Artery to Vein Differences in Nitric Oxide Metabolites Are Diminished in Sepsis

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SUPPLEMENTAL DIGITAL CONTENT

MATERIALS AND METHODS

Measurement of NO Metabolites

In sepsis patients, arterial blood was obtained from indwelling radial, femoral or axillary arterial catheters, and venous blood was obtained from central venous catheters (n=84) or by peripheral venipuncture if catheters were absent (n=3). Catheter samples were collected after aspirating an initial 3 cc dead space volume during momentary cessation of intravenous infusions. The dead space volume was returned after sample acquisition and the catheter was then slowly flushed with isotonic saline. Venipuncture of antecubital arm veins was performed using 21 gauge needles. Arterial blood was obtained by radial artery puncture using 23 gauge needles in control subjects. Venous and arterial blood specimens were obtained in rapid succession and each processed immediately after collection as described below.

All reagents were purchased from EMD Chemicals Inc., Gibbstown, NJ unless otherwise specified. Blood samples were collected in empty 5 cc volume Vacutainer® tubes modified by the addition of 90 μL of 1,000 USP units/ml heparin and 200 mM N-ethylmaleimide (NEM). One-hundred μL aliquots of arterial and venous whole blood were immediately placed in separate amber Eppendorf tubes containing 900 μL S-nitrosohemoglobin (SNOHb) stabilization solution (1) (4 mM ferricyanide, 10 mM NEM, 100 μM dietheylenetriaminepentaacetic acid [DTPA], and 1% NP-40 detergent in Dulbecco's phosphate buffered saline [PBS, Mediatech, Inc., Herndon, VA, pH=7.0]) at the bedside to minimize processing time and SNOHb decay (2). Four-hundred μL aliquots of arterial and venous whole blood were immediately added to separate Eppendorf tubes containing 100 μL whole blood nitrite stabilization solution (3) (160 mM potassium ferricyanide, 0.2% NP-40, and 2 mM NEM in PBS) at the bedside. Remaining whole blood samples were centrifuged at 1,500g for 10 minutes at 4°C in the dark. Plasma

supernatant (500 μL) was added to two amber Eppendorf tubes, one containing 55 μL acidified sulfanilamide and the other containing 55 μL PBS and incubated for 3 minutes. These samples were separately added to a purge vessel containing 5 ml I_3^- solution, in line with a Sievers 270B chemiluminescence analyzer (GE Instruments, Boulder, CO). Tri-iodide solution was replaced after each sample. Tri-iodide converts nitrite, metal-nitrosyls, nitrosothiols, and nitrosamines to NO gas (1). Gaseous NO is then carried via a nitrogen gas purge stream to the chemiluminescence analyzer for measurement. The chemiluminescence analyzer generates ozone that reacts with NO to form excited NO_2*, which in turn releases photons in proportion to the NO concentration. The NO concentration is obtained by integration of the area under the curve and comparison with a nitrite standard curve as recommended (1). Nitrite standard curves were constructed daily and were highly linear and reproducible (Supplemental Figure E1). There was minimal nitrite contamination of blood collection tubes, assessed by measuring the nitrite concentration in 5 ml Nanopure water samples that were added to the tubes (7 ± 9 nM [s.d.], n=20).

The NO signal obtained after addition of plasma + PBS was a mixture of plasma nitrite, nitrosothiols, metal-nitrosyl compounds and nitrosamines (4, 5). The NO signal obtained after addition of plasma + AS represents plasma nitrosothiols + metal nitrosyls + nitrosamines. The concentration of plasma nitrite is the difference in NO signal between the tube containing PBS and the tube containing AS.

Whole blood (WB) nitrite was measured in 500 μL samples incubated for 5 minutes in WB nitrite stabilization solution. These samples were deproteinated by the addition of 500 μL methanol, then vortexed for 1 minute and centrifuged at 16,000g for 2 minutes. Centrifugation was repeated as necessary until supernatant was clearly distinguishable from the underlying

pellet. Six-hundred μL of supernatant was added to 5 ml I_3^- solution in the purge vessel, with the resulting signal representing WB nitrite concentration. Red blood cell (RBC) nitrite levels were calculated from the levels of plasma nitrite, WB nitrite and hematocrit (Hct) using the following equation (6):

$$\text{Nitrite}_{RBC} = \frac{\text{Nitrite}_{WB} - (\text{Nitrite}_{PLASMA} \times [1 - \text{Hct}])}{\text{Hct}} \quad \text{(Equation 1)}$$

The lower limit of RBC nitrite was fixed at zero since negative results are mathematically but not physiologically possible.

The samples in SNOHb stabilization solution were incubated for 10 minutes and then passed through a Sephadex G25 sizing column to remove nitrite, low-molecular weight thiols and cyanide. The hemoglobin (Hb) fraction was collected and 700 μL was added to two Eppendorf tubes, one with 77 μL PBS and one with 77 μL of 50 mM mercuric chloride ($HgCl_2$, reduces S-nitrosothiols to nitrite) and allowed to incubate for 2 minutes. Acidified sulfanilamide was then added to both tubes and allowed to incubate for 3 minutes to eliminate nitrite. The samples with and without $HgCl_2$ were separately added to a purge vessel containing 5 mL fresh I_3^- solution. The NO signal from the sample without $HgCl_2$ corresponds to the sum of SNOHb and RBC XNO (the latter consists of iron-nitrosyl-Hb [FeNOHb] but also may include N-, C-, or O-nitrosamines) (1, 4, 5). The signal obtained from the sample with $HgCl_2$ represents RBC XNO. The difference between these signals yields a sensitive and specific measurement of SNOHb (7). A representative tracing of arterial and venous NO metabolite signals using this methodology is shown in Supplemental Figure E2. Heme concentration of the G25 column effluent was determined by mixing 50 μL effluent with 950 μL Drabkin's solution (Pointe Scientific, Inc.,

Canton, MI) and measuring light absorbance via spectrophotometry at 540 nm wavelength. The amount of SNOHb and FeNOHb can be expressed as percent NO per heme via the formula:

$$\frac{[\text{mM NO}] \text{ measured from } I_3^-}{[\text{mM heme}]} \times 100 \quad \text{(Equation 2)}$$

The quantities of these NO-Hb adducts were converted to nanomolar concentration in blood. To accurately reflect the concentration of NO in the subject's blood, the result must be normalized to the patient's Hb concentration, according to the formula:

$$\frac{x \text{ gm Hb / L}}{64{,}800 \text{ gm Hb / mole Hb}} \times \frac{\text{\% moles NO / mole heme}}{100} \times \frac{4 \text{ heme}}{\text{Hb molecule}} \times \frac{10^9 \text{ nmoles}}{\text{mole}} \quad \text{(Equation 3)}$$

Where 64,800 is the molecular weight of Hb and % moles NO / mole heme subunit is obtained from Equation 2.

Nitrate Measurements

Plasma was immediately snap frozen in a dry ice and ethanol slurry, stored at minus 80°C, and thawed on the day of analysis. The analytic method involved reduction of nitrate in plasma to nitrite, and subsequent reductive chemiluminescence analysis. One hundred µL plasma samples are mixed with 45 µL nitrate reductase (Roche Diagnostics / Boehringer Mannheim, Indianapolis, IN) and 55 µl NADPH solution, resulting in the following reaction:

$$NADPH + H^+ + NO_3 \xrightarrow{\text{nitrate reductase}} NADP^+ + NO_2^- + H_2O \quad \text{(Equation 4)}$$

Samples were then added to the purge vessel containing 10 ml acidified potassium iodide (KI) solution, reducing nitrite to NO according to the following chemistry (8):

$$2HNO_2 + 2KI \longrightarrow 2NO\cdot + I_2 + 2H_2O \quad \text{(Equation 5)}$$

NO release is quantified with the chemiluminescence analyzer. The signal resulting from injection of the plasma sample treated with nitrate reductase and NADPH therefore represents the total concentration of plasma nitrate and nitrite. The plasma nitrite concentration (obtained

using I_3^--based chemiluminescence as described above) is subtracted from this value to determine the plasma nitrate concentration. Nitrate standard curves were constructed daily and compared with equivalent nitrite standards on each day of measurement. The results were linear and reproducible, and nitrate reduction was complete with quantitative nitrite recovery (Supplemental Figure E3).

STATISTICAL ANALYSIS

A constant of 1 was added to all plasma nitrite, RBC nitrite, SNOHb, and RBC XNO values to eliminate zero values prior to log transformation.

Variables potentially confounding the relationships between NO metabolites and sepsis or between NO metabolites and hospital mortality were identified using classical and collapsibility criteria (9). These criteria hold that the relationship between a NO metabolite and the outcome of interest may be confounded by another variable if that variable is: 1) associated with the outcome (specifically in subjects with lower levels of the NO metabolite); and 2) associated with the NO metabolite. All variables that were distributed differently ($p \leq 0.10$) between control subjects and sepsis patients, or between survivors and non-survivors, were assessed as potential confounders using these criteria (Tables E1-E2 and E4-E5). For each NO metabolite, all variables meeting both of these criteria were included in two-way analysis of variance (ANOVA) models. Variables that were insignificant by Wald test ($p>0.10$) were removed from the model if the adjusted R^2 increased and the root error mean square decreased in the reduced, nested model. The outcome of interest (sepsis vs. control or survivor vs. non-survivor) was then added to the reduced model and the analysis was repeated, thereby determining whether significant

relationships between NO metabolites and the outcomes of interest persisted after adjusting potential confounding variables (Tables E3 and Table E6).

Sample Size

We planned a sample size of 52 control subjects and approximately 80 sepsis patients. Assuming a hospital mortality rate of 30% (10), this would provide 24 non-survivors for comparison with 56 survivors. Using the published standard deviation for plasma nitrite of 35 nmol/L (3), our projected sample size permitted detection of a 23 nmol/L difference in venous plasma nitrite between cases and controls with α=0.05 and 90% power (11). This sample size also allowed detection of a 33 nmol/L difference in venous plasma nitrite between survivors and non-survivors (α=0.05 and 90% power). We aimed to detect AV differences in NO metabolite concentrations in 4 separate groups of subjects: control subjects, sepsis patients, sepsis survivors, and sepsis non-survivors. Assuming the standard deviation for the plasma nitrite AV difference is also 35 nM, this sample size allowed detection of an AV difference of 16 nM in the 50 controls, 13 nM in the 80 sepsis subjects, 24 nM in the projected 24 non-survivors, and 16 nM in the projected 56 sepsis survivors (α=0.05 and 90% power).

ADDITIONAL RESULTS

Venous blood samples were obtained in 45 sepsis patients just prior to hospital discharge, either peripherally (n=27) or centrally (n=18) depending on available venous access. There were no significant differences in plasma nitrite (central = 95 (59-151) nM vs. peripheral=80 (64-99) nM p = 0.44), WB nitrite (central =258 (202-331) nM vs. peripheral = 217 (183-257) nM, p = 0.22), RBC nitrite (central = 452 (198-1036) nM vs. peripheral =476 (387-586) nM, p = 0.88) or

SNOHb (central = 132 (71-243) nM vs. peripheral =79 (56-111) nM, $p = 0.11$). These data provide additional reassurance that sampling site does not bias the study findings.

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Figure Legends

Figure E1. Nitrite Standard Curve. Panel A: Representative real-time computer display showing sequentially increasing concentrations of sodium nitrite standards diluted in Nanopure water that are added to the purge vessel containing 5 ml of tri-iodide (I_3^-). Output from the NO analyzer is read in millivolts (mV) on the y-axis. Time is represented on the x-axis. Panel B: Linear regression showing the integrated peaks in panel A (mV x seconds) on the y-axis and nitrite concentration on the x-axis. Standard curves were performed daily, and were consistently linear ($R^2 = 0.998 \pm 0.005$ [s.d.], n=158) and reproducible (slope = 0.384 ± 0.064 [s.d.], n=158).

Figure E2. Tri-iodide based reductive chemiluminescence. A representative real-time tracing of NO metabolite analysis is shown. The red blood cell analyses are performed after incubation of whole blood in SNOHb stabilization solution for 5-10 minutes and passage through a G25 sizing column. Venous (V) and arterial (A) samples incubated with acidified sulfanilamide (AS) and without (-Hg) or with (+Hg) mercuric chloride are injected into the purge vessel containing 5 ml I_3^-. The difference between the areas under the curves of these two signals represents the SNOHb concentration. Venous and arterial plasma is incubated without or with AS and injected into the I_3^--containing purge vessel. The difference between the areas under the curves of these two signals represents the plasma nitrite concentration. Venous and arterial whole blood is incubated with whole blood nitrite stabilization solution, deproteinated with methanol, and centrifuged. The supernatant is injected into the I_3^--containing purge vessel, and the area under the curve represents the whole blood nitrite concentration.

Figure E3. Nitrate standard curve. Panel A: Representative real-time computer display showing sequentially increasing concentrations of sodium nitrate standards diluted in Nanopure water and incubated with NADPH and nitrate reductase. Samples are added to the purge vessel containing 5 ml of potassium iodide (KI). Output from the NO analyzer is read in millivolts (mV) on the y-axis. Time is represented on the x-axis. Panel B: Representative real-time computer display showing sequentially increasing concentrations of sodium nitrite standards diluted in Nanopure water and incubated with NADPH and nitrate reductase. The nitrite concentrations of these standards are equal to the nitrate concentrations shown in Panel A. Panel C: Linear regression showing the integrated peaks in panels A and B (mV x seconds) on the y-axis and nitrite or nitrate concentration on the x-axis. The curves are nearly superimposable, demonstrating complete reduction and recovery of nitrite from the nitrate standards. Standard curves were performed on each day of nitrate measurement, and were consistently linear ($R^2 = 0.995 \pm 0.009$ [s.d.], n=11) and reproducible (slope = 0.162 ± 0.049 [s.d.], n=11).

Table E1. Association between candidate confounders and sepsis[a]

NO metabolite	Covariate	% (number) of sepsis patients	% (number) of control subjects	*p* value
Arterial nitrate				
	Race[b]	23% (10/44)	4% (1/26)	0.04
	Creatinine[c]	59% (26/54)	4% (1/26)	<0.001
	Hypertension	52% (23/44)	19% (5/26)	0.006
	Smoking	27% (12/44)	0% (0/26)	0.002
Venous nitrate				
	Race	20% (8/39)	6% (2/31)	0.17
	Creatinine	54% (21/39)	0% (0/31)	<0.001
	Hypertension	46% (18/39)	22% (7/31)	0.04
	Smoking	31% (12/39)	3% (1/31)	0.004
Venous WB nitrite				
	Race	12% (4/34)	3% (1/37)	0.15
	Creatinine	68% (23/34)	8% (3/37)	<0.001
	Hypertension	62% (21/34)	16% (6/37)	<0.001
	Smoking	20% (7/34)	3% (1/37)	0.02
Venous RBC nitrite				
	Race	15% (4/27)	2% (1/44)	0.06
	Creatinine	74% (20/27)	4% (2/44)	<0.001
	Hypertension	52% (14/27)	16% (7/44)	0.001
	Smoking	42% (18/43)	3% (1/29)	<0.001
Venous SNOHb				
	Race	23% (10/43)	7% (2/29)	0.11
	Creatinine	60% (26/43)	7% (2/29)	<0.001
	Hypertension	60% (26/43)	21% (6/29)	<0.001
	Smoking	42% (18/43)	3% (1/29)	<0.001

[a]For each NO metabolite, this analysis is limited to those subjects with NO metabolite concentrations < median value, constituting a group with lesser "exposure" to the independent variable of interest.
[b]Race category coded dichotomously, 1=non-Caucasian, 0=Caucasian
[c]Creatinine coded dichotomously according to median creatinine concentration in all subjects (1 > 0.9 mg/dL, 0 ≤ 0.9 mg/dL).

Table E2. Association between candidate confounders and relevant NO metabolite

NO metabolite[a]	Covariate	Risk factor absent	Risk factor present	*p* value
Arterial nitrate				
	Race[b]	37 (31 - 44)	46 (25 – 74)	0.35
	Creatinine[c]	28 (23 - 34)	52 (40 – 66)	<0.001
	Hypertension	33 (25 - 41)	45 (36 – 55)	0.06
	Smoking	35.7 (29.0 - 43.1)	47 (35 – 61)	0.14
Venous nitrate				
	Race	35 (29 – 41)	40 (20 – 68)	0.52
	Creatinine	24 (20 – 29)	53 (41 – 66)	<0.001
	Hypertension	29 (23 - 36)	43 (34 – 54)	0.02
	Smoking	33.5 (27.2 – 40.6)	42.7 (32 - 55)	0.21
Venous WB nitrite				
	Race	171 (154 - 189)	227 (180 - 287)	0.04
	Creatinine	157 (140 -177)	209 (181 - 241)	0.003
	Hypertension	154 (137 - 173)	211 (183 - 243)	0.0008
	Smoking	166 (150 - 185)	226 (188 - 272)	0.007
Venous RBC nitrite				
	Race	258 (196 - 342)	310 (114 - 844)	0.65
	Creatinine	255 (193 -337)	279 (165 - 470)	0.76
	Hypertension	220 (154 - 313)	332 (217 -509)	0.14
	Smoking	239 (177 - 323)	381 (200 - 726)	0.16
Venous SNOHb				
	Race	100 (80 – 124)	62 (27 - 141)	0.11
	Creatinine	99 (77 - 127)	87.2 (60 - 127)	0.56
	Hypertension	114 (90 - 143)	75 (51 - 109)	0.05
	Smoking	102 (80 - 129)	70 (41 - 118)	0.15

[a]All metabolite concentrations are nM, except nitrate which is μM.
[b]Race category coded dichotomously (1=non-Caucasian, 0=Caucasian)
[c]Creatinine coded dichotomously according to median creatinine concentration in all subjects (1 > 0.9 mg/dL, 0 ≤ 0.9 mg/dL).

Table E3. Associations between NO metabolites and sepsis: univariate and multivariate analysis of variance (ANOVA)

	Univariate ANOVA		Multivariate ANOVA	
NO metabolite	**Regression coefficient (95% CI)**	***p* value**	**Regression coefficient (95% CI)**	***p* value**
Arterial plasma nitrate	32.413 (-0.198 – 65.024)	0.051	-7.868 (-45.892 – 30.155) [a]	0.683
Venous plasma nitrate	50.927 (19.979 – 81.875)	< 0.001	9.092 (-26.918 – 45.102) [a]	0.618
Venous whole blood nitrate	0.168 (0.090 – 0.247)	< 0.001	0.113 (0.023 – 0.204) [b]	0.014
Venous SNOHb	-0.187 (-0.378 – 0.003)	0.054	-0.135 (-0.341 – 0.070) [c]	0.194

[a] Quartiles of creatinine met the criteria for inclusion in the multivariate model

[b] History of hypertension (yes vs. no) and current tobacco use (yes vs. no) met the above criteria for inclusion in the multivariate ANOVA

[c] History of hypertension (yes vs. no) met criteria for inclusion in the multivariate model

Table E4. Association between candidate confounders and non-survival[a]

NO metabolite	Covariate	% (number) of non-survivors	% (number) of survivors	*p* value
Venous plasma nitrite				
	Age[b]	60% (6/10)	29% (10/34)	0.08
	Creatinine[c]	70% (7/10)	38% (13/34)	0.08
	Hypertension	80% (8/10)	31% (17/34)	0.09

[a]For each NO metabolite, this analysis is limited to those subjects with NO metabolite concentrations < median value, constituting a group with lesser "exposure" to the independent variable of interest.
[b]Age coded dichotomously according to median age of sepsis patients (1 > 62 years, 0 ≤ 62 years)
[c]Creatinine coded dichotomously according to median creatinine concentration in sepsis subjects (1 > 1.3 mg/dL, 0 ≤ 1.3 mg/dL).

Table E5. Association between candidate confounders and relevant NO metabolite

NO metabolite	Covariate	Risk factor absent	Risk factor present	*p* value
Venous plasma nitrite (nM)				
	Age[a]	54 (41 - 71)	82 (61 -112)	0.04
	Creatinine[b]	61 (48 - 76)	72.4 (51 - 103)	0.39
	Hypertension	62 (51 - 76)	72.1 (58 - 90)	0.31

[a]Age coded dichotomously according to median age of sepsis patients (1 > 62 years, 0 ≤ 62 years)
[b]Creatinine coded dichotomously according to median creatinine concentration in sepsis subjects (1 > 1.3 mg/dL, 0 ≤ 1.3 mg/dL).

Table E6. Associations between NO metabolites and hospital mortality: univariate and multivariate analysis of variance (ANOVA)

	Univariate ANOVA		Multivariate ANOVA[a]	
NO metabolite	**Regression coefficient (95% CI)**	***p* value**	**Regression coefficient (95% CI)**	***p* value**
Venous plasma nitrite	0.202 (0.0192 – 0.385)	0.031	0.154 (-0.0390 – 0.347)	0.116

[a] age met criteria as a potential confounding variable (Tables E4 –E5) and was included in the multivariate model

Table E7. NO Metabolite concentrations by tertiles of the time interval between diagnosis and Measurement

NO Metabolite [a]	0-22 hours (n=33)	23-33 hours (n=25)	34-48 hours (n=29)	*p* value [b]
Arterial plasma nitrite	90 (60 – 134)	126 (86 – 182)	110 (76 – 161)	0.46
Venous plasma nitrite	66 (47 – 94)	57 (44 – 74)	75 (48 – 115)	0.58
Arterial WB nitrite	224 (182 – 275) [c]	236 (193 – 290) [d]	252 (210 – 303)	0.66
Venous WB nitrite	191 (153 – 240) [c]	202 (166 – 246) [d]	228 (190 – 274)	0.44
Arterial RBC nitrite	420 (327 – 541) [c]	301 (139 – 651) [d]	394 (239 – 649)	0.76
Venous RBC nitrite	313 (168 – 582) [c]	525 (415 – 663) [d]	295 (135 – 644)	0.83
Arterial SNOHb	56 (31 – 103)	52 (27 – 99)	60 (44 – 83)	0.93
Venous SNOHb	64 (35 – 115)	100 (56 – 178)	86 (53 – 142)	0.53
Arterial RBC XNO	15 (8 – 27)	16 (8 – 36)	27 (18 – 40)	0.52
Venous RBC XNO	36 (24 – 54)	35 (18 – 69)	28 (17 – 46)	0.76
Arterial nitrate	39 (26 - 55)	42 (27 - 60)	48 (31 – 69)	0.72
Venous nitrate	36 (25 - 49)	37 (23 - 53)	58 (40 – 81)	0.08

[a] All metabolite concentrations are nM, except nitrate which is μM
[b] One-way ANOVA or Kruskal-Wallis test
[c] 31 subjects; [d] 24 subjects

Figure E1.

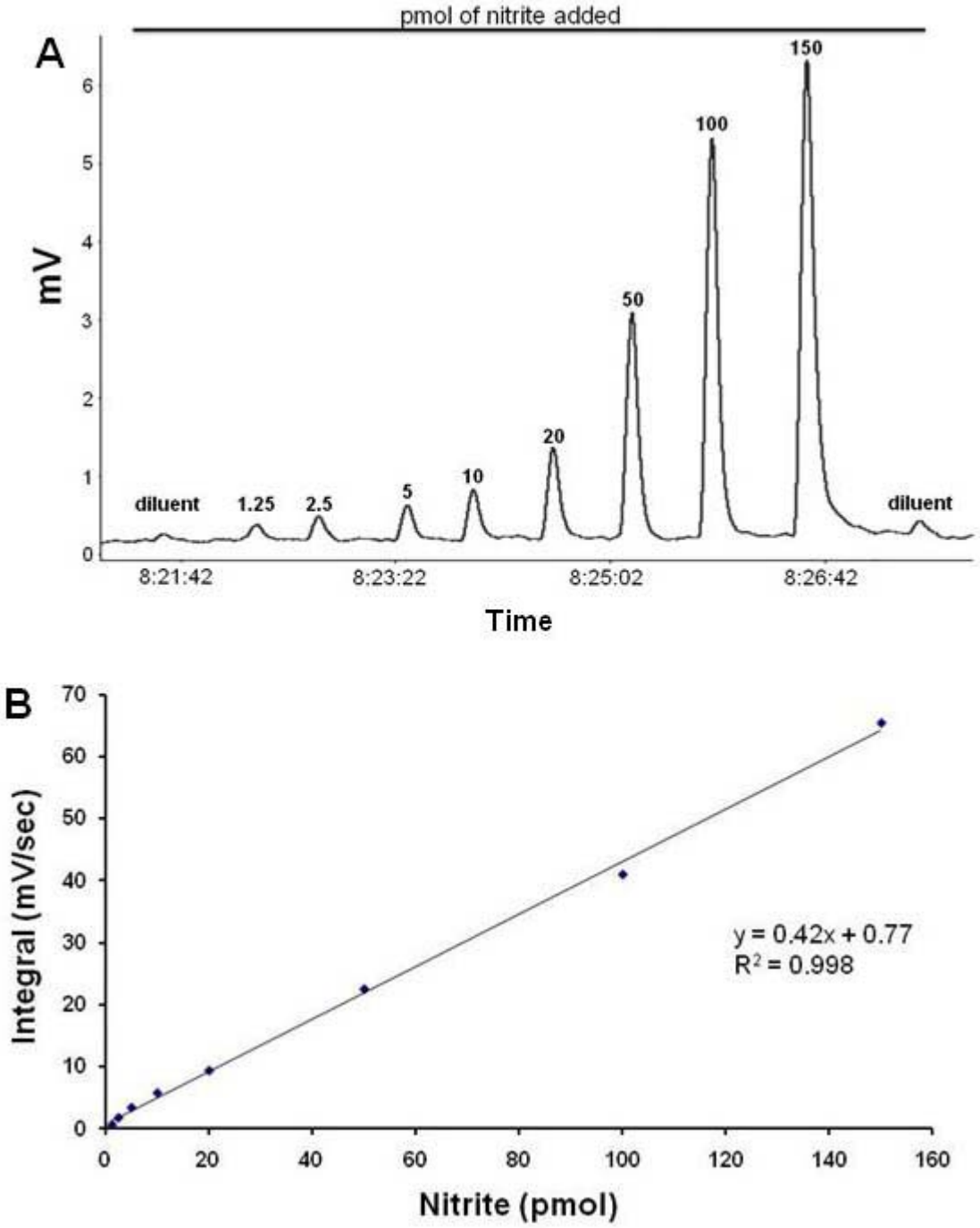
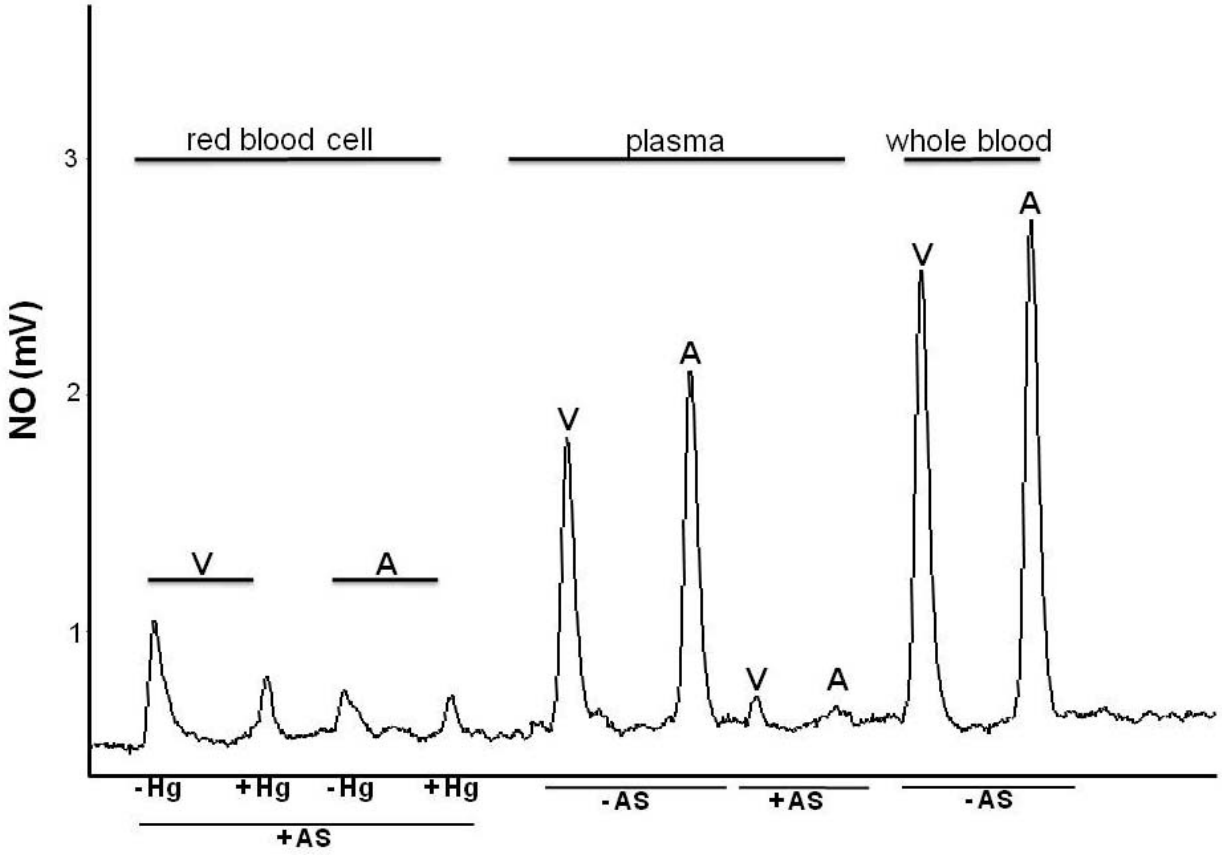

A
pmol of nitrite added
mV
diluent
1.25
2.5
5
10
20
50
100
150
diluent
8:21:42
8:23:22
8:25:02
8:26:42
Time
B
Integral (mV/sec)
y = 0.42x + 0.77
R² = 0.998
Nitrite (pmol)

Figure E2.


red blood cell
plasma
whole blood
NO (mV)
3
2
1
V
A
V
A
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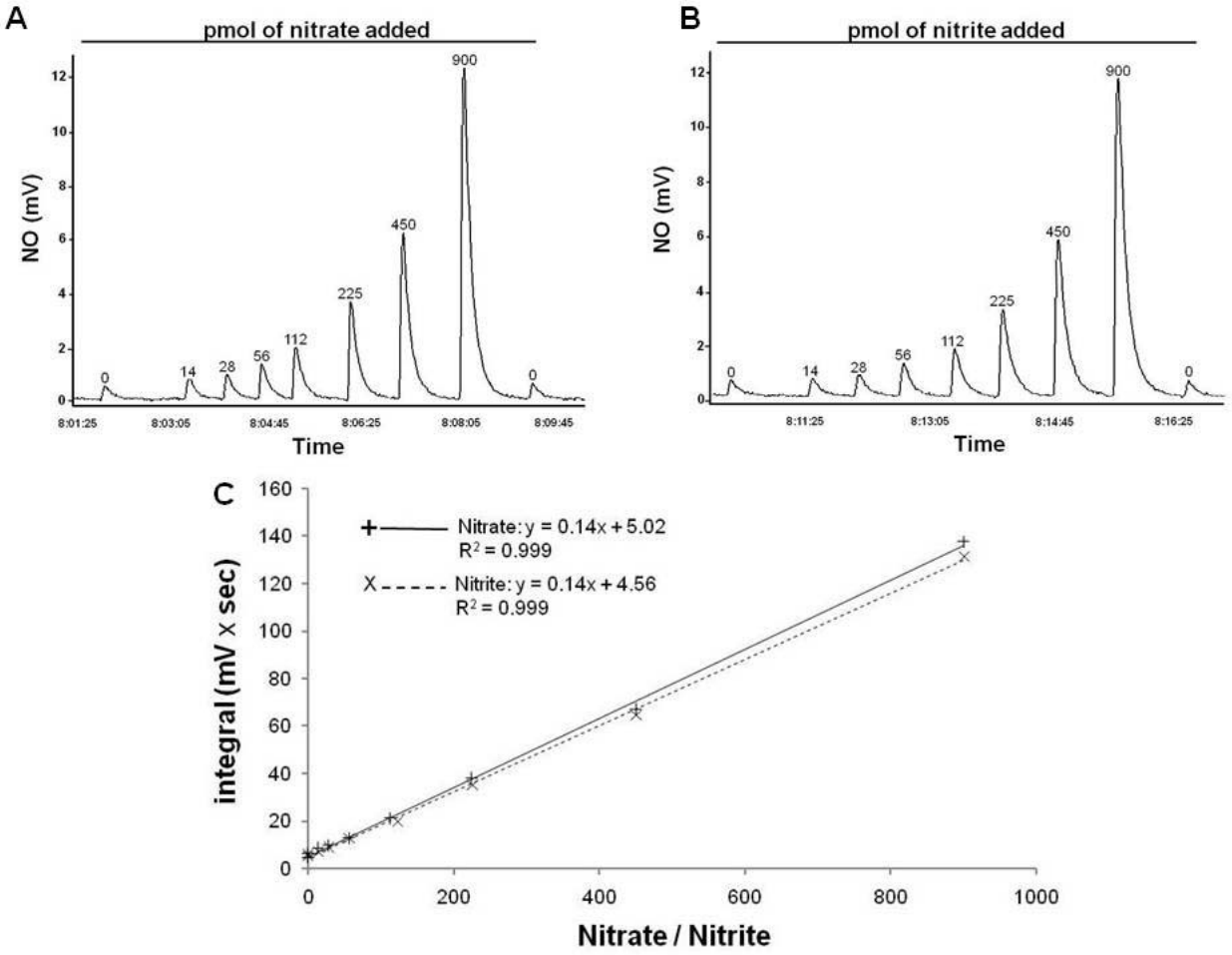
A
V
A
-Hg
+Hg
-Hg
+Hg
+AS
-AS
+AS
-AS

Figure E3.


A
pmol of nitrate added
NO (mV)
Time
0
14
28
56
112
225
450
900
0
8:01:25
8:03:05
8:04:45
8:06:25
8:08:05
8:09:45
B
pmol of nitrite added
NO (mV)
Time
0
14
28
56
112
225
450
900
0
8:11:25
8:13:05
8:14:45
8:16:25
C
Nitrate: y = 0.14x + 5.02
R² = 0.999
Nitrite: y = 0.14x + 4.56
R² = 0.999
integral (mV x sec)
Nitrate / Nitrite