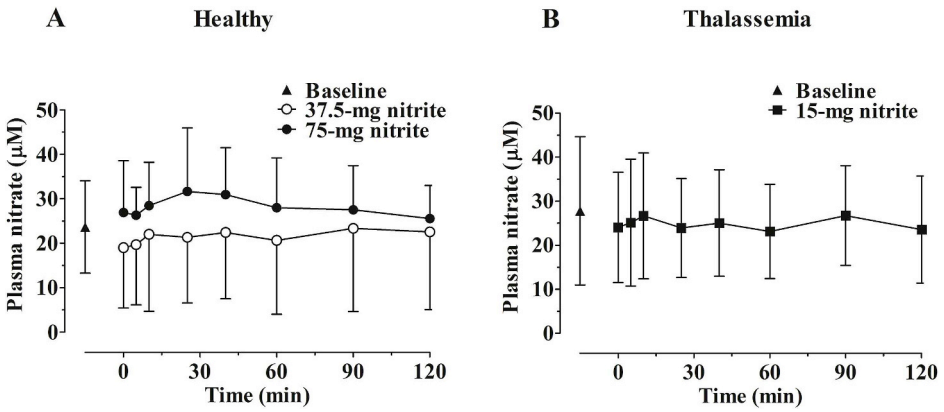

Fig. 2. Concentration-time profiles of plasma nitrate in healthy subjects (A, n = 10) and hemoglobin E/β-thalassemia patients (B, n = 8). Each point represents mean ± SD.

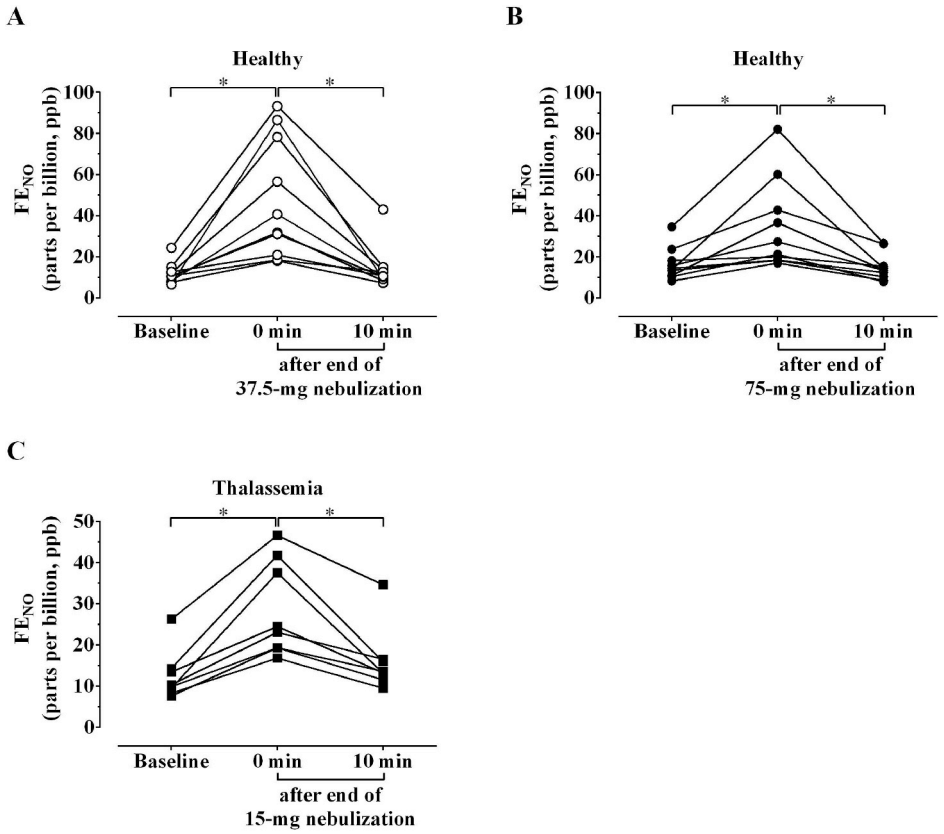

Fig. 3. Changes in the fraction exhaled nitric oxide (FE_{NO}) after the end of sodium nitrite nebulization in healthy subjects (A, n = 10), and hemoglobin E/β-thalassemia patients (B, n = 8). *$P < 0.05$ by paired *t*-test.

inhalation at these doses. In our study, the oxidative and nitrosative stress markers were not changed by nitrite. *In vitro* incubation of RBC with nitrite at high concentration (0.2–1 mM) increased methemoglobin but had no effect on oxidative stress in RBC [30]. Nitrite at doses used here does not appear to produce oxidative and nitrosative stress.

Nitrite reduction to NO occurs in blood and tissues through the reactions catalyzed by deoxygenated heme proteins such as hemoglobin and molybdopterin-containing enzymes [11,31–33]. RBC could uptake nitrite rapidly and then released NO gas for long period of time, in which the process was enhanced by hypoxia and hyperthermia [34]. Relaxation of aortic rings [11,35] and platelet inhibition [12,36] in the presence of nitrite and deoxygenated RBC support the notion that RBC can produce NO from nitrite. From our data, the higher V_d and CL of nitrite in RBC is consistent with the data showing that RBC are the major storage sites of circulating nitrite and that RBC bioactivate nitrite to NO [20]. In blood, most nitrite is transported into RBC and interacts with hemoglobin to form nitrate and NO, depending on oxygen levels. Our findings suggest that the major pathway for elimination of blood nitrite is by interaction of nitrite with hemoglobin in RBC, while urinary excretion is minor.

The absolute bioavailability of nitrite is 98% after oral administration in healthy subjects [37]. The low bioavailability (18%) seen in our study could be due to the nebulizer output for the most part and breathing pattern. Following absorption, nitrite reached C_{max} within 5 min. The absence of difference in V_d, CL and $t_{1/2}$ of nitrite between two doses (37.5 and 75 mg) and dose proportionality of C_{max} and AUC implies that the nitrite elimination follows the linear pharmacokinetics with dose-independent manner, as described previously [17]. The lower CL and longer $t_{1/2}$ of nitrite in RBC of HbE/β-thal patients can be

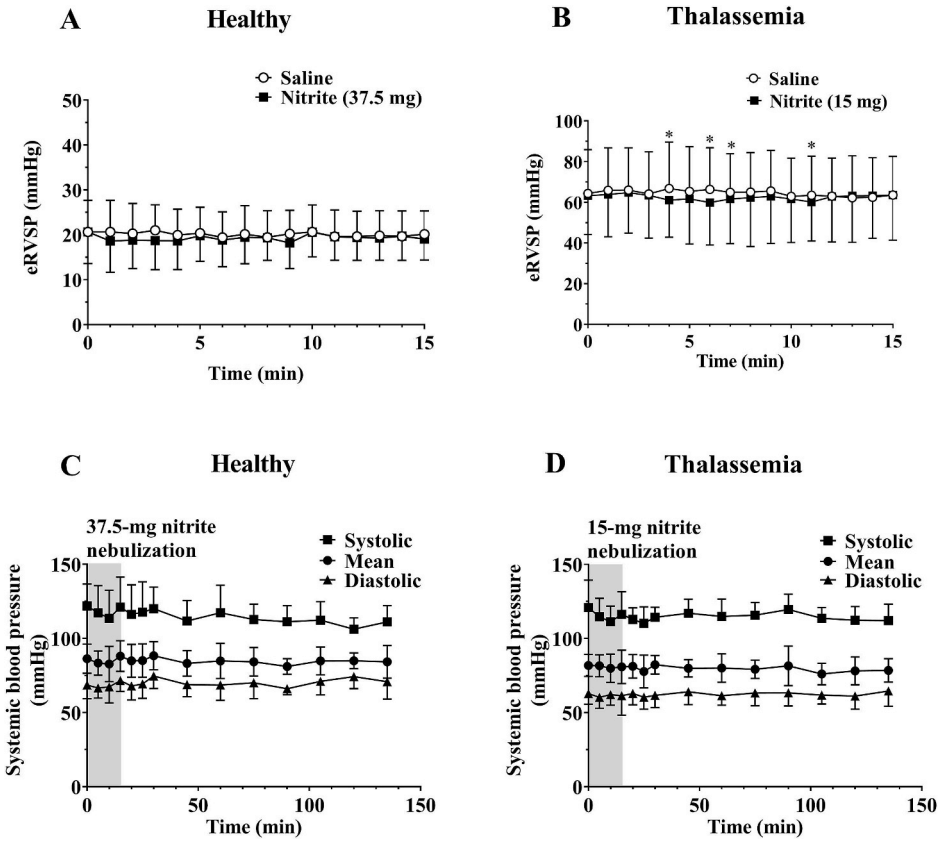

Fig. 4. Changes in the estimated right ventricular systolic pressure (eRVSP) in healthy subjects (A, n = 10) and hemoglobin E/β-thalassemia patients (B, n = 8) during sodium nitrite inhalation, and systemic blood pressure in heathy subjects (C, n = 10) and hemoglobin E/β-thalassemia patients (D, n = 8) during and after sodium nitrite inhalation. Each point represents mean ± SD. *$P < 0.05$ by paired *t*-test comparing between saline and nitrite.

explained by a decrease in nitrite reductase activity of hemoglobin E [18,19]. No change in V_d suggests the distribution into RBC is not affected in HbE/β-thalassemia. Reduction in maintenance dose of nitrite is thus required in HbE/β-thal patients to avoid systemic adverse effect.

In this study, none of HbE/β-thal patients who had elevated eRVSP received treatment for pulmonary hypertension. Despite recognized increased prevalence of pulmonary hypertension in β-thalassemia [1], there is no standard treatment or randomized trial for this complication, leaving most patients untreated. Although the effect of inhaled nitrite on eRVSP measured by echocardiography was inconsistent, it was shown to decrease mean pulmonary artery pressure by 13.5% from the baseline as determined by right heart catheterization [10]. Our study was limited to the use of noninvasive echocardiography which was considered as screening tool for pulmonary hypertension.

Nitrite is a pulmonary vasodilator through NO. Apart from nitrite reduction to NO in blood, nitrite is reduced to NO at tissue sites by

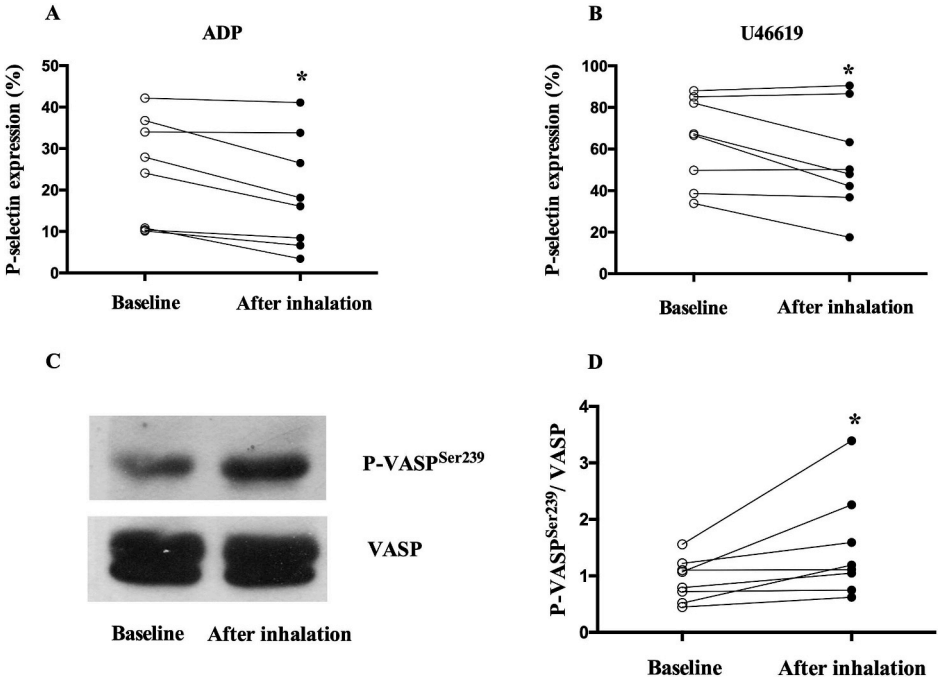

Fig. 5. Decrease in platelet activity of hemoglobin E/β-thalassemia patients (n = 8) after 15-mg sodium nitrite inhalation. Platelet activity was determined as the expression of P-selectin (A and B) in response to stimulation with 20 μM adenosine diphosphate (ADP) and 20 μM U46619 (thromboxane A_2 receptor agonist). Intra-platelet P-VASP^{Ser239} was measured by western blot (C) and normalized to total P-VASP (D). *$P < 0.05$ by paired *t*-test.

Table 4
Effects of inhaled nebulized sodium nitrite on oxidative and nitrosative stress markers.

	Baseline	60 min after the end of inhalation
Healthy (37.5 mg), n = 10		
Plasma malondialdehyde, μM	0.23 ± 0.1	0.23 ± 0.1
Plasma 8-isoprostane, pg/ml	19.2 ± 11.4	29.6 ± 20.2
Plasma nitrotyrosine, ng/ml	66.0 ± 38.6	62.3 ± 34.2
Thalassemia (15 mg), n = 8		
Plasma malondialdehyde, μM	0.25 ± 0.1	0.24 ± 0.1
Plasma 8-isoprostane, pg/ml	32.6 ± 20.9	34.4 ± 19.2
Plasma nitrotyrosine, ng/ml	63.4 ± 23.5	62.7 ± 27.2

Values are means ± SD.

several tissue nitrite reductases including myoglobin, xanthine oxidase, aldehyde oxidase, and endothelial nitric oxide synthase [31–33,38,39]. These proteins have increased nitrite reductase activity during hypoxia. NO production from nitrite at tissues was suggested in a study with healthy volunteers that pulmonary vasodilation occurred in subjects who were in hypoxic chamber, but not normoxic subjects [40]. The pulmonary vasodilation by nitrite was independent of its plasma levels, suggesting nitrite deposition and metabolism in tissues. The fact that pulmonary artery has lower oxygen levels than systemic circulation and that pulmonary artery relaxes at lower nitrite concentrations than systemic artery [41] could explain the potential effect of nitrite in thalassemia patients who have chronic hypoxemia.

Platelet hyperactivity is acknowledged in thalassemia and associated with vascular disorders including pulmonary hypertension. Thalassemia patients with elevated pulmonary artery pressure had increase in P-selectin and activated GPIIb/IIIa expression on platelets [19,42]. In the presence of deoxygenated RBC, nitrite via NO inhibits platelets through cGMP/protein kinase G pathway [43]. The inhibitory effect of inhaled nitrite on platelets has been demonstrated in healthy subjects [16]. In these subjects, nitrite inhalation decreased platelet aggregation, P-selectin expression and platelet-leukocyte aggregates, and increased P-VASP^{Ser239}, a marker of NO/soluble guanylate cyclase/protein kinase G pathway. Here, we provide further evidence that inhaled nitrite can also decrease platelet activation in β-thalassemia. Nitrite use may benefit HbE/β-thal patients for treatment of pulmonary hypertension and platelet hyperactivity.

In conclusion, we report the pharmacokinetics, pharmacodynamics on the estimated pulmonary artery pressure and platelet activity, and safety of inhaled sodium nitrite in healthy subjects and HbE/β-thal patients with pulmonary hypertension. Neither decrease in systemic blood pressure nor increase in methemoglobin was observed at 75-mg dose for healthy subjects and 15-mg dose for thalassemia patients. HbE/β-thal patients have decreased nitrite CL in RBC, suggesting requirement for dosage reduction. Sodium nitrite at 15-mg dose has no effect on the estimated pulmonary artery pressure, but can decrease platelet activity in β-thalassemia. Our study provides pharmacologic information for development of inhaled sodium nitrite.

Statement of conflict of interest

The authors declared no conflict of interest.

Author contribution

The authors' contributions are as follows: KP, SF, GR, PS, AS, and NS contributed to conceptualization, study design and supervision; KS, TS and NS were responsible for data curation; KS, TS, JK, TP, SS, and PV performed investigation. All authors contributed to writing the article and approved the final version of the manuscript.

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