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Monitoring Dose Response of Cyanide Antidote Dimethyl Trisulfide in Rabbits Using Diffuse Optical Spectroscopy

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Abstract

Introduction Cyanide (CN) poisoning is a serious chemical threat from accidental or intentional exposures. Current CN exposure treatments, including direct binding agents, methemoglobin donors, and sulfur donors, have several limitations. Dimethyl trisulfide (DMTS) is capable of reacting with CN to form the less toxic thiocyanate with high efficiency, even without the sulfurtransferase rhodanese. We investigated a soluble DMTS formulation with the potential to provide a continuous supply of substrate for CN detoxification which could be delivered via intramuscular (IM) injection in a mass casualty situation. We also used non-invasive technology, diffuse optical spectroscopy (DOS), to monitor physiologic changes associated with CN exposure and reversal.

Methods Thirty-six New Zealand white rabbits were infused with a lethal dose of sodium cyanide solution (20 mg/60 ml normal saline). Animals were divided into three groups and treated with saline, low dose (20 mg), or high dose (150 mg) of DMTS intramuscularly. DOS continuously assessed changes in tissue hemoglobin concentrations and cytochrome c oxidase redox state status throughout the experiment.

Results IM injection of DMTS increased the survival in lethal CN poisoning. DOS demonstrated that high-dose DMTS (150 mg) reversed the effects of CN exposure on cytochrome c oxidase, while low dose (20 mg) did not fully reverse effects, even in surviving animals.

Conclusions This study demonstrated potential efficacy for the novel approach of supplying substrate for non-rhodanese mediated sulfur transferase pathways for CN detoxification via intramuscular injection in a moderate size animal model and showed that DOS was useful for optimizing the DMTS treatment.

Keywords Chemical and biological weapons · Cyanide toxicity reversal · Optical hemodynamic monitoring · Dimethyl trisulfide · Lethal cyanide poisoning · Diffuse optical spectroscopy

Abbreviations

DOS Diffuse optical spectroscopy
FD Frequency-domain
SS Steady-state

OxyHb Oxyhemoglobin
DeoxyHb Deoxyhemoglobin
CcO Cytochrome c oxidase

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Introduction

Mass casualty CN poisoning is a major threat and a priority concern of the US chemical defense program for civilians, as well as military personnel. Mass CN exposures can occur from a broad range of sources including industrial accidents, inhalation of combustion products, and acts of military aggression or terrorism [1–4]. Doses of as small as 50 mg may be fatal to humans [5]. CN is inexpensive and readily accessible, with more than 5.2 billion pounds produced annually worldwide [6]. Ingestion or inhalation of CN can cause irreversible injury or death within minutes of exposure [7]. While effective antidotes are available for treating individual or a small number of victims, no antidote presently exists that could be administered in a mass casualty scenario, since current antidotes must be given intravenously in large volumes of fluid. We have been investigating potential novel antidote agents that could be readily administered in mass casualty settings. Ideal agent properties for mass casualty antidote applications would be safe, rapidly acting agents, preferably capable of being administered by intramuscular injection, or a similarly rapid route of administration.

Clinical treatments of CN poisoning include three general classes of agents: methemoglobin generators and NO donors (sodium nitrite, amyl nitrite, and dimethyl aminophenol), direct binding agents (hydroxocobalamin, dicobalt edetate), and sulfur donors (sodium thiosulfate and glutathione) [4, 8]. Sulfur donors convert CN to thiocyanate, primarily through enzymatic catalysis with sulfur transferases, including rhodanase. The conversion rate for detoxification of CN can be accelerated by increasing the sulfur donor pool. Dimethyl trisulfide (DMTS) is a simple trisulfide donor with a number of attributes that would theoretically be favorable for use as a CN antidote [9, 10]. DMTS is a natural product (found in garlic, and decaying organic matter) that the FDA has approved to be used in the food industry as a flavor enhancer. As for the intramuscular injection, LD₅₀ of DMTS was estimated to be 598.5 mg/kg in mice [11]. This shows that, at the dose we are using as antidote, it is safe, inexpensive, and can serve as a substrate in sulfurtransferase enzymatic reactions. However, it can also react with CN in high efficiency even without rhodanase. The low molecular weight and lipophilicity of DMTS should enable rapid transfer following intramuscular injection and potential for good penetration across the blood-brain barrier for possible use as a CN antidote [12, 13]. Demonstration of the ability of intramuscular DMTS formulations to effectively treat lethal CN poisoning should help serve as the basis for subsequent mass casualty antidote development, either as a single agent, or in combination with other antidotes. Direct intramuscular injection of DMTS results in local muscle tissue damage. We have shown that formulation into micelles using polysorbate 80 preserves the effectiveness, while eliminating local tissue toxicity ([http://](http://www.dtic.mil/get-tr-doc/pdf?AD=ADA594849)

www.dtic.mil/get-tr-doc/pdf?AD=ADA594849) [12, 13]. Our prior work has shown effectiveness of DMTS to prevent lethal CN toxicity in a mouse model [10].

Since antidote development for lethal exposures of compounds such as CN cannot be tested for efficacy in humans, animal studies are the FDA-designated path to efficacy approval. We have extensively used rabbit models and non-invasive diffuse optical spectroscopy (DOS) to assess tissue hemoglobin concentrations under severe physiological stresses. Especially in clinical care monitoring, DOS has been successfully employed in monitoring hemorrhage and fluid resuscitation [14, 15], the formation and treatment of methemoglobinemia [16], and cyanide poisoning by quantifying the tissue concentrations of hemoglobin species and relevant chromophores [17, 18].

Main objectives of this study are (1) to investigate whether the reversal of lethal CN toxicity in a rabbit model is dependent upon intramuscular injection dose of DMTS and (2) to demonstrate that diffuse optical spectroscopy can verify underlying metabolic changes using cytochrome c oxidase redox state changes during CN poisoning and reversal with DMTS.

Materials and Methods

General Preparation

Pathogen-free New Zealand White rabbits weighing 3.5–4.5 kg (Western Oregon Rabbit Supply) were used. There are number of reasons that we selected the rabbit model for these studies [17–20]. Rabbits are large enough to be readily intubated and instrumented with arterial and venous catheters, and have sufficient muscle mass that is suitable for DOS measurements. This enables assessment of the rate of CN toxicity effect development and of the rate of CN toxicity reversal based on intervention [17–20]. Rabbits are more facile to work with than pigs, dogs, or other larger vertebrate animals, making them more practical for moderate-scale studies. All procedures were reviewed and approved by the University of California, Irvine, Institutional Animal Care and Use Committee (IACUC).

Animal Preparation

Animals were anesthetized with an intramuscular injection of a mixture of 2 ml of ketamine HCl (100 mg/ml, Ketaject, Phoenix Pharmaceutical Inc., St. Joseph, MI) and 1 ml of xylazine (20 mg/ml, Anased, Lloyd Laboratories, Shenandoah, IA) using a 23 gauge 5/8-in. needle. After the intramuscular injection, a 23 gauge 1-in. catheter was placed in the animals' marginal ear vein to administer continuous IV anesthesia. The animals were intubated with a 3.0 cuffed endotracheal tube secured by a gauze tie and were mechanically

ventilated (dual phase control respirator, model 32A4BEPM-5R, Harvard Apparatus, Chicago, IL) at a respiratory rate of 20 to 22 breaths/min, a tidal volume of 60 cc, and fraction of inspired oxygen (FiO₂) of 100%. A pulse oximeter (Biox 3700 Pulse Oximeter, Ohmeda, Boulder, CO) with a probe placed on the tongue was used to measure arterial blood oxygen saturation (SpO₂) and heart rate. Blunt dissection was performed to isolate the femoral artery and vein on the left thigh for blood sampling, CN infusion, and systemic pressure monitoring.

Lethal Level Cyanide Poisoning

Sodium cyanide (NaCN, 20 mg; MilliporeSigma, St. Louis, MO) was dissolved in 60 ml of 0.9% saline in a 60-cc plastic syringe and was given intravenously by IV pump at the rate of 1 ml/min (0.33 mg/ml of NaCN). One hundred percent O₂ supply was switched to atmospheric air after 30 min of cyanide infusion, and the respiratory rate on the ventilator was reduced down to 18–20 breaths/min. NaCN solution was continuously infused until the systolic blood pressure fell below 60 mmHg, at which time the treatment was prepared and administered. After administration of the treatment, cyanide infusion was continued for an additional 30 min. This protocol resulted in rabbits receiving 22–26 mg of NaCN across 1 h, which, in this model, was lethal in 80% of rabbits not given cyanide antidote. On completion of the experiment (90 min), the surviving animals were euthanized with an intravenous injection of 1.0 cc Euthasol (390 mg pentobarbital sodium, 50 mg phenytoin sodium, Vibrac AH, Inc., Fort Worth, TX) administered through the marginal ear vein. Figure 1 illustrates key time points of the experiment.

DMTS Formulation, Dose, and Administration

DMTS was formulated with 15% aqueous polysorbate 80 (PS 80, Sigma-Aldrich, St. Louis, MO) to make up a stable solution for intramuscular injection as described previously [13, 21, 22]. Briefly, DMTS antidotal treatment formulation was prepared by adding 1.5 g PS 80, to injectable sterile water (Hospira, Lake Forest, IL) for a total weight of 10 g. This mixture was then covered with parafilm and allowed to sit on a stir plate overnight. Following this preparation, 250 mg of neat dimethyl trisulfide (Sigma-Aldrich, St. Louis, MO) was then diluted to 5 ml with the 15% PS 80 solution. The mixture was vortexed until clear, yielding a 15% DMTS solution by weight, with a final concentration of 50 mg/ml. The solution was stored in sealed glass vials.

Data Collection

Venous blood CN levels were measured at baseline and 25 min of CN infusion. Additional blood samples were collected after DMTS injection. After the injection was performed, 5, 15, 30, 45, 60, and 90 min post-injection samples were collected. Throughout the experiment, DOS measurements were taken continuously every 12 s.

Study and Control Groups

DMTS injections were performed intramuscularly in the left latissimus dorsi muscle. A total of 36 animals were studied. As controls, 12 animals received saline. For the treatment groups, two groups (12 animals each) received an intramuscular injection of DMTS, 20 mg and 150 mg, respectively (50 mg/ml in saline). With animals weighing 3.5–4.5 kg, the

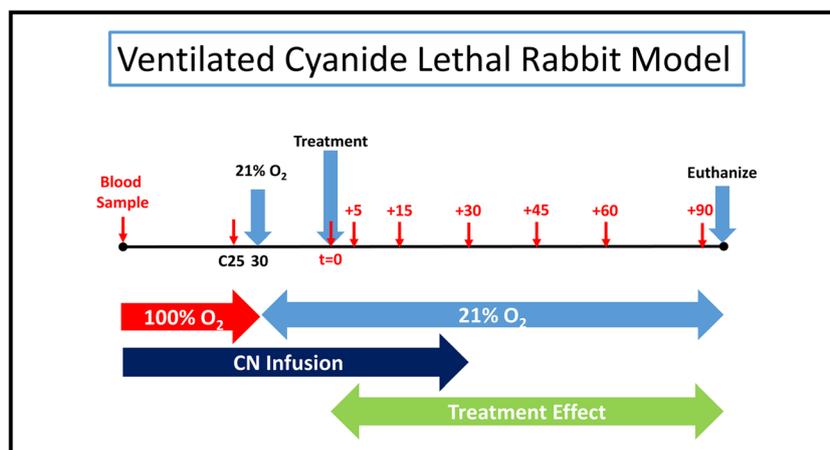


Fig. 1 Timeline for the ventilated cyanide lethal rabbit model. Key time points of the measurement are baseline (bsln), CN25, $t=0$ min, and $t=30$ min. CN infusion started at bsln and CN25 represents 25 min of CN infusion under 100% O₂. One hundred percent inspired O₂ was switched to atmospheric air (21% O₂) 30 min after initiation of CN infusion. The

systolic blood pressure as the primary trigger to decide the antidote injection. At $t=0$ min, the treatment (saline or DMTS) was prepared and administered. At $t=30$ min, an additional CN intravenous infusion after antidote injection stopped

dose range in the low DMTS group was 4.4–5.7 mg/kg, and for the high DMTS group, the dose range was 33.3–42.9 mg/kg. Low and high doses of DMTS were calculated based on molecular weight of DMTS (126.26 g/mol) and NaCN (49.01 g/mol). With rabbits receiving 22–26 mg of NaCN, the molar ratio of low (20 mg DMTS) and high (150 mg DMTS) to NaCN was approximately 1/3- and 3-fold, respectively. Table 1 summarizes three study groups.

Non-invasive Measurements Using Diffuse Optical Spectroscopy (DOS)

Diffuse optical spectroscopy (DOS) measurements were obtained through a fiber optic probe with a light diode emitter and detector at a fixed distance (10 mm) from the source fiber, which was placed on the shaved surface of the right inner thigh of the animal. The DOS system we constructed combines multi-frequency domain photon migration with time-independent near-infrared spectroscopy to accurately measure bulk tissue absorption and scattering spectra [16]. It employs five laser diodes at discrete wavelengths (661, 681, 783, 823, and 850 nm), and a fiber coupled avalanche photo diode (APD) detector (Hamamatsu high-speed APD module C5658, Bridgewater, NJ) for the frequency domain measurements. The APD detects the intensity-modulated diffuse reflectance signal at modulation frequencies between 50 and 300 MHz after propagation through the tissue. Absorption and reduced scattering coefficients are measured directly at each of the five laser diode wavelengths using frequency-dependent phase and amplitude data. Steady-state acquisition is accomplished using a broadband reflectance measurement from 650 to 1000 nm that follows frequency domain measurements using a tungsten-halogen light source (Ocean Optics HL-2000, Dunedin, FL) and a spectrometer (BWTEK BTC611E, Newark, DE). Intensity of the steady-state reflectance measurements is calibrated to the frequency domain values of absorption and scattering to establish the absolute reflectance intensity. DOS monitoring of CcO redox states changes during cyanide poisoning has been previously described [23]. In brief, reduced scattering coefficients are calculated as a function of wavelength throughout the near-infrared region by fitting a power-law to five reduced

scattering coefficients at the baseline and are set as constant values in subsequent measurements. In addition, only the changes in chromophore concentrations as well as changes in CcO redox states are calculated from the difference in absorption spectra from baseline values. In contrast, tissue concentrations of oxy- and deoxyhemoglobin, water, and CcO redox state changes (changes from baseline of the differential between oxidized and reduced CcO concentrations) are calculated by a linear least squares fit of the wavelength-dependent extinction coefficient spectra of each chromophore. We used published absorption spectra for hemoglobins [24], water [25], lipid [26], and difference spectra of CcO [27] (downloaded from <http://www.ucl.ac.uk/medphys/research/borl/intro/spectra>) for the subsequent fitting and analysis.

Measurement of Respiratory Exchange Ratio (RER)

In order to confirm and correlate DOS assessment of cytochrome c oxidase (CcO) redox state changes with anaerobic metabolism during CN poisoning, gas exchange measurements were used. Respiratory exchange rate (RER) is the ratio between rate of CO₂ exhaled and O₂ uptake (VCO₂/VO₂), where VO₂ is the difference between O₂ inhaled minus exhaled per unit time (reflecting rate of O₂ consumption) and VCO₂ is the volume of CO₂ exhaled per unit time (reflecting the rate of CO₂ production). It is a dimensionless number used in calculations of basal metabolic rate when estimated from carbon dioxide production. Since CO₂ is a byproduct of the cellular metabolic process, most of the exhaled CO₂ results from cellular respiration. The range of RER for organisms in metabolic balance is usually from 0.7 (pure fat oxidation) to 1.0 (pure carbohydrate oxidation). RER during the lethal CN poisoning was measured using a Datex–Ohmeda S/5 compact anesthesia monitor (Madison, WI) with a continuous gas exchange/metabolic monitor module.

Results

Table 1 shows the three treatment groups used in the study. Saline, 20 mg DMTS, and 150 mg DMTS were used as treatments, respectively. Each group consisted of 12 animals with

Table 1 Three treatment groups in the study. Group 1 = controls (CN alone), Group 2 = CN with low-dose IM DMTS (20 mg), Group 3 = CN with high-dose IM DMTS (150 mg). Each group consists of 12 animals.

Group	<i>n</i>	Weight (kg)	Total NaCN (mg)	Treatment	Survived (%)
1	12	3.90 ± 0.08	22.0 ± 1.11	Saline	16.7
2	12	3.95 ± 0.08	25.5 ± 0.94	20 mg DMTS	41.7
3	12	4.02 ± 0.06	22.6 ± 0.86	150 mg DMTS	75.0

The weight, total NaCN received, and survival rate are listed. Data are listed as mean ± SEM (standard error of mean)

average weight from 3.90 to 4.02 kg. The total amount of NaCN in all three groups was comparable, with 20-mg DMTS group with highest amount (25.5 mg). Figure 2 shows DMTS treatment survival curves of all three groups following a lethal CN exposure. All non-survival animals died within 32 min after treatment injection. Survival analysis was conducted using a Kaplan-Meier estimator with equality over three groups examined with a log rank test (OriginPro 8.1, OriginLab Corp., Northampton, MA 01060). The log rank test ($\chi^2 = 11.247$, $p = 0.00361$) indicates that IM DMTS treatment was associated with a significant difference in survival functions during the study. Pairwise comparison of survival curves showed that control and high-dose survival curves were significantly different ($p = 0.0022$), while low-dose and high-dose ($p = 0.133$), and control and low-dose ($p = 0.0552$) survival functions were not significantly different. As shown in the Table 1, the survival rate was the highest with 150 mg DMTS group (75% survival).

Table 2 lists blood pressure, heart rate, arterial blood gas values of animals that survived following DMTS intramuscular injection after receiving the lethal dose of CN (group 2 and 3). Arterial oxygen partial pressure, arterial carbon dioxide partial pressure, base excess, and pH were measured with blood gas analysis. Key time points of the measurement are baseline (bsln), CN25, $t = 0$ min, and $t = 30$ min. CN infusion started at bsln and CN25 represent 25 min of CN infusion under 100% O₂. Animals underwent a transitional period when the 100% inspired O₂ was switched to atmospheric air (21% O₂) 30 min after initiation of CN infusion resulting in both physiological and optical data variability in this period, prior to DMTS injection. DMTS intramuscular injection was

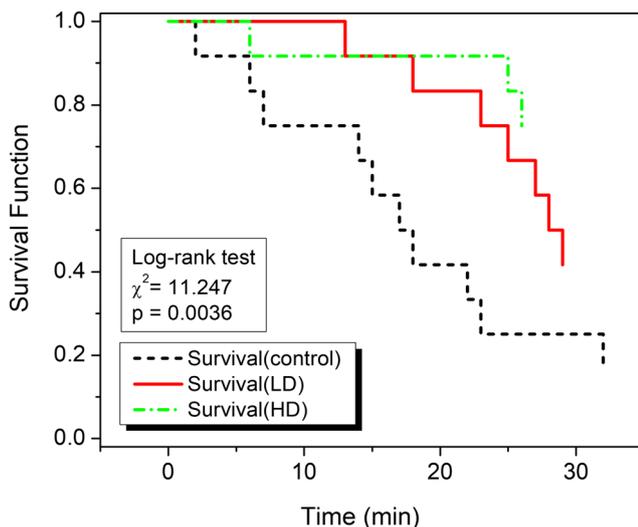


Fig. 2 Survival curve for control, low (20 mg), and high (150 mg) dose DMTS intramuscular injection groups following lethal CN exposure. Control animals were injected with saline. Survival analysis was accomplished using a Kaplan-Meier estimator with equality over three groups examined with a log rank test ($\chi^2 = 11.247$ and $p = 0.0036$)

designated as $t = 0$ and 30 min after DMTS injection signaled the discontinuation of CN infusion, respectively (Fig. 1).

Arterial partial pressure of oxygen in both low-dose (20 mg) and high-dose (150 mg) DMTS-treated animals remained high (>470 mmHg) during CN infusion under 100% O₂ and declined sharply after switching to room air and continued to decline during and following DMTS treatment. No distinct differences between two treatments were seen in the progression of arterial oxygen partial pressure. Both groups started with slightly alkaline pH, which continuously declined throughout the CN infusion and DMTS treatment. The blood pH for the high (150 mg) and low (20 mg) DMTS treatments decreased by 0.37 and 0.59 units, respectively, from the baseline to 90 min post-treatment. For the low-dose treatment, base excess (Beb) changes, reflecting production of lactic acid, declined continuously from 7.83 at the baseline to -22.3 at 90 min post-treatment, without recovery. Beb in the high-dose treatment group declined from baseline (5.36) to -17.3 at 45 min post-treatment and then recovered to -11.8 at 90 min post-treatment. Non-parametric Friedman ANOVA tests (OriginPro 8.1, OriginLab Corp., Northampton, MA 01060) showed that there was a difference in all physiological parameters presented in Table 2 at post-IM injection time points. A p value <0.05 was considered significant.

To examine whether DOS oxy- and deoxyhemoglobin and cytochrome c oxidase redox state (CcO_{rdx}) changes track the effect of a lethal CN poisoning and reversal, pairwise-related changes of two or more time points were assessed by a paired nonparametric tests (Wilcoxon signed rank test or Friedman ANOVA test, OriginPro8.1) with significant difference at $p < 0.05$. Figure 3 shows oxyhemoglobin, deoxyhemoglobin, and cytochrome c oxidase redox state (CcO_{rdx}) changes from animals that did not survive following DMTS intramuscular treatment. Non-surviving animals from groups 2 and 3 were combined together ($n = 10$). During CN infusion under 100% O₂, hemoglobin concentrations at C25 did not change significantly from baseline values. However, under these conditions, changes in cytochrome c oxidase redox state (ΔCcO_{rdx}) became significantly negative (Wilcoxon signed rank test, $p = 0.0078$). This shift toward the negative values indicates the reduction of CcO_{rdx} . CcO_{rdx} became reduced further following the FiO_2 decrease. Oxyhemoglobin concentrations also significantly decreased when FiO_2 was decreased from 100 to 21% O₂ (Wilcoxon signed rank test, $p = 0.0078$). After DMTS was injected intramuscularly, no further significant changes were observed in deoxyhemoglobin and CcO_{rdx} values among $t = 0, 5,$ and 15 min, while there was a statistical difference in oxyhemoglobin ($p = 0.0022$, Friedman ANOVA, OriginPro 8.1) among non-surviving animals.

Figure 4 shows changes in oxyhemoglobin, deoxyhemoglobin, and CcO_{rdx} of surviving animals with DMTS intramuscular injection in groups 2 and 3. The

Table 2 Blood pressure, heart rate, and arterial blood gas values of animals that survived with DMTS intramuscular injection after receiving the lethal dose of CN. PO₂, PCO₂, Beb, HR, and MAP represent arterial oxygen partial pressure, arterial carbon dioxide partial pressure, base excess, heart rate, and mean arterial pressure, respectively. Bslin and CN25 represent baseline and 25 min into cyanide infusion under 100% inspired oxygen fraction. Data are listed as mean \pm SEM (standard error of mean). Number of animals is nine and five for 150 and 20 mg DMTS IM injection, respectively

	Dose	bsln	CN25	0 min	5 min	15 min	30 min	45 min	60 min	90 min
PO ₂ (mmHg)	150 mg	435.0 \pm 33.78	514.0 \pm 37.56	105.8 \pm 4.93	90.9 \pm 5.60	81.1 \pm 3.58	79.3 \pm 3.62	67.7 \pm 3.66	63.5 \pm 3.87	61.4 \pm 4.48
	20 mg	478.0 \pm 33.78	470.7 \pm 28.72	87.7 \pm 2.69	82.9 \pm 2.93	79.8 \pm 2.94	78.7 \pm 1.19	69.8 \pm 0.63	69.8 \pm 1.91	60.8 \pm 1.73
PCO ₂ (mmHg)	150 mg	35.3 \pm 1.78	31.3 \pm 1.97	28.4 \pm 1.82	33.7 \pm 2.18	41.8 \pm 2.20	47.0 \pm 1.85	48.8 \pm 2.52	48.8 \pm 2.52	47.0 \pm 1.56
	20 mg	44.7 \pm 2.01	46.2 \pm 3.77	40.3 \pm 3.84	45.3 \pm 3.55	50.6 \pm 3.22	50.6 \pm 1.31	66.8 \pm 8.08	64.1 \pm 7.51	60.2 \pm 3.71
pH	150 mg	7.51 \pm 0.02	7.51 \pm 0.03	7.37 \pm 0.03	7.26 \pm 0.03	7.16 \pm 0.02	7.16 \pm 0.02	7.03 \pm 0.04	7.04 \pm 0.05	7.14 \pm 0.05
	20 mg	7.46 \pm 0.02	7.43 \pm 0.03	7.26 \pm 0.05	7.21 \pm 0.05	7.08 \pm 0.04	6.99 \pm 0.04	6.90 \pm 0.03	6.89 \pm 0.04	6.87 \pm 0.03
Beb (mEq/L)	150 mg	5.36 \pm 0.96	3.83 \pm 0.97	-6.99 \pm 1.23	-10.3 \pm 1.15	-13.9 \pm 0.93	-17.0 \pm 1.16	-17.3 \pm 1.74	-16.0 \pm 2.28	-11.8 \pm 2.90
	20 mg	7.83 \pm 1.02	5.78 \pm 0.98	-8.13 \pm 1.19	-9.10 \pm 1.92	-14.5 \pm 1.45	-18.8 \pm 1.50	-21.4 \pm 1.33	-22.5 \pm 1.24	-22.3 \pm 1.10
HR (bpm)	150 mg	144.9 \pm 4.06	173.6 \pm 6.08	170.2 \pm 4.37	155.3 \pm 11.15	156.3 \pm 8.37	162.3 \pm 7.95	171.6 \pm 7.92	176.2 \pm 6.91	180.8 \pm 6.99
	20 mg	156.2 \pm 14.38	169.7 \pm 10.83	160.8 \pm 6.13	165.6 \pm 4.82	160.0 \pm 3.48	141.8 \pm 5.95	155.2 \pm 5.55	147.6 \pm 13.08	171.2 \pm 9.72
MAP (mmHg)	150 mg	65.8 \pm 2.31	41.7 \pm 3.57	43.4 \pm 2.38	45.9 \pm 4.40	44.7 \pm 3.51	35.9 \pm 3.47	36.7 \pm 1.83	38.3 \pm 3.31	36.4 \pm 1.32
	20 mg	79.3 \pm 2.80	50.9 \pm 3.80	39.7 \pm 1.68	39.0 \pm 0.54	37.3 \pm 1.14	28.9 \pm 2.01	36.1 \pm 1.08	31.5 \pm .73	30.2 \pm 1.95

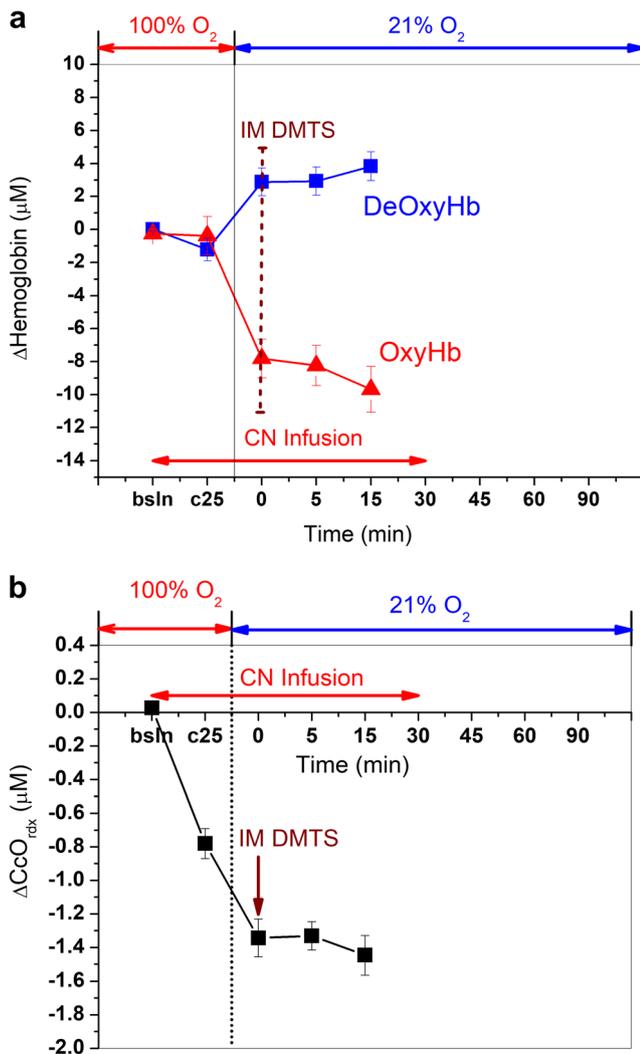


Fig. 3 **a** Hemoglobin and **b** cytochrome c oxidase redox state (CcO_{rdx}) changes (in micromolar units) of CN-exposed animals that did not survive following DMTS intramuscular injection. Data are listed as mean \pm SEM (standard error of mean). Non-surviving animals from groups 2 and 3 were combined together ($n = 10$). Two animals, which died before 15 min post-DMTS injection, were excluded from the non-parametric Friedman ANOVA analysis

progression of hemoglobins and CcO_{rdx} until $t = 0$ (when DMTS was injected intramuscularly) are very similar to those shown in Fig. 3 among non-survival animals. IM DMTS injection did not result in further significant changes in oxyhemoglobin in both groups 2 and 3 (Fig. 4a, c) according to Friedman ANOVA test ($p = 0.070$ and 0.288 , respectively). This result suggests that the hemoglobin concentration changes alone may not be sufficient to monitor for the optimal dose response of DMTS and its therapeutic effectiveness. Friedman ANOVA test indicates that there were differences in ΔCcO_{rdx} values with time after IM DMTS injection at $t = 0$ in both groups 2 and 3 ($p = 0.039$ and $p < 0.0005$, respectively, Fig. 4b, d). However, in group 3, median ΔCcO_{rdx} values rose progressively toward the baseline from -1.87 ($t = 0$) to $-$

0.124 ($t = 90$). On the contrary, median ΔCcO_{rdx} values in group 2 fluctuated around $t = 0$ value (-1.226) and did not recover back toward the baseline value.

Figure 5 shows examples of ΔCcO_{rdx} and respiratory exchange ratio during lethal CN infusion and intramuscular DMTS treatment in this rabbit model. RER values during CN infusion in the first 30 min were not recorded since RER values cannot be obtained under 100% O_2 . Figure 5a shows typical ΔCcO_{rdx} and RER from an animal in group 3. At the beginning of the study, prior to CN infusion, O_2 was switched to 21% to measure the baseline ΔCcO_{rdx} and RER values. FiO_2 levels have no significant effect on baseline ΔCcO_{rdx} values when 50 sample measurements each from 21 and 100% O_2 were compared ($p = 0.196$, paired sample t test, OriginPro 8.1). RER values are < 1 indicating that the animal is in a stable aerobic metabolic state. After 30 min of CN infusion under 100% O_2 , O_2 was then returned to 21%, RER was measured again, and intramuscular DMTS was administered. At the time of DMTS injection, RER was much greater than 1 (~ 1.6). RER values clearly show that the animal is experiencing anaerobic metabolism resulting from CN infusion. After DMTS injection, ΔCcO_{rdx} increases back toward the baseline values. Concomitantly with recovering ΔCcO_{rdx} , RER also continues to fall and reached the baseline level at $t = 90$ min. From the same animal in group 3 (150 mg DMTS), RER and ΔCcO_{rdx} values were closely correlated during high-dose DMTS injection ($R = -0.94$). However, in contrast, with low-dose (20 mg) DMTS injection, ΔCcO_{rdx} did not recover to baseline values and RER stayed above the normal value of 1 from an animal in group 2 (Fig. 5b). This animal was chosen as an example because neither RER nor ΔCcO_{rdx} recovered back to the baseline values despite the animal survived with low-dose DMTS. However, the progression of RER parallels ΔCcO_{rdx} in both low- and high-dose animals.

Discussion

In order to develop more effective antidotes for acute CN poisoning that can be applicable to mass casualty exposure treatment scenarios, we investigated the potential for augmenting rhodanese-dependent enzymatic trans-sulfuration treatment pathways for neutralization of CN. We utilized DMTS to provide sulfur to combine with CN, since it has minimal toxicity risks and is rapidly absorbed following intramuscular injection with the formulation used. Animals were administered a CN dose that leads to 83% mortality. Intramuscular administration of DMTS led to a dose-dependent increase in survival (42% with 20 mg and 75% with 150 mg DMTS, respectively). These findings in our moderate-size rabbit animal model further support the findings of recently published studies demonstrating effectiveness of DMTS in improving survival in small animal models [9, 10].

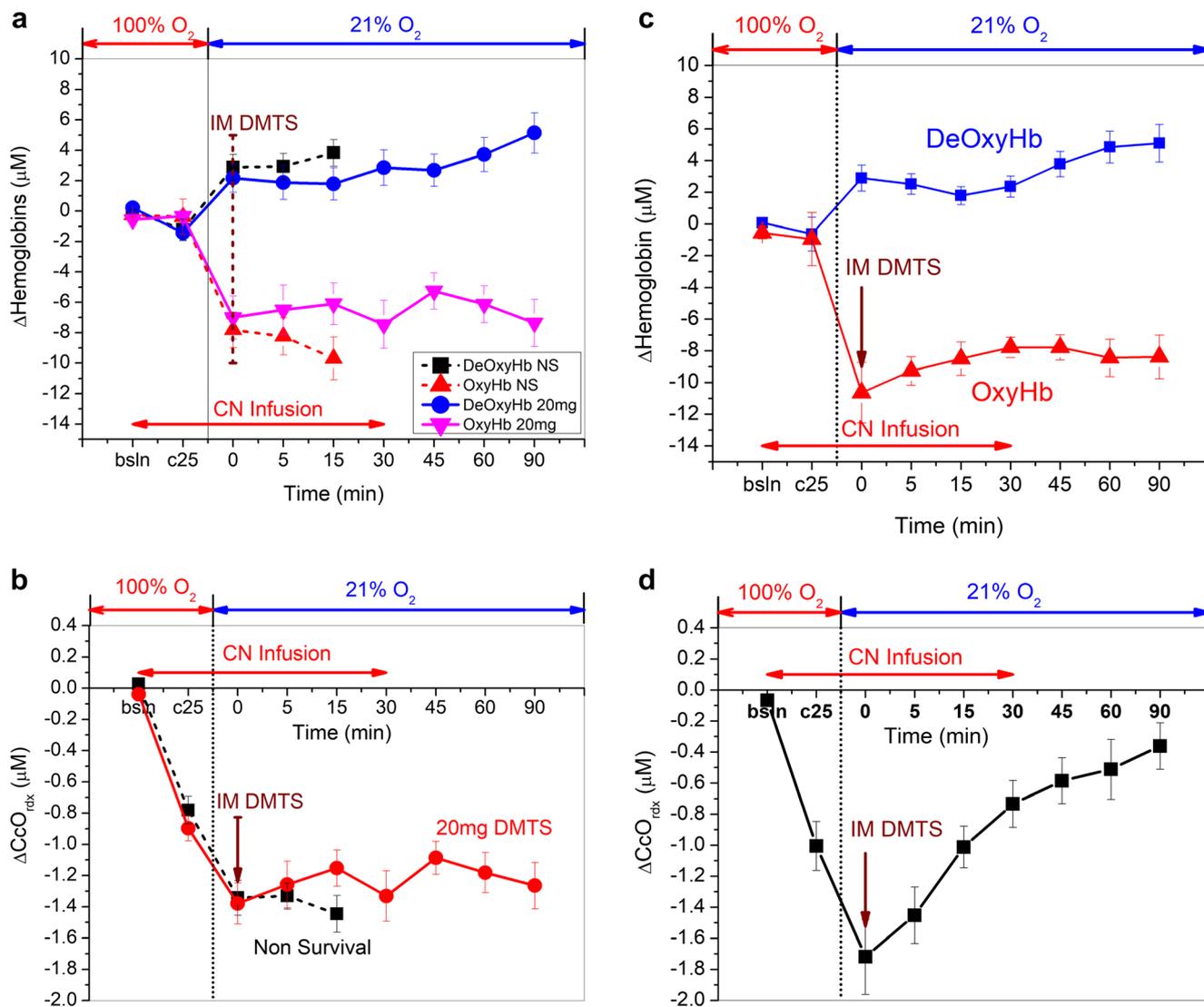


Fig. 4 Hemoglobins and cytochrome c oxidase redox state (CcO_{rdx}) changes of animals that survived lethal dose CN infusion with DMTS intramuscular injections (20 and 150 mg). Data are listed as mean \pm SEM (standard error of mean). Number of animals was five and nine for treatment with 20 and 150 mg DMTS IM injection, respectively. **a** Oxy- and deoxyhemoglobin concentration changes of five surviving

animals in group 2. **b** CcO_{rdx} changes of five surviving animals in group 2. Hemoglobins and CcO_{rdx} changes of non-survival animals in group 1 were superimposed for the comparison. **c** Oxy- and deoxyhemoglobin concentration changes of nine surviving animals in group 3. **d** CcO_{rdx} changes of nine surviving animals in group 3

In addition to improvements in survival, we investigated the rate and extent of reversal of the pathophysiologic effects of CN poisoning with DMTS treatment. We have previously reported the use of DOS measurements of tissue hemoglobin concentrations during CN poisoning and treatment phases in order to determine the efficacy of various CN antidote candidates [19, 20, 28]. While DOS and continuous wave near-infrared spectroscopy measurements have proven valuable to determine the effectiveness of CN antidotes, we have relied primarily on the kinetics of oxy- and deoxyhemoglobin concentration changes such as decay time constants after the antidote injection [19, 20, 29]. However, the changes in tissue hemoglobin concentrations

at the onset of CN poisoning also incorporate other confounding factors, including hemodynamic, cardiovascular changes, and severe metabolic acidosis which affect tissue oxy- and deoxyhemoglobin concentrations. Ultimately, the ability to monitor more direct measurements of tissue level cellular metabolic inhibition by CN appears more ideal to help assess the extent of injury, the response to treatment, and to guide therapeutic resuscitation. In this case, quantitative assessment of CcO redox state potentially reveals cellular metabolic status during CN poisoning and the effects of CN antidotes more directly since CcO redox status is indicative of adequacy of tissue perfusion, oxygenation, and cellular metabolic status at the mitochondrial level.

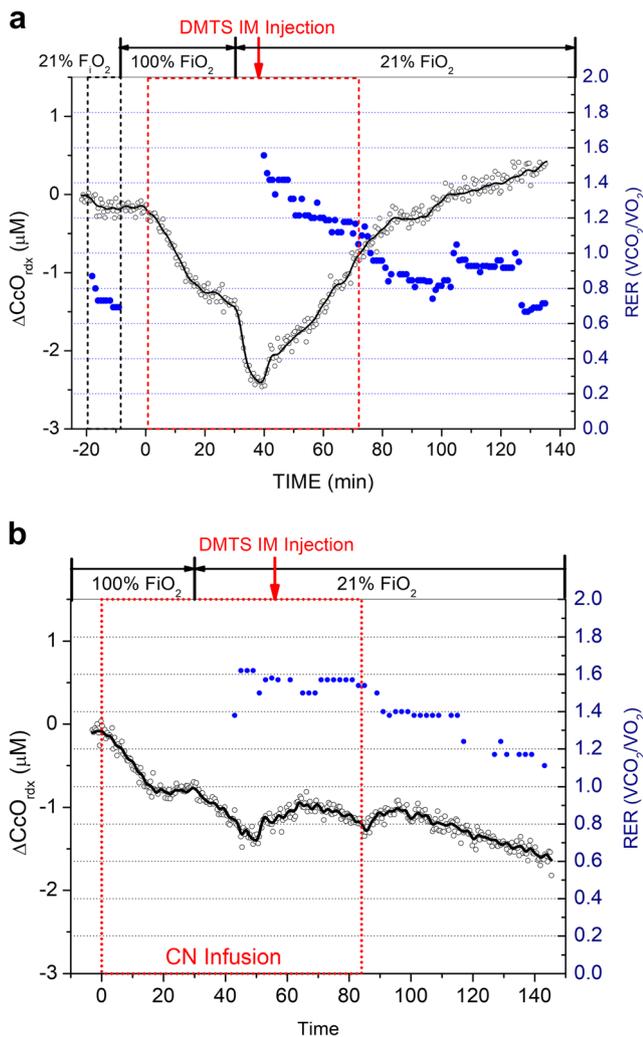


Fig. 5 Changes in cytochrome c oxidase (CcO_{rdx}) and respiratory exchange ratio during lethal CN infusion and intramuscular DMTS treatment model. RER was not measured at times when the animals were receiving 100% O_2 . **a** CcO_{rdx} and RER from an animal in group 3. Prior to CN infusion, O_2 was 21% to measure the baseline CcO_{rdx} and RER. RER value was below 1 (time -20 to -10 min). **b** Example of the CcO_{rdx} and RER from an animal in group 2

CN poisoning presents a relatively unique situation with regard to CcO and hemoglobin oxygenation, in which hemoglobin oxygenation and cytochrome chain redox state diverge. During CN poisoning, tissues are unable to extract oxygen from hemoglobin, while principal electron transport chain cytochromes are reduced [30, 31]. By using a combination of CN poisoning, hemorrhage, and alterations in inspired oxygen concentration in the rabbit models, we have previously demonstrated that DOS noninvasively tracks changes in CcO redox states in vivo, and this CcO redox state change measurement is independent from hemoglobin (or myoglobin) “optical crosstalk” [23].

In the current study, DOS measurement of cytochrome c oxidase redox state is used to determine the effectiveness of the potential CN antidote, DMTS, at two different doses. DOS

data from both low- (20 mg DMTS) and high-dose (150 mg DMTS) survival groups showed that the changes in oxy- and deoxyhemoglobin stabilized after DMTS injection but did not reverse back to baseline. In contrast, high-dose DMTS fully reversed the cytochrome c oxidase redox changes back to baseline, while the low dose did not. This result demonstrates that oxy- and deoxyhemoglobin change alone is not sufficient to fully determine the dose response of DMTS at the onset of lethal CN poisoning. This is most likely because the oxy- and deoxyhemoglobin concentrations are dependent on many physiologic variables including tissue oxygen consumption, oxygen delivery, cardiac output, and blood pressure/vascular resistance that are changing during CN exposure. However, at the tissue metabolic level, DOS was able to demonstrate significant changes in cytochrome c oxidase redox state with antidote dose dependency. Further support for the fidelity of DOS measurement of cytochrome c oxidase redox state reflecting the state of aerobic metabolism is its correlation with negative base excess production (from lactic acidosis) as well as respiratory exchange ratio. Inside cells, CN attaches itself to ubiquitous metalloenzymes, rendering them inactive. A major mechanism of cyanide toxicity is inhibition of cytochrome c oxidase, a key component of complex IV of the mitochondrial electron transport system. Inhibition of the electron transport process leads to cellular hypoxia, even in the presence of adequate oxygen stores. Cellular metabolism shifts from aerobic to anaerobic, with the consequent production of lactic acid. The amount of lactic acid in the blood is related to the degree to which the subject needed to supplement aerobic energy production with anaerobic energy production.

During CN poisoning, as cytochrome oxidase activity is inhibited, cells shift to anaerobic metabolism, creating lactic acid, leading to metabolic acidosis as reflected in the negative base excess shown in Table 2. In conjunction with this, increased anaerobic metabolism leads to RER values greater than 1.0 due to excess CO_2 production.

There are a number of limitations of this study. All animals were anesthetized, as required by our animal review committee; therefore, the effects of anesthetic cannot be determined. However, anesthetic agents would not likely have a substantial CN protective effect, given the extremely large doses of CN administered. This is a short-term survival study, and the animals were not awakened from anesthesia prior to sacrifice. Whether neurologic sequelae or long-term recovery issues will be present requires further investigation. Additional large animal efficacy and toxicity studies performed under GLP conditions will be required before use in humans can be considered. From a practical standpoint, stability of the DMTS formulation under more vigorous environmental conditions will also require investigation for clinical applicability.

Conclusion

In conclusion, we have demonstrated the therapeutic effect of intramuscular DMTS injection on lethal CN poisoning in this moderate-sized rabbit model, corroborating previous efficacy reports in rodents. Noninvasive *in vivo* measurement of cytochrome c oxidase redox state changes using DOS delineated the metabolic changes during CN poisoning and treatment, as a sensitive, non-invasive indicator of effectiveness of DMTS at the cellular metabolic level. DOS monitoring of CcO redox state is a promising approach to guide the development and optimization of antidote formulation during CN exposure.

Compliance with Ethical Standards

All procedures were reviewed and approved by the University of California, Irvine, Institutional Animal Care and Use Committee (IACUC).

Conflict of Interest Statement None.

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