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Investigation of the Reaction of Myosmine with Sodium Nitrite in vitro and in Rats

Stephen S. Hecht^{*}, Shaomei Han, Patrick M.J. Kenney, Mingyao Wang, Bruce Lindgren, Yong Wang, Yanbin Lao, J. Bradley Hochalter, and Pramod Upadhyaya

The Cancer Center, University of Minnesota, Minneapolis, MN 55455

Abstract

Previous studies have shown that the minor tobacco alkaloid myosmine (5) reacts with NaNO₂ in the presence of acid to yield 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, 8) via 4-(3-pyridyl)-4-oxobutanediazohydroxide (7). Intermediate 7 is also formed in the metabolism of the tobacco-specific nitrosamines *N*'-nitrosornicotine (NNN, 1) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 2), resulting in pyridyloxobutylation of DNA and Hb. These pyridyloxobutyl adducts can be quantified by analyzing HPB released upon acid treatment of DNA or base treatment of Hb. Quantitation of HPB-releasing DNA and Hb adducts has been used to assess the metabolic activation of NNN and NNK in smokers and smokeless tobacco users. Since myosmine is found in the diet as well as in tobacco products, it has been suggested that nitrosation of myosmine could lead to the formation of HPB-releasing adducts in people not exposed to tobacco products. We investigated the nitrosation of myosmine in vitro and in vivo in rats. Reaction of myosmine with NaNO₂ under acidic conditions produced HPB, as previously reported. A new product was identified as 3'-oximinomyosmine (11) based on its spectral properties. NNN was not detected. Groups of rats were treated with NNN, NNK, myosmine, NaNO₂, or combinations of myosmine and NaNO₂. HPB-releasing Hb and DNA adducts were clearly detected in the rats treated with NNN or NNK, but we found no evidence for production of these adducts from the combination of myosmine plus NaNO₂. The results of this study do not support the hypothesis that exposure to dietary myosmine could lead to HPB-releasing DNA or Hb adducts in humans.

Introduction

The tobacco-specific nitrosamines *N*'-nitrosornicotine (NNN, 1, Scheme 1) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 2) are “carcinogenic to humans” according to a working group of the International Agency for Research on Cancer (1). NNN and NNK are likely to play a major role as causes of cancers of the lung, head and neck, and pancreas in people who use tobacco products (2,3). NNN and NNK require metabolic activation to express their carcinogenic properties, which are clearly observed in laboratory animals treated with low doses of these compounds (4). The major metabolic activation pathway is α -hydroxylation, initiated with catalysis by cytochrome P450 enzymes (4,5). NNN undergoes α -hydroxylation at its 2'- and 5'-positions, and NNK at its methyl and methylene carbons. As shown in Scheme 1, 2'-hydroxylation of NNN and methyl hydroxylation of NNK produce the same intermediate, 4-(3-pyridyl)-4-oxobutanediazohydroxide (7), which reacts with DNA and Hb to give pyridyloxobutyl (POB) adducts. Considerable structural information on the POB adducts with DNA and Hb is available (6–8). When this DNA is treated with acid, or the Hb with base, 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, 8) is released (9–11). The released HPB

^{*}To whom correspondence should be addressed: The Cancer Center, University of Minnesota, MMC 806, 420 Delaware St SE, Minneapolis, MN 55455, USA. ph: (612) 626-7604 fax: (612) 626-5135 e-mail: hecht002@umn.edu.

can be derivatized with pentafluorobenzoyl chloride (9) yielding HPB-pentafluorobenzoate (10), which can be quantified with high sensitivity and specificity by GC-NICI-MS (9–11). Several studies have used this method to measure POB adducts in DNA or Hb of people who used tobacco products or were exposed to secondhand tobacco smoke (9,10,12–17). The results of these studies have not been as definitive as expected. In some, overlap was observed between exposed and reportedly non-exposed humans. This overlap was unexpected based on the tobacco-specificity of NNN and NNK, and has not been observed in studies of urinary metabolites of NNN and NNK in humans (18,19). This has led to the hypothesis that there may be sources of HPB releasing adducts other than NNN or NNK. One potential source, which has been discussed in a series of studies, is myosmine (5) (20–26).

Myosmine is a minor tobacco alkaloid found in both tobacco and tobacco smoke (27). In aqueous acid, myosmine is in equilibrium with 4-(3-pyridyl)-4-oxobutylamine (6) which, upon reaction with nitrite in the presence of acid, could produce intermediate **7**, identical to that formed in the metabolism of NNN and NNK (Scheme 1). Zwicknagl demonstrated that nitrosation of myosmine did indeed produce HPB, consistent with Scheme 1, and also reported formation of NNN in this reaction (23). Further studies then showed that trace amounts of myosmine are found in a number of dietary substances such as nuts, cereals, fruits, vegetables, and milk, in addition to tobacco (21,28). Therefore, these researchers hypothesized that in vivo nitrosation of dietary myosmine could lead to the formation of HPB-releasing Hb or DNA adducts in non-tobacco users not exposed to secondhand tobacco smoke (22). This could then explain some of the reported observations of HPB-releasing DNA and Hb adducts in non-tobacco exposed humans (13), and would call into question the tobacco specificity of this biomarker. In this paper, we further test this hypothesis by studying the reaction of myosmine with nitrite and quantifying HPB-releasing DNA and Hb adducts in rats treated with myosmine and nitrite.

Experimental Procedures

Caution

NNK and NNN are carcinogenic. They should be handled in a well-ventilated hood with extreme care and personal protective equipment.

Chemicals—Myosmine, HPB, NNN and NNK were synthesized (29–31). NaNO₂ was obtained from Sigma-Aldrich Chemical Co (Milwaukee, WI).

Reaction of myosmine (5) and NaNO₂: A mixture of myosmine (5.5 mM) and NaNO₂ (41 mM) in 1.0 mL KHC₈O₄H₄ (phthalate) buffer (50 mM) was adjusted to pH 1.5 or pH 3.57 with HCl and incubated at 37° C for 24 h. One µL of the reaction mixture was directly analyzed by HPLC using a Waters Associates (Milford, MA) system equipped with a UV detector (Shimadzu SPD – 10A, Columbia, MD) operated at 254 nm, and a 250 × 4.6 mm Luna 5 µ C18 (2) column (Phenomenex, Torrance, CA) eluted at 0.7 mL/min with pH 7.0 phosphate buffer (5 mM) and CH₃CN using a gradient as follows (% CH₃CN): 0–3 min (0); 3–30 min (22); 30–50 min (55). Retention times of standard HPB (8), NNN (1) and myosmine (5), were 35.2, 40.6, and 43 min, respectively. LC-ESI-MS for identification of products was carried out with an Agilent 1100 Series LC/MSD capillary flow HPLC/ion trap mass spectrometer equipped with a diode array detector (Agilent Technologies, Palo Alto, CA) and a 250 × 0.5 mm 5 µ C18 column (Agilent Zorbax SB-C18). The column was eluted with CH₃CN and 15 mM ammonium acetate buffer (pH 7.0). The solvent elution program was a 10 µL/min gradient from 5 to 55% CH₃CN in 60 min at 30 °C. The material eluting in the first 15 min was diverted to waste to prevent salts from entering the mass spectrometer. The ESI source was set in the positive ion mode as follows: spray voltage, 3.5 kV; source temperature, 200 °C; nebulizing

gas (N₂) pressure, 15 psi; and drying gas flow rate, 5 L/min. The skimmer voltage was 40 V and the capillary exit voltage was 96 V. Full scan experiments were conducted by scanning from *m/z* 50 – 450 with a maximum accumulation time of 300 ms, with selected ion monitoring at *m/z* 166 (HPB), *m/z* 176 (3'-oximinomyosmine) and *m/z* 147 (myosmine).

Direct inlet MS and HRMS were obtained on a Finnigan-MAT 90/95 instrument (Thermo Finnigan MAT, Bremen, Germany). NMR spectra were run on Varian Inova 500 and 600 MHz instruments (Varian, Inc., Palo Alto, CA).

Rat study: The study was approved by the University of Minnesota Research Subjects Protection Programs Institutional Animal Care and Use Committee. Fifty-six male F-344 rats (8 weeks old) were obtained from Charles River Laboratories (Kingston, NY). They were housed two animals per micro-isolator cage with corn cob bedding in the Research Animal Resources facility of the University of Minnesota under the following conditions: temperature 20 ± 24 °C; relative humidity 50 ± 10%; 12 h light/dark cycle. They were given NIH-07 diet (Harlan, Madison, WI) and tap water *ad libitum*. They were allowed to acclimate to the animal facility for 2 weeks prior to the beginning of the experiment. They were randomized by weight to groups and given NIH-07 diet and tap water, or diet and tap water containing test compounds (Table 1). Aqueous solutions of NNN, NNK and NaNO₂ were prepared every 2 weeks and stored at 4 °C, conditions under which they are known to be stable. Diets containing myosmine were prepared every 2 weeks and stored in air tight bags at 4 °C. The diet in the cages was changed every 4 days, and myosmine was stable under these conditions, based on analysis of the diet. Food and water consumption were measured twice weekly. At the end of the 8 week treatment period, the rats were anesthetized with isoflurane and sacrificed by cardiac puncture. Blood was obtained and red blood cells were isolated (32). Tissues were removed, rinsed with saline, and stored at –80 °C until isolation of DNA.

Analysis of HPB-releasing adducts—Hb and DNA were isolated as described previously except that NaBH₃CN was not used in the DNA isolation (32,33). HPB-releasing adducts in Hb and DNA were analyzed as described previously (34).

Statistical analysis—For both weight and food consumption, a repeated measures analysis was performed using a generalized linear mixed model, which includes both fixed and random effects. The groups and time intervals were considered fixed effects and the measurements over time within an animal or cage were random. The variance/covariance structure for the data was estimated using restricted maximum likelihood. One-way analysis of variance was used to compare the net weight gain among groups. Adjustments for multiple comparisons among groups were made according to the Tukey method. Comparisons of adduct levels were done with the t-test. P-values less than 0.05 were considered statistically significant.

Results

Reaction of myosmine and NaNO₂

A chromatogram obtained upon HPLC analysis of the reaction of myosmine and NaNO₂ is illustrated in Figure 1. The peak eluting at 35.2 min was identified as HPB (8) by comparison of its retention time and MS to those of a standard. The yields of HPB were 79% and 6% of the material eluting from HPLC, when the reactions were carried out at pH 3.57 and pH 1.5, respectively, consistent with previous results (22). The retention time of NNN in this system was 40.6 min. NNN was not detected as a product of this reaction at either pH. The detection limit was less than 0.1% of the material eluting from HPLC. The peak eluting at 38.5 min in Figure 1 was identified as 3'-oximinomyosmine (11) by the spectral properties described below.

The UV spectra of 3'-oximinomyosmine (**11**), myosmine (**5**), and HPB (**8**) are illustrated in Figure 2. The spectrum of 3'-oximinomyosmine (Figure 2A) was similar to that of myosmine (Figure 2B) except for a bathochromic shift of about 5 nm. The spectrum of HPB (Figure 2C) was quite different from those of the cyclic imines.

The EI-MS of **11** had a molecular ion of m/z 175 and a base peak of m/z 158 ($M - 17$). HRMS of these peaks confirmed their elemental compositions as $C_4H_9N_3O$ (calculated, 175.0745; found, 175.0762) and $C_9H_7N_3$ (calculated 158.0718, found 158.0713), respectively.

NMR data are summarized in Table 2. The ^1H -NMR spectrum of **11** showed four signals corresponding to a 3-substituted pyridine (9.23, 8.66, 8.40 and 7.50 ppm), consistent with those of myosmine. Two triplets appeared at 2.77 ppm and 4.14 ppm, coupled with each other as shown by COSY. Compared to the spectrum of myosmine, two pyrrolidine protons were missing and an additional exchangeable signal appeared at 11.70 ppm. Thus, the ^1H -NMR data indicated that the new compound was a substituted myosmine with substitution occurring on the pyrrolidine ring. There were two likely positions of substitution: 3'- and 5'. The latter would produce 5'-oximinomyosmine (**12**). The NMR data were consistent with 3'-substitution. The low-field proton signal at 4.14 ppm was close to the 5' methylene protons of myosmine (3.95 ppm), indicating that it was adjacent to a nitrogen. In the HMQC spectrum, this proton correlated with the carbon signal at 57 ppm, which is similar to C5' of myosmine (61 ppm). This low-field carbon signal indicated that the carbon was linked to a nitrogen. Therefore, the peak at 4.14 ppm was assigned as the 5' methylene protons. The proton signal at 2.77 ppm was assigned as the 4' methylene protons, and this correlated with C4' at 26.5 ppm in the HMQC spectrum.

The ^{13}C -NMR spectrum of **11** gave nine carbon signals. Five signals (151, 148.5, 135, 128 and 123 ppm) corresponded to a 3-substituted pyridine, consistent with those of myosmine. A peak at 164 ppm was assigned as the imine carbon (C2') based on the HMBC spectrum, in which it correlated with H4 (8.40 ppm) as well as H4' (2.77 ppm) and H5' (4.14 ppm). Its chemical shift was similar to that of C2' of myosmine (170.5 ppm). An additional imine carbon signal at 160.5 ppm correlated with H4' (2.77 ppm) in the HMBC spectrum, and was assigned as C3'. Together with the MS data, the NMR data demonstrated that the 3' position was substituted with an oximino group. The exchangeable signal at 11.70 ppm in the ^1H -NMR spectrum was assigned as the hydroxyl proton of oximino group.

Rat study

Weight gain is summarized in Figure 3. Repeated measures analysis showed that the overall mean body weight of the rats in group 5 was significantly lower than those in groups 3, 6, and 7 ($p < 0.05$) but there were no significant differences in net weight gain among the groups. Rats in group 5 also consumed significantly less food per day (overall mean 15.2 ± 0.63 g per day) than controls (16.7 ± 0.81 g per day) ($p = 0.015$). Therefore, the lower mean body weights in group 5 may have been due to low palatability of the diet containing the higher dose of

myosmine together with drinking water containing NaNO₂. There were no signs of toxicity during the study and all rats survived the 8 week treatment period.

The results of the analyses of HPB-releasing Hb and DNA adducts are summarized in Table 3. Hb adduct levels in the rats treated with NNN and NNK were clearly higher than in any of the other groups ($p < 0.001$). Small peaks co-eluting with HPB-pentafluorobenzoate (10) were observed in Hb samples from most of the rats treated with myosmine (group 3) or the higher dose of myosmine plus NaNO₂ (group 5) and the mean values were significantly greater than in controls (group 7), ($p < 0.03$), but not greater than in the rats treated with NaNO₂ alone (group 4). Small peaks were also observed in some samples from groups 4 and 6 but the mean values were not significant compared to controls.

HPB-releasing DNA adducts were detected only in the tissues of the rats treated with NNN and NNK.

Discussion

Our results confirm those of others (22,23) demonstrating that myosmine can be nitrosated in vitro to produce pyridyloxobutylating species **7**, the same intermediate that is formed in the metabolism of NNN and NNK (Scheme 1). However, we found no evidence that this reaction occurs in rats treated with myosmine in the diet plus NaNO₂ in the drinking water. Furthermore, we did not detect NNN as a product of myosmine nitrosation in vitro, but rather identified a new product, 3'-oximinomyosmine (11).

The formation of **7** upon nitrosation of myosmine in vitro is fully consistent with literature precedent. In aqueous solutions, myosmine is known to be in equilibrium with aminoketone **6**, a primary amine (35). The nitrosation of primary amines to produce intermediate diazohydroxides and diazonium ions is well established (36). Thus, the formation of HPB (**8**) as a product of myosmine nitrosation is expected. While this reaction could also occur in vivo, for example in the acidic environment of the stomach, where myosmine and NaNO₂ might both be present, it seemed unlikely to us that the pyridyloxobutylating intermediate **7**, an unstable compound, would be able to migrate from the site of its formation and alkylate cellular DNA or Hb. Consistent with this, the results of our rat studies provide no support for the formation of HPB-releasing DNA or Hb adducts from the in vivo nitrosation of myosmine. Whereas the expected HPB-releasing Hb and DNA adducts of NNN and NNK were clearly detected, resulting from intracellular generation of **7**, there was little or no evidence for their formation from myosmine plus NaNO₂, which would have generated **7** extracellularly. HPB-releasing DNA adducts were not detected in esophagus, liver, or lung of rats treated with myosmine or NaNO₂, alone or in combination. We did observe small, and statistically significant (compared to controls), amounts of HPB-releasing Hb adducts in the rats treated with the higher dose of myosmine, and in those treated with the higher dose of myosmine plus NaNO₂, but these were not significantly different from each other, nor were they different from the levels in the rats treated with NaNO₂ alone. The doses of myosmine in groups 5 and 6 were 10 times higher than those of NNN and NNK. If 10% of myosmine had been nitrosated and the resulting intermediate could alkylate Hb or DNA, then we would have expected to find adduct levels at least as high as those seen in the rats treated with NNN or NNK. Instead, the adduct levels found in groups 5 and 6 indicate that, at most, 0.004 – 0.6% of myosmine was involved in adduct formation in vivo. These results do not support the proposal that the minute amounts of myosmine (1 – 6 ppb) found in several foods (21), more than 1,000 times lower than the quantities used here, could contribute significantly to the HPB-releasing Hb and DNA adducts found in some reported non-smokers. Instead, it is likely that these adducts may have arisen by exposure to secondhand tobacco smoke or from unreported use of tobacco products.

We did detect low but significant levels of HPB-releasing Hb adducts in the rats treated with myosmine (50 ppm in the diet) or myosmine (50 ppm) plus NaNO₂ (1500 ppm). While the peaks in these chromatograms were small, they were detected in six of eight samples in these groups, and the overall levels were significantly higher than in controls. We considered the possibility that myosmine could react directly with aspartate or glutamate in Hb to give **13**, as illustrated in Scheme 2. HPB-releasing Hb adducts are formed by reaction of intermediate **7** with aspartate or glutamate in globin (6). Treatment of the hypothetical adduct **13** with base could give amino ketone **6** via 2'-hydroxynornicotine (14). Derivatization of **6** with pentafluorobenzoyl chloride (9) would yield pentafluorobenzoate **15**, which has a molecular weight of 358, one unit less than that of HPB-pentafluorobenzoate (10). If this were formed in large amounts and had identical chromatographic properties to those of **10**, its M + 1 peak could be detected in our analysis. However, selected ion monitoring of the samples from groups 3 and 5 for *m/z* 358 did not reveal a peak at the correct retention time. These results do not support the hypothesis shown in Scheme 2 as an explanation for the observed peaks. It appears that the small *m/z* 359 peaks in the base treated Hb from groups 3 and 5 may have resulted from low level HPB background which is frequently seen in this assay.

The peak eluting at 38.5 min in Figure 1 was identified as 3'-oximinomysosmine (**11**). This was most likely produced by C-nitrosation adjacent to the carbonyl group of compound **6**, which is in equilibrium with myosmine in aqueous acid (35,37) (Scheme 3). C-Nitrosation adjacent to a carbonyl group is a well-established reaction (38). The initially formed C-nitroso compound tautomerizes to an oxime. In the case of **6**, this produces **16**, which cyclizes giving **11**. It is possible that in earlier reports compound **11** may have been mistakenly identified as NNN, since they have similar polarities and HPLC retention times, and little spectral data were presented (23).

In summary, the results of this study provide new insights on the nitrosation of the minor tobacco alkaloid myosmine. The pyridyloxobutylating intermediate **7** is readily formed in vitro, but we found no evidence for alkylation of Hb or DNA in rats treated with myosmine plus NaNO₂. These results suggest that dietary myosmine does not contribute significantly to HPB-releasing DNA or Hb adducts in humans.

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Abbreviations

HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
NNN	N'-nitrososornicotine
NNK	4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone
POB	pyridyloxobut-1-yl

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Figure 1. Chromatogram obtained upon HPLC analysis of the reaction of myosmine and NaNO_2 . The reaction was carried out at pH 3.57.

Figure 2.
UV spectra (20% CH₃CN in H₂O) of A) 3'-oximinomyosmine (11), B) myosmine (5), and C) HPB (8).

Figure 3.

Body weights of the rats treated with NNN, NNK, myosmine, NaNO_2 , and myosmine + NaNO_2 according to the study design outlined in Table 1. Symbols: ◇ NNN (group 1); ■ NNK (group 2); △ myosmine (group 3); ○ NaNO_2 (group 4); □ NaNO_2 + myosmine (high dose) (group 5); ● NaNO_2 + myosmine (low dose) (group 6); ⊗ control (group 7).

Scheme 1.

Metabolism of NNN and NNK, and nitrosation of myosmine, to produce intermediate 7.

Scheme 2.

Hypothetical reaction of myosmine with Hb yielding **6** upon base hydrolysis.

Scheme 3.

Formation of 3'-oximinomyosmine in the nitrosation of myosmine.

Table 1 Study design for treatment of rats with NNN, NNK, myosmine, and NaNO₂

Group	Treatment and dose	Average daily measured dose
1. NNN	5 ppm in drinking water	92.2 ± 5.4 µg
2. NNK	2 ppm in drinking water	37.2 ± 2.6 µg
3. Myosmine	50 ppm in diet	820 ± 44 µg
4. NaNO ₂	1500 ppm in drinking water	20.2 ± 2.0 mg
5. Myosmine plus NaNO ₂	50 ppm in diet 1500 ppm in drinking water	780 ± 56 µg 19.2 ± 2.2 mg
6. Myosmine plus NaNO ₂	20 ppm in diet 600 ppm in drinking water	300 ± 24 µg 9.6 ± 1.1 mg
7. Control	None	

Table 2NMR data (600 MHz in DMSO-*d*₆) for myosmine (5) and 3'-oximinomysomine (11)

11 12			
	Position	Chemical shift (δ)	Chemical shift (δ)
¹ H (ppm)	2	8.97 (s, 1H)	9.23 (s, 1H)
	4	8.17 (d, <i>J</i> =7.8 Hz, 1H)	8.40 (d, <i>J</i> =7.8 Hz, 1H)
	5	7.47 (dd, <i>J</i> =4.8, 7.8Hz, 1H)	7.50 (dd, <i>J</i> =4.2, 7.2Hz, 1H)
	6	8.64 (d, <i>J</i> =5.4 Hz, 1H)	8.66 (d, <i>J</i> =4.2 Hz, 1H)
	3'	2.94 (t, <i>J</i> =7.8 Hz, 2H)	
	4'	1.95 (m, 2H)	2.77 (t, <i>J</i> =5.4 Hz, 2H)
	5'	3.95 (t, <i>J</i> =7.8 Hz, 2H)	4.14 (t, <i>J</i> =5.4 Hz, 2H)
	OH		11.7 s, 1H
¹³ C (ppm)	2	148.5	148.5
	3	129.5	128
	4	134.5	135
	5	123	123
	6	151	151
	2'	170.5	164
	3'	34	160.5
	4'	22	26.5
	5'	61	57

Table 3

HPB-Releasing Hb and DNA adducts in rats treated with NNN, NNK, NaNO₂, myosmine, or myosmine and NaNO₂.

Group	Hb Adducts (fmol HPB/ mg Hb) ^a	DNA Adducts (fmol HPB/mg DNA) ^b		
		Liver	Lung	Esophagus
1. NNN	5.3 ± 3.6	1960 ± 1500	183 ± 135	3460 ± 590
2. NNK	93.7 ± 38.4	1740 ± 760	1090 ± 251	660 ± 480
3. Myosmine (50 ppm) ^c	0.33 ± 0.37	ND ^e	ND	ND
4. NaNO ₂ (1500 ppm) ^d	0.11 ± 0.20	ND	ND	ND
5. Myosmine (50 ppm) plus NaNO ₂ (1500 ppm) ^c	0.30 ± 0.27	ND	ND	ND
6. Myosmine (20 ppm) plus NaNO ₂ (600 ppm) ^d	0.036 ± 0.082	ND	ND	ND
7. Controld	0.007 ± 0.019	ND	ND	ND

^a Mean ± S.D., N=8 rats. Values in groups 1 and 2 are significantly greater than all other groups, p<0.001

^b Mean ± S.D., N=4 rats. Values in groups 1 and 2 are significantly greater than all other groups, p<0.001

^c 2 of 8 samples were below LOQ (0.05 fmol/mg Hb). A value of 0 was used for these samples. Mean values are significantly greater than group 7 (p < 0.03)

^d 6 of 8 samples were below LOQ. A value of 0 was used for these samples.

^e ND, all samples below LOQ (3 fmol/mg DNA)