On-line supplement for:

Intravascular neutrophil activation due to carbon monoxide poisoning

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METHODS

Patients: The research protocol was approved by the Institutional Review Board to obtain blood for laboratory analysis from patients undergoing evaluation for suspected acute CO poisoning. Analyses were carried out on citrate-anticoagulated blood samples obtained from 50 consecutive patients. Twenty-seven women and twenty-three men with an average age of 38.5 $\pm$ 2.9 (SE) years (range 22 months to 86 years) were evaluated. These were individuals suspected of suffering CO poisoning either due to faulty home heating systems, internal combustion engines, or smoke from house fires. Patients were considered to have been exposed to environmental CO if they had a COHb level above 1 % and they did not smoke or 10 % if they smoked cigarettes. Table E1 in this supplement outlines details for these individuals. Among the group, CO exposure was confirmed in all but 8 patients.

Animals and reagents: Wistar male rats (Harlan Laboratories, Indianapolis, IN) weighing 220-240 g were fed a standard diet and water *ad libitum*. House mice (*Mus musculus*) of the C57B6J strain were either wild type or lacked a functional myeloperoxidase gene (MPO -/-). Wild type mice were purchased from Jackson Laboratories, Bar Harbor, ME and knock-out mice were a generous gift from Dr. Charles Jennette, University of North Carolina at Chapel Hill. All aspects of the animal investigation were reviewed and approved by the Institutional Animal Care and Use Committee. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animal manipulations: CO poisoning was performed according to published protocol in a 7-liter Plexiglas chamber (E1). Animals breathed 1,000 parts per million (ppm) CO for 40 minutes, then 3,000 ppm for up to 20 minutes, until they lost consciousness, and then they were removed to breathe room air and regain consciousness. The mean COHb, 63±3 % (SE, n=24), and arterial blood gas values at termination of the exposure have been reported (E2). We have also reported that cerebral blood flow increases by 54% within minutes of CO exposure and remains elevated until loss of consciousness when, due to transient cardiac compromise, blood pressure drops to 50 % below normal for ~ 4 minutes (E1, 3, 4). There is never a time when blood flow ceases, but brain mitochondrial function is mildly disturbed during unconsciousness. Assessed by reflectance spectroscopy, CO poisoning causes a transient 11% increase in the NADH/NAD ratio (E3, 4). Where indicated neutropenia (neutrophil counts < 100 cells/μl) was achieved by injecting anti-neutrophil antiserum (Inter-cell Technologies, Hopewell, NJ), and thrombocytopenia (platelet count reduced to less than 30 % of normal) was achieved by injecting anti-rat platelet antiserum (Inter-cell Technologies, Hopewell, NJ) as previously described (E5). Some rats were injected with 40 mg/kg L- nitroarginine methyl ester (L-NAME) ip 30 minutes prior to CO poisoning, or with 125 μg tirofiban iv plus 500 μg tirofiban ip immediately prior to CO. At 90 minutes after CO poisoning, the rats were anesthetized, a midline sternotomy was performed and blood was removed by cardiac puncture into heparinized syringes for cell and plasma studies. The descending aorta was then cross-clamped, the ventricles opened, a cannula was inserted in the left ventricular outflow track and intravascular blood was removed by injecting PBS containing 100 U/ml heparin until the right ventricular effluent was clear (~ 100 ml injected).

Tissue preparation for chemical assays: Brain tissue was prepared and processed for Western blotting as described previously (E6, 7). Blots were probed using 1:1000 dilutions of rabbit anti-human myeloperoxidase (Dako Corp., Carpinteria, CA), mouse anti-neuronal nitric oxide synthase (nNOS), anti-inducible NOS (iNOS), anti-neuronal NOS (nNOS), and anti-human CD66 (BD Pharmingen, San Jose, CA), and mouse monoclonal anti-GLUT3 (Calbiochem, La Jolla, CA) followed by appropriate secondary antibodies and analyzed using an Odyssey infra-red imaging system (Lincoln, NE). Where indicated brain tissue was processed for column chromatography exactly as described in prior publications (E6-8).

Western blot band densities for the three isoforms of NOS was assessed and normalized to the band density for glucose transporter-3 (GLUT3). GLUT-3 is abundantly expressed within neurons (E9). Results demonstrated no significant differences between brains from control rats and rats killed 90 minutes after CO poisoning (Table E2). It should also be noted that the control rat MBP chromatography pattern was slightly different from the pattern observed in previous trials (E6, 7). The reason for this is not known, but may be related to the fact that the rats were obtained from a different vendor than in the previous studies.

Histochemistry: Immunohistochemistry was performed on paraffin-embedded sections after perfusion/fixation of anesthetized rats following published methods (E5-8).

Analysis of blood: Blood from patients or anesthetized animals was handled in a similar manner. Within 5 minutes after obtaining blood from rats and mice, or 30 minutes for humans, 0.5 ml was combined with an equal volume of 0.5 % paraformaldehyde in PBS, and stored at 4^{o}C until stained with antibodies for flow cytometry (typically within 12 hours for clinical samples and within 30 minutes for animal samples). The remainder of the blood was centrifuged at 250 X g for 10 minutes and supernatant plasma was frozen at –20^{o}C until analysis. Plasma MPO from rats and the first eight patients was assessed by Western blots. Plasma protein (50 μg) was subjected to SDS-PAGE, transfer to nitrocellulose and probed with a 1:1000 dilution of rabbit anti-human MPO (Dako Corp.) followed by the appropriate secondary antibody and images were analyzed as described above for brain tissue. Thereafter, plasma from patients was analyzed using a MPO ELISA from Northwest Life Science Specialists, Vancouver, WA.

To assess MPO content of rat neutrophils, cells were isolated from anticoagulated blood by density gradient centrifugation using Robbins PMN Prep (Robbins Scientific, Sunnyvale, CA) following the manufacturer's instructions. Cells were washed once in buffer (Hanks balanced salts solution containing 20 mM Hepes, 2 mg/ml endotoxin-free bovine serum albumin, pH 7.4) and then suspended in 0.5 ml buffer for counting in a Z2 Coulter Counter. Cells were frozen in liquid nitrogen, thawed and sonicated twice for 20 seconds each in an ice-cold water bath using a Heat Systems-Ultrasonics (Plainview, NJ) sonicator at a setting of 7. Samples were centrifuged at 1000 X g for 5 minutes and MPO activity assessed in 0.2 ml aliquots of the supernatant. Enzyme activity was measured in 50 mM potassium phosphate (pH 6.0) containing 0.5 mM *ortho*-diansidine and 0.6 mM

H_2O_2 by monitoring the increase in 460 nm light absorbance in a dual beam spectrophotometer (Hitachi Model 3210) for four minutes.

Flow cytometry: Formalin-fixed blood samples from rats, mice and humans were processed in a similar fashion following the method of Li, *et al.* (E10) with minor modifications. Aliquots of formalin-fixed blood samples (0.5 ml for humans and rats, 0.2 to 0.4 ml for mice) were incubated with saturating concentrations of antibodies for 30 minutes at room temperature. Antibodies included [R-phycoerythrin (RPE) conjugated mouse anti-human MPO (Acris Antibodies obtained via Novus Biologicals, Littleton, CO), fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD18 (BD Pharmingen), FITC mouse anti-rat CD18 or RPE conjugated rat anti-mouse CD18 (Accurate Chemical, Westbury, NY), RPE conjugated mouse anti-human CD61 (BD Pharmingen), or rabbit anti-rat platelet antiserum (Inter-cell Technologies) followed by 1:1000 anti-rabbit IgG conjugated to FITC, or FITC conjugated PAC1 (a mouse IgM that recognizes only the activated form of human platelet glycoprotein $\alpha_{IIb}\beta_3$ integrin from BD Pharmingen). For the last 10 minutes of antibody incubation, 1 μl DRAQ5 (Alexis Chemicals, San Diego, CA) a cell permeable anthraquinone to stain cell DNA, was added. The blood was then diluted with 2 ml PBS and kept at 4^oC until flow cytometry analysis. Blood cells could be analyzed in the whole-blood suspension, and on occasion the leukocytes were concentrated by density gradient centrifugation. For this processing the 2.5 ml blood-PBS suspension was placed on top of 3 ml Histopaque 1119 (Sigma), centrifuged at 400 x g for 30 minutes and the cells washed twice in PBS prior to flow cytometry analysis.

Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson) at the Abramson Cancer Center Flow Cytometry Core facility. Nucleated cells were identified by DRAQ5 staining, and neutrophils gated based on forward and side laser light scattering (Figure E1). Isotype matched mouse immunoglobulin served as control for non-specific antibody binding. Studies were always coupled with analyses of control cells to assess cell activation due to CO. Thus, on each day of study, blood obtained from air-breathing animals or, for evaluation of human cell surface markers, blood obtained from a normal healthy human volunteer was processed in exactly the same manner as the blood from patients.

Statistics: Statistical significance was determined by ANOVA followed by the Dunn's test (SigmaSTAT, Jandel Scientific). The level of significance was taken as $P< 0.05$. Results are expressed as mean$\pm$SE.

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Figure E1. Flow cytometry gating paradigm followed for all trials. Nucleated cells in formalin-fixed blood were identified by DRAQ5 staining using the cytometer FL3 channel and displayed based on forward and side light scatter. Neutrophils were identified based on light scatter characteristics (circled population), and histograms for surface markers were generated for these cells.

Table E1: Patients evaluated for this trial:

#	AGE	M/F	SOURCE	BURN	DURATION	COHb	LOC?	F/U
1	22	F	Smoke	No	0.5 hour	0.3%	No	ok
2	66	M	Furnace	No	0.5 hour	0.3%	No	ok
3	21	M	Smoke	No	0.5 hour	0.7%	No	ok
4	26	M	Smoke	Yes (knee)	0.5 hour	0.3%	No	ok
5	25	F	Smoke	Airway soot	0.6 hour	0.7%	No	ok
6	45	M	Furnace	No	1 hour	0.6%	No	ok
7	24	M	Furnace	No	3 hour	0.8%	No	ok
8	59	M	Auto	No	1 hour	0.5%	No	ok
9	22	M	Smoke	No	0.6 hour	1%	No	ok
10	21	F	Smoke	No	0.5 hour	3.1%	No	ok
11	22	M	Smoke	Yes (hands)	0.5 hour	5.3%	No	ok
12	26	F	Smoke	No	0.6 hour	36%	No	ok
13	5	M	Smoke	No	0.9 hour	26%	Yes	ok
14	81	F	Smoke	Airway soot	0.4 hour	31%	Yes	ok
15	7	F	Smoke	Yes (facial)	0.9 hour	31%	Yes	ok
16	55	M	Smoke	No	0.8 hour	4.7%	No	ok
17	11	F	Smoke	15% BSA	0.9 hour	36%	Yes	‘Slow’
18	36	M	Auto	No	3 hour	30.3%	Yes	ok
19	38	M	Smoke	No	1.3 hour	50.7%	Yes	ok
20	22	M	Smoke	5% BSA	1.2 hour	21%	No	ok
21	21	F	Smoke	No	2 hour	1.3%	No	ok
22	47	F	Smoke	No	2 hour	1.5%	No	ok

23	28	M	Motor	No	2.5 hour	21%	Yes	ok
24	46	F	Motor	No	1.5 hour	41%	Yes	ok
25	34	M	Motor	No	1.2 hour	26.3%	Yes	ok
26	64	F	Furnace	No	2 hour	21%	No	ok
27	50	F	Furnace	No	4 hour	22%	No	ok
28	42	F	Furnace	No	3.5 hour	31%	No	lost
29	51	M	Furnace	No	3.5 hour	23%	Yes	ok
30	25	M	Motor	No	3.4 hour	27%	No	ok
31	41	F	Smoke	4% BSA	3.1 hour	4%	Yes	ok
32	1	F	Furnace	No	3.5 hour	12.2	Yes	ok
33	42	M	Auto	No	4 hour	35%	Yes	lost
34	35	F	Furnace	No	4 hour	21%	Yes	ok
35	41	M	Furnace	No	4 hour	23%	Yes	ok
36	19	M	Smoke	No	6.1 hour	15%	Yes	ok
37	25	F	Furnace	No	6.5 hour	25%	Yes	ok
38	41	M	Furnace	No	8 hour	27%	Yes	ok
39	35	M	Auto	No	6.2 hour	28%	Yes	lost
40	3	M	Furnace	No	6.2 hour	18%	Yes	ok
41	48	F	Furnace	No	6.5 hour	16%	No	ok
42	33	M	Auto	No	8 hour	21%	Yes	lost
43	46	M	Furnace	No	6.5 hour	35%	Yes	ok
44	47	M	Furnace	No	8.5 hour	38%	Yes	ok
45	75	F	Smoke	No	6.5 hour	44%	Yes	ok
46	20	M	Furnace	No	13 hour	11.6%	No	ok

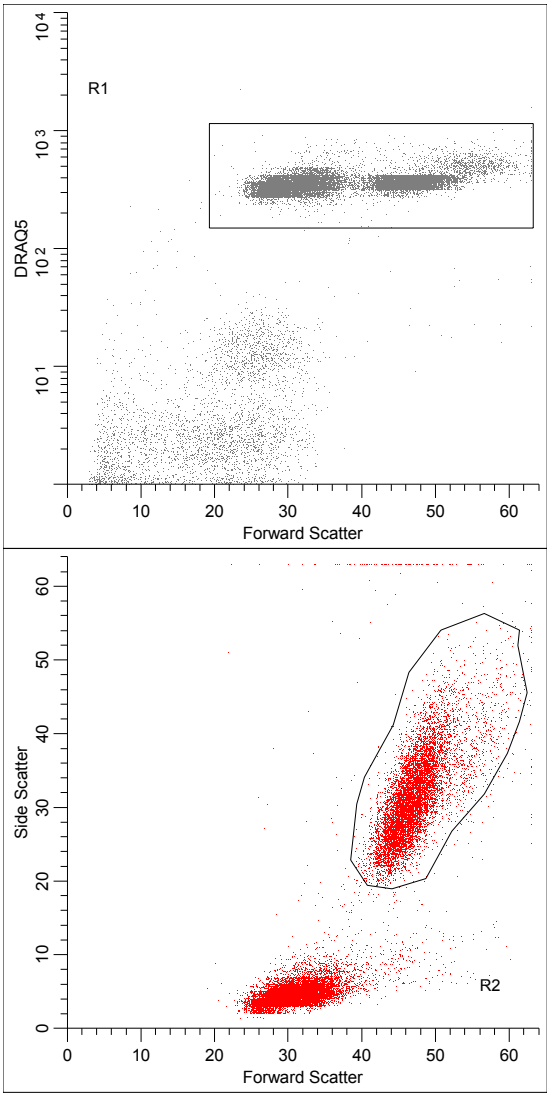
47	35	F	Furnace	No	13 hour	14%	No	ok
48	16	F	Furnace	No	13 hour	15%	Yes	ok
49	86	M	Furnace	No	14 hour	55%	Yes	ok
50	84	F	Furnace	No	14 hour	57%	No	ok

Numbers in the table were used to identify patients in each category of the figures in the main text. Shown are the age of the patients, sex (male/female), source of CO (smoke from house fire, or combustion products from an automobile, malfunctioning water heater or furnace, or a gasoline-driven motor such as a generator or power saw in a confined space), whether a patient also suffered burns, the estimated duration of exposure to CO, carboxyhemoglobin (COHb) level on admission to the Emergency Department, and whether they suffered an interval of unconsciousness during the CO exposure. Outcome of the patients was assessed by telephone contact with the patients or in the case of pediatric cases, with the patient's parent or guardian. Notation of 'ok' indicates absence of abnormalities on an itemized list which was administered between 6 and 8 weeks after exposure. Inquiry was made about whether following CO poisoning any of these new abnormalities had occurred: headache, fatigue, nausea, dizziness, trouble thinking, trouble sleeping, heart pounding, decreased vision, chest pain, shortness of breath, abdominal pain, numbness or tingling, unusual spells characterized as difficulty with concentration or thinking, weakness, or poor balance. One patient was felt to be mentally 'slow' by her parent. This child exhibited no abnormalities on examination in our clinic 3 months after the CO exposure, and the parent declined to have the patient undergo formal neuropsychological evaluation. Four patients were lost to follow-up, and this is reflected by the term 'lost' in the table.

Table E2. Relative band density for nitric oxide synthase (NOS) isoforms relative to GLUT3 in brain homogenates from air-breathing control rats and rats killed 90 minutes after CO poisoning. Data are mean ± SE, n=4 for each group.

	CONTROL	CO
Neuronal NOS /GLUT3	0.48 ± 0.03	0.50 ± 0.03
Inducible NOS/GLUT3	0.019 ± 0.001	0.020 ± 0.001
Endothelial NOS/GLUT3	0.027 ± 0.002	0.028 ± 0.002

FIGURE E1


R1
DRAQ5
10 4
10 3
10 2
10 1
0
10
20
30
40
50
60
Forward Scatter
Side Scatter
R2
Forward Scatter