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A Comparison of the Relative Propensities of Isoamyl Nitrite and Sodium Nitrite to Ameliorate Acute Cyanide Poisoning in Mice and a Novel Antidotal Effect Arising from Anesthetics

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Abstract

Isoamyl nitrite has previously been considered acceptable as an inhaled cyanide antidote, so the antidotal utility of this organic nitrite compared with sodium nitrite was investigated. To facilitate a quantitative comparison, doses of both sodium nitrite and isoamyl nitrite were given intraperitoneally in equimolar amounts to sub-lethally cyanide-challenged mice. Righting recovery from the knockdown state was clearly compromised in the isoamyl nitrite-treated animals, the effect being attributable to the toxicity of the isoamyl alcohol produced during hydrolysis of the isoamyl nitrite to release nitrite anion. Subsequently, inhaled aqueous sodium nitrite aerosol was demonstrated to ameliorate sub-lethal cyanide toxicity, when provided to mice after the toxic dose, by the more rapid recovery of righting ability compared to control animals given only the toxicant. Aerosolized sodium nitrite has thus been shown by these experiments to have promise as a better alternative to organic nitrites for development as an inhaled cyanide antidote. The inhaled sodium nitrite led to production of NO in the bloodstream as determined by the appearance of EPR signals attributable to nitrosylhemoglobin and methemoglobin. The aerosol delivery was performed in an unmeted inhalation chamber and, in this study, no attempt was made to optimize the procedure. It is argued that administration of an effective inhaled aqueous sodium nitrite dose in humans is possible, though just beyond the capability of current individual metered-dose inhaler designs, such as those used for asthma. Finally, working at slightly greater than LD₅₀ NaCN doses, it was fortuitously discovered that (i) anesthesia leads to significantly prolonged survival compared to unanesthetized animals and (ii) the antidotal activity of nitrite anion was completely abolished under anesthesia. Plausible explanations for these effects in mice and their practical consequences in relation to testing putative cyanide antidotes are discussed.

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Keywords

cyanide antidote; drug interactions; electron paramagnetic resonance; nitrite; nitrosohemoglobin

Introduction

The combined administration of sodium nitrite plus sodium thiosulfate solutions has been available as a treatment for cyanide poisoning for over seventy years and this combination has recently been approved by FDA (NDA 201444, “Nithiodote,” Hope Pharmaceuticals). However, isoamyl nitrite, the other component of the old Cyanide Antidote Kit (originally produced by Eli Lilly and later marketed by several other companies) has many undesirable side effects¹ and is not currently approved for use as an inhaled cyanide antidote. Since Nithiodote and Cyanokit (*i.e.* hydroxocobalamin) the other currently available antidote, both require intravenous injection, the need remains for some rapidly acting alternative to be used while waiting for qualified medical assistance. We have recently shown that intraperitoneally administered sodium nitrite alone can ameliorate sub-lethal cyanide toxicity in mice when given from ~1 hour before until 20 minutes after the toxic dose². In this paper, we compare the relative antidotal effectiveness of sodium nitrite with that of isoamyl nitrite in mice and investigate the possibility of administering sodium nitrite as an inhaled aqueous vapor.

We have previously demonstrated antagonism of cyanide inhibition of cytochrome c oxidase by NO^{3,4} and, subsequently, argued that the NO donor capability of nitrite is the crucial antidotal activity rather than its methemoglobin-forming action². Production of NO in the bloodstream can be monitored by electron paramagnetic resonance (EPR) spectroscopy due to the dose-dependent appearance of signals attributable to nitrosylhemoglobin (HbNO) and methemoglobin (metHb)^{2,5}. Herein, we use this method to quantitatively compare the NO-donor capabilities of sodium nitrite and isoamyl nitrite in the bloodstream of mice.

During the course of these studies, we have used a behavioral assessment of mice sub-lethally intoxicated with cyanide^{2,6} to evaluate antidotal effectiveness. Preliminary experiments with lethal doses of cyanide given to anesthetized animals revealed an

unexpected ameliorative effect of anesthesia alone, without any nitrite antidote having been given. Additionally surprising was the subsequent observation of a pronounced antagonism of nitrite action by anesthesia toward amelioration of cyanide intoxication. These findings have significant implications regarding protocols for testing antidotes in anesthetized cyanide-intoxicated animals.

Experimental Methods

Chemicals

All reagents were ACS grade, or better, used without further purification and unless stated to the contrary, were purchased from Fisher or Sigma-Aldrich. Avertin (99% 2,2,2-tribromoethanol) and isoamyl nitrite (97%) were obtained from Acros Organics; Nembutal (50 mg/mL sodium pentobarbital, USP) was from Ovation Pharmaceuticals; saline solution (0.9% NaCl, USP) was from Baxter Healthcare. Carbon dioxide (4.0 grade, 99.99%) and argon (4.8 grade, 99.998%) gases were obtained from Matheson Incorporated.

Animals, Exposure and Blood Collection

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Numbers 0808101 & 1008725). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss Webster (CFW) mice weighing 35-45 g were purchased from Charles River Laboratories, Wilmington, MA. All animals were 16-20 weeks old and were housed four per cage. The mice were allowed access to food and water *ad libitum*. Experiments commenced after the animals were allowed to adapt to their new environment for one week. In general, a group of at least 3 mice was tested for each experimental point. At the end of exposures and tests, any surviving mice were euthanized by CO₂ asphyxiation and subsequent cervical dislocation.

All sodium nitrite, isoamyl nitrite and sodium cyanide solutions were prepared by dilutions into sterilized saline and administered through ~0.1 mL intraperitoneal (*i.p.*) injections. Avertin (tribromoethanol), an injectable anesthetic, was chosen for its ability to produce good skeletal muscle relaxation with only a moderate degree of cardiopulmonary suppression. Avertin solutions were produced and administered following the University of California, San Diego IACUC recommendations, "Guidelines for Use of Avertin." Sterilized solutions of Avertin (306 mg/kg) were prepared by dilutions into saline and administered through *i.p.* injections. We also performed a limited number of experiments with Nembutal (sodium pentobarbital, 70 mg/kg) in a similar fashion. Typically, induction of anesthesia (confirmed by lack of toe-pinch response) took about 5 minutes and righting ability returned in approximately 40-60 minutes.

A series of experiments were performed in order to determine the effect of inhaled sodium nitrite on cyanide toxicity. Sodium nitrite dissolved in water (4.8 – 480 mg/mL) was placed in the solution holder of a mouse specific in-house manufactured inhalation chamber. Nebulized NaNO₂ was delivered to the inhalation chamber for several minutes using an Omron comp air XLT (model NE-C25) by Omron Healthcare prior to placing mice inside.

Mice were injected with cyanide (5.0 mg/kg) and placed within the holding chamber 2 minutes following the NaCN dose. Efficacy was tested through recovery of righting ability (observed *in situ* within the chamber) and pulse oximetric measurements (see below). To compare the efficacies of sodium nitrite and isoamyl nitrite as antidotes to cyanide toxicity, either sodium nitrite (12 mg/kg, 0.17 mmol/kg, *i.p.*) or isoamyl nitrite (20 mg/kg, 0.17 mmol/kg, *i.p.*) were administered two minutes following a NaCN (5.0 mg/kg, 0.1 mmol/kg, *i.p.*) dose. Efficacy was tested through recovery of righting ability and pulse oximetric measurements (see below).

For collection of blood samples, mice were first euthanized in an atmosphere of carbon dioxide, the thoracic cavities opened and blood drawn by cardiac puncture. The blood was expelled into the bottom of a quartz EPR tube containing 10% (w/v) EDTA through a Teflon “needle” and then frozen by immersion in liquid nitrogen. This entire process could comfortably be completed in 2 minutes. The cryogenically preserved sample was stored and subsequently transferred to the EPR spectrometer without ever having been thawed.

Righting Recovery Procedure

Lengths of time required for recovery of righting ability were determined based on some of the recommendations of Crankshaw *et al.*⁶ regarding their measurement of the righting reflex, but adopting a simpler procedure². Following the procedures outlined above for NaCN injections and antidote administration, mice were placed in a transparent but dark colored plastic tube in a supine position. This arrangement enables the mice to be easily rolled onto their backs again following an initial righting and a subsequent righting observed. The time duration from the cyanide injection until the mouse flipped from the supine to a prone position in the plastic tube was taken as the endpoint. To verify the righting ability endpoint, following the initial flip the mice were again placed in a supine position and expected to flip to a prone position within 1 minute of the initial flip. If unable to do so, the righting ability test continued until 2 consecutive flips within 1 of each other were demonstrated – thereby avoiding false positives due to muscle spasms. While this was the standard procedure, mice given isoamyl nitrite or isoamyl alcohol never righted twice in quick succession, exhibiting increasingly lengthy delays between successive rightings as described under Results. In order to determine the effect of inhaled sodium nitrite on cyanide toxicity, animals were first injected with NaCN and then placed in an in-house manufactured nebulizer chamber with concentrations of sodium nitrite ranging from 4.8 to 480 mg/mL. The time duration from the cyanide injection until the mouse flipped from the supine to a prone position in the nebulizer chamber was taken as the endpoint.

Measurement of Oxygen Saturation, Heart Rate and Respiratory Rate

A MouseOx® Pulse Oximeter (manufactured by STARR Life Sciences Corp.) was employed with a subset of mice to record physiologic data in response to administered solutions. The data were recorded and processed using the software supplied by the manufacturer. The procedure was non-invasive, requiring only the placement of a wrap-around collar clip-sensor (designed to fit) around the neck of the mouse (unanesthetized and unshaven). The mouse was then free to roam in his cage while the sensor on the collar constantly monitored oxygen saturation, heart rate and breathing rate. The collar was placed

on the mouse to record baseline data, removed during *i.p.* injections and then replaced. To record physiologic data on Avertin anesthetized mice, the same oximeter was used except a thigh clip was employed *in lieu* of the collar clip. Prior to the administration of Avertin, baseline data was recorded on the unanesthetized mouse using the collar clip. This clip was removed prior to the Avertin injection and once anesthetized, the thigh clip was placed on the mouse to monitor oxygen saturation, heart rate and breathing rate. The thigh clip was the more robust and sensitive experimental monitor of the two, but it was not possible to use this with unanesthetized animals. The collar or thigh clip was removed (experiment terminated) approximately 45 min to 1 hr after the initial injection.

Electron Paramagnetic Resonance (EPR) Spectroscopy

X-band (9 GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments ESR 910 cryostat for ultra-low-temperature measurements (typically 20 K). The microwave frequency was calibrated by a frequency counter and the magnetic field was calibrated with a gaussmeter. This instrument and the software (SpinCount) used to analyze the EPR spectra were graciously provided by Professor Mike Hendrich, Carnegie Mellon University. Quantification of EPR signals was performed by simulating the spectra using known (or determined) parameters for each sample in question. Simulations employed a least-squares fitting method to match the lineshape and signal intensity of a selected spectrum. Simulated spectra were expressed in terms of an absolute intensity scale, which could then be related to sample analyzed using KaleidaGraph a Cu^{II} (EDTA) spin standard of known concentration.

Data Analysis

Statistical data was analyzed using KaleidaGraph™ software by ANOVA with a Tukey *post-hoc* test. A *p*-value of < 0.05 was considered statistically significant and results are reported as mean values $\pm$ standard errors.

Results

Isoamyl Nitrite as an Antidote to Cyanide Toxicity

The relative effectiveness of molar-equivalent doses of sodium nitrite and isoamyl nitrite were quantitatively compared (Figure 1). We have previously shown ² that EPR spectra of control blood samples from animals given neither cyanide nor nitrite, exhibit only weak signals arising from transferrin at $g \sim 1600$ gauss (*e.g.* Figure 1A, upper trace). In the case of samples prepared with 12 mg/kg NaNO_2 (0.17 mmol/kg, given *i.p.*) both metHb ($g \sim 1100$ gauss) and HbNO ($g \sim 3400$ gauss) EPR signals can be observed (*e.g.* Figure 1A, lower trace) at the earliest time point (2 min). The signals are dose dependent (0-16 mg/kg NaNO_2) and, in general, the amount of HbNO in the blood (a maximum of 0.5 mM, or $\sim 6\%$ of the total hemoglobin) is roughly 2-3 times the amount of metHb (0.15 mM, or $\sim 2\%$ of the total hemoglobin). Followed over time, the signal intensities peaked at 5-10 minutes, were persistent up to 1 hour after administration of the nitrite dose and, while both diminished by $\sim 1/2$, they were still present in the case of cyanide-challenged animals ². The EPR spectrum of blood from mice given isoamyl nitrite (20 mg/kg, 0.17 mmol/kg, *i.p.*) and examined over a 5 to 60 minute time span clearly contains less (10-20%) of the HbNO

signal than that of mice given a molar equivalent of sodium nitrite (Figure 1A, middle trace), whereas the intensities of the metHb signals observed were similar for the two antidotes. These results demonstrate the two agents to be NO donors having different characteristics as previously suggested by others ⁷ and sodium nitrite to be the better NO donor.

When the relative efficacies of sodium nitrite and isoamyl nitrite were compared by righting recovery in sub-lethally cyanide-challenged mice, both sets of animals initially righted at the same time (Figure 1B, main panel, columns 1 and 2). However, when the animals were quickly turned on their backs again, the sodium nitrite-treated mice righted more quickly than the isoamyl nitrite-treated mice (columns 4 and 5) and the effect became more pronounced when the process was repeated yet again (columns 7 and 8). The resulting products of the hydrolysis of isoamyl nitrite are nitrite anion and isoamyl alcohol (3-methyl-1-butanol) ⁸. We also examined the righting recovery of cyanide-intoxicated animals simultaneously administered sodium nitrite plus isoamyl alcohol 2 minutes following the cyanide dose. These results were essentially the same as those obtained with isoamyl nitrite alone (Figure 1B, columns 3, 6 and 9). When the righting recovery of animals given cyanide and then either saline or isoamyl alcohol were compared (Figure 1B, inset) it was clear that the isoamyl alcohol significantly increased the duration of righting recovery after cyanide intoxication. Thus, we conclude that the antidotal activity of isoamyl nitrite is measurably compromised by the toxicity of the hydrolysis product isoamyl alcohol. The concern has been raised by others that sub-lethally cyanide-intoxicated individuals might be making poor escape decisions in emergency situations involving fire smoke and be perishing as a result ^{9,10}. It follows that any antidote able to ameliorate the effect of a potentially lethal cyanide dose, but itself be otherwise incapacitating, is certainly far from ideal and might be hazardous in some conceivable circumstances.

Effect of Inhaled Nitrite

The possibility of employing aqueous aerosolized sodium nitrite rather than isoamyl nitrite as an inhaled cyanide antidote was briefly investigated in mice (Figure 2). Conscious mice were first administered 5 mg/kg sodium cyanide (0.1 mmol/kg, *i.p.*) inducing a knockdown state within two minutes. Mice used to generate antidotal dose-response data then received aqueous nebulized NaNO₂ starting at 2 minutes following the cyanide injection (Figure 2A). The mice were placed in a supine position in the nebulizer chamber and the times at which they were subsequently able to right themselves noted (see Experimental Methods). All righting recovery times are quoted with respect to the initial cyanide injection (*i.e.* given at *t* = 0). As shown by the dose response (Figure 2A) sub-lethally cyanide-challenged mice recovered their righting ability sooner when placed in an atmosphere of nebulized sodium nitrite (concentration 48 mg/mL) compared to controls given the cyanide dose but no antidote. These findings clearly demonstrate aqueous aerosolized sodium nitrite to be a potential alternative to isoamyl nitrite as an inhaled cyanide antidote.

Interactions of inhaled sodium nitrite with hemoglobin in blood

We wished to compare the intensities of these biomarker signals obtained with inhaled and injected (*i.p.*) sodium nitrite. However, it proved impossible to get mice that were not immobilized into the inhalation chamber, since it was designed principally with anesthetized

animals in mind. Consequently, it was necessary to undertake the comparison with NaCN-incapacitated mice. When aqueous NaNO₂ aerosol was inhaled by mice (480 mg/mL solution in nebulizer) starting 2 min after sodium cyanide (i.p.) administration, the same biomarkers for NO/ NO₂⁻ exposure were observed in EPR samples of the mouse blood (Figure 2B, lower trace) in a time- and dose-dependent manner as those obtained with the injected NaNO₂ (12 mg/kg, i.p.) (Figure 2B, upper trace). Care must be exercised in comparing the the nitrite-dependent signal intensities in these two traces, because several hundred-fold more sodium nitrite was delivered to the animals continuously in the inhalation chamber (but only a fraction of this would have been inhaled) compared to those given the antidote essentially instantaneously by injection (i.p.). In the present (proof-of-concept) study, no attempt was made to further refine the aerosol delivery. It should be realized that the antidotal NaNO₂ (12 mg/kg, i.p.) dose was previously shown to be effective in mice between one hour before and up to 20 minutes after the toxic cyanide dose. If given at about the same time as the cyanide, only 3 mg/kg NaNO₂ was required². Therefore, when considering the requirements for a device suitable for self-administration (or assisted-administration) of the antidote in close proximity to the place and time of the toxic exposure, the lower figure is reasonable. The solubility of NaNO₂ in water is just over 800 mg/mL, so to quickly administer a dose of 3 mg/kg to a 70-80 kg human would require 0.26-0.30 mL of near-saturated solution. Nebulization of ~0.1 mL of solution by devices such as personal metered-dose inhalers is currently routine¹¹, but for use in life-or-death situations, double-shot and/or larger volume- delivery devices are conceivable. Consequently, further effort toward developing inhaled sodium nitrite as a practical cyanide antidote is warranted.

Anesthetized animals and cyanide toxicity

During a preliminary series of experiments that were to involve the use of increasingly lethal cyanide doses given to anesthetized mice, we observed an unexpected ameliorative effect of anesthetics on cyanide intoxication (Figure 3). Avertin (tribromoethanol, 306 mg/kg given 2 min before toxicant) significantly prolonged survival time (~5-fold increase) in lethally-challenged (6.4 mg/kg NaCN) animals (Figure 3A, columns 1 and 2). The magnitude of the effect was much smaller, but a still significant improvement in survival was found (~2-fold increase) when Nembutal (sodium pentobarbital, 70 mg/kg given 2 min before toxicant) was used as the anesthetic (columns 1 and 4). Also, surprisingly, the administration of sodium nitrite did not result in any further improvement in survival of the anesthetized mice (columns 3 and 5). In the absence of anesthesia, the *bona fide* antidote sodium nitrite (10 mg/kg NaNO₂ given 1 min before toxicant) was fully protective (5 out of 5 mice surviving 2 hr until experimental ethanasia). Consequently, it was very surprising that anesthesia appeared to completely abolish any measurable antidotal effect of sodium nitrite toward cyanide intoxication. A decision was made to perform additional experiments with Avertin only, since the effect was larger.

As we have previously reported², in sub-lethally challenged mice (5 mg/kg NaCN, *i.p.*) that have not been anesthetized, the righting recovery time is much shorter in animals given sodium nitrite (12 mg/kg NaNO₂, *i.p.*) 2 min after the cyanide dose (*e.g.* Figure 3B, columns 1 and 2). In the case of mice anesthetized with Avertin, administration of sodium nitrite increased the recovery time (columns 3 and 4) and, when NaCN was also given, the recovery

time increased still further with no measurable antidotal effect of NaNO_2 being observed (columns 5 and 6). Therefore, these data also clearly demonstrate that the anesthetic substantially eliminates the antidotal effectiveness of sodium nitrite in the anesthetized animals. Interestingly, physiological data obtained by oximetric telemetry (see Experimental Methods) indicated that while there was a measureable drop in O_2 saturation, heart and breath rate for Avertin injected mice, this did not appear to be much different for mice which were administered both Avertin and sodium nitrite (Figures 3C, D and E, columns 2 and 3 in each panel). As expected, sodium nitrite alone had no significant effect (column 4 in each panel). It should be noted that the changes observed in the response of both breathing rate and pulse rate to the anesthetic and/or the antidote lie within the range of values reported to be normal for the species: respectively, 110-330 breaths/min^{12,13} and 400-800 beats/min¹⁴ depending on mouse strain.

Discussion

It has begun to emerge that the antidotal activity of nitrites toward cyanide poisoning is not primarily due to generation of methemoglobin (metHb) and subsequent cyanide scavenging through formation of cyanomethemoglobin (metHbCN). The evidence for this includes: (i) nitrite anion is still fully effective as an antidote if metHb generation is suppressed¹⁵; (ii) significant metHb generation lags behind the antidotal action of isoamyl nitrite¹⁶; (iii) sodium nitrite is still clearly protective in hemoglobin/myoglobin-free cultured cells¹⁷; (iv) sodium nitrite is maximally protective in mice when the induced metHb level is an order of magnitude lower than the cyanide dose². Undoubtedly, if there is an increase in metHb in the presence of cyanide, there will also be a concomitant increase in the metHbCN present in the blood – but this is a collateral process rather than the main antidotal action. It has been observed that NO reverses the cyanide inhibition of cytochrome *c* oxidase^{3,4} and, consequently, nitrite anion acting as an NO donor is the probable mechanism through which nitrites are antidotal to cyanide. It follows that NO-dependent EPR signals in the blood, such as those arising from metHb and HbNO, can be regarded as biomarkers indicating the probable effectiveness of nitrites and other NO donors as cyanide antidotes.

Hemoglobin considerations

There are (at least) four reactions of hemoglobin that need to be considered in relation to the present data, these and their rate constants¹⁸⁻²¹ are:

where the EPR detectable products have been underlined. The NO product of reaction 2 may then react with Hb (reaction 4) and so, overall, the reaction between Hb and NO_2^- can be expected to generate 1 metHb and 1 HbNO per reacting anion. There is a growing literature suggesting reaction 2 to be a physiologically significant process in relation to vasodilation of hypoxic tissues²²⁻²⁴. However, this idea is not necessarily helpful in analyzing the present results as we are dealing with either normoxia (in controls given only antidote) or hyperoxia (in cyanide-challenged subjects). In physiologically normal individuals, the HbO_2 content of arterial blood is ~95%, while the HbO_2 content of venous blood is ~70%²⁵⁻²⁹. *In vivo*, if sodium nitrite in the bloodstream were to enter erythrocytes and the anion react directly with the available hemoglobin species, because $[\text{HbO}_2] > [\text{Hb}]$ and reaction 1 has a rate constant that is 5 orders of magnitude faster than reaction 2, then reaction 1 must dominate overwhelmingly and metHb would be essentially the *only* EPR-detectable product. However, this is obviously not the case regarding the present data; in fact, ~3 times more HbNO is formed than metHb (Figures 1A and 2B) which is, seemingly, not possible unless NO is the predominant species reacting with hemoglobin in the interior of the erythrocyte. That is, in these experiments, the substantial majority of the nitrite anion administered must have become reduced to NO either before, or during entry into the erythrocytes. Interestingly, in a study with rabbit erythrocytes exposed to external NaNO_2 under deoxygenated conditions in which HbNO and metHb were determined, a product ratio of ~4:1 (HbNO:metHb) was found³⁰ instead of the 1:1 predicted from reaction 2 followed sequentially by reaction 4. That is, this earlier (hypoxic) experiment is in full agreement with the present findings that NO formed before or during entry into the erythrocyte appears to be the predominant species reacting with hemoglobin rather than nitrite anion.

While there may be some argument regarding the mechanism(s), there appears to be a consensual viewpoint that erythrocytes take up nitrite anion³¹ – in contrast to the present findings. The pre-existing idea has emerged based on measurements with isolated erythrocytes in which the *disappearance of external nitrite* has been monitored and correlated with metHb formation, sometimes without accounting for HbNO formation. There is some species dependence, but the rate at which metHb is reported to accumulate in isolated erythrocytes exposed to millimolar external added nitrite concentrations varies from $t_{1/2} = 11 \text{ min}$ ³² to ~40 minutes³⁰. The metHb formation we monitor is kinetically distinct with $t_{1/2} < 2 \text{ min}$ at 1.3 mM NaNO_2 in the mouse blood and the product ratio of ~3:1 (HbNO:HbO₂) persists if euthanasia is delayed for more than 10 min following administration of sodium nitrite². These kinetic observations strongly suggest that the hemoglobin reactions with NO must substantially be taking place *in vivo*, although the large excess of HbNO over metHb we observe (Figures 1A and 2B) may be partly explained by increasing hypoxia and consequent availability of deoxyhemoglobin during the euthanasia.

It is additionally intriguing that the ratio [HbNO]:[metHb] obtained in our experiments is nearly the same irrespective of whether the NaNO_2 was administered *i.p.* or through inhalation (Figure 2B). Inhaled nitrite is absorbed into the microvasculature of the lung

where the HbO₂ level will be around 95%, whereas nitrite given *i.p.* will be absorbed into the microvasculature of the peritoneum and quickly be transported to a venous environment where the HbO₂ level will be closer to 70%. It follows that the bulk of the nitrite must become distributed throughout the vasculature more quickly than any nitrite-dependent hemoglobin reactions take place. Thus, the observed [HbNO]:[metHb] product ratio probably reflects the extent of reaction 4 to that of reaction 3 throughout the vasculature as a whole, or in localized regions other than the lung and peritoneum. The idea that transport around the vasculature must be fast compared to reactions with hemoglobin (a sink) is important for the hypothesis that the antidotal action of nitrite toward cyanide intoxication hinges upon its ability to supply NO to CNS mitochondria ²⁻⁴.

Isoamyl nitrite versus sodium nitrite

Isoamyl nitrite was first synthesized over 100 years ago and subsequent studies noted its potential usefulness as a therapy for relief of angina pectoris ³³. However, to achieve similarly satisfactory results, the drug was needed at much higher amounts than, for example, nitroglycerin ³⁴. There can be no reasonable doubt that this lesser effect is at least partly due to the lower amount of nitrite that is released by isoamyl nitrite and, which we now know, results in therapeutic NO production. Approximately 60% of isoamyl nitrite is converted to nitrite and isoamyl alcohol in circulating plasma ⁸. We would, therefore, expect to see roughly half the amount of metHb + HbNO produced when isoamyl nitrite is administered compared to when sodium nitrite is used. This expectation is, indeed, reflected by the data. We estimate 3-4% of total hemoglobin as metHb + HbNO (2% + 1-2%, respectively) with isoamyl nitrite and 7-8% of total hemoglobin as metHb + HbNO (2% + 5-6%, respectively) with a molar equivalent dose of sodium nitrite. The ratio [HbNO]:[metHb] obtained with isoamyl nitrite is < 1:1 compared with ~3:1 for sodium nitrite (by quantitation of Figure 1A signals) – an intriguing observation that may indicate significant hydrolysis of isoamyl nitrite occurs within the erythrocyte, resulting in release of nitrite anion and stimulation of reaction 1. This matter is currently under investigation and we do not consider it further here. More pertinently with regard to the purposes of the present study, the lower level of NO produced by isoamyl nitrite suggests that it should be a poorer cyanide antidote than sodium nitrite. Consequently, the finding that sub-lethally cyanide intoxicated mice given either isoamyl nitrite or sodium nitrite as the antidote undergo initial (“first”) righting recovery at the same time (Figure 1B, columns 1 and 2) was something of a surprise. The primary disadvantageous toxic effect of isoamyl nitrite, evident in the repeated righting-recovery test (Figure 1B, columns 2, 5 and 8), is clearly due to its other hydrolysis product, isoamyl alcohol (Figure 1B, columns 3, 6 and 9, plus inset), rather than changed NO₂⁻ or NO production. It is well known that alcohols are hypotensive and it was documented over 100 years ago that, when given at the same concentration, isoamyl alcohol lowered blood pressure ~4 times as much as ethyl alcohol ³⁵. This is obviously a potential problem as NO is also vasodilatory and the combined action of these two compounds on murine blood pressure may have been responsible for the increasingly lengthy sequential righting recoveries observed.

In addition to the toxicity issue associated with the hydrolysis product isoamyl alcohol that we demonstrate here (Figure 1B), there are other documented problems associated with the

use of isoamyl nitrite as a therapeutic. Recently, Lavon *et al.*¹ reviewed the available literature concerning the use of isoamyl nitrite as an antidote to cyanide poisoning and concluded that some of the potentially serious side effects were hypotension, syncope, excessive methemoglobinemia, and hemolysis in glucose-6-phosphate dehydrogenase-deficient patients. Some of the protocols that used isoamyl nitrite as an antidote included repeated dosings of the drug which induced lactic acidosis and very high levels of methemoglobinemia indeed³⁶. In summary, we conclude that isoamyl nitrite is somewhat less effective than sodium nitrite as a cyanide antidote, but more seriously, isoamyl nitrite has some undesirable side effects that do not seem to be associated with the use of sodium nitrite, at least not nearly to the same extent.

Anesthesia: a complication

While Avertin (1,1,1-tribromoethane) is an injectable veterinary anesthetic, it is chemically related to volatile (inhalation) anesthetics such as halothane, isoflurane and sevoflurane. The properties of these agents include cardiac depression, cardiac sensitization to norepinephrine, and bronchial relaxation. In the past, their lack of any pronounced tendency to induce airway irritation made them useful for adult and pediatric anesthesia. The mechanisms by which such anesthetics work remain unclear, but interaction with receptors on CNS membranes is one hypothesis^{37,38}. Also, however, interactions with the mitochondrial electron transport chain appear to play a role in anesthetic sensitivity^{39,40}. In particular, isoflurane has been shown to inhibit complex I and, possibly, also complexes II and III⁴⁰. Nembutal, on the other hand, is a barbiturate that depresses the central nervous system and does not have the same chemical properties as Avertin. Nembutal is thought to exert its activity on the benzodiazepine-GABA_A-chloride receptor complex by enhancing chloride flux and causing neural inhibitory effects^{41,42}. Given the different chemical properties of Avertin and Nembutal, the findings that they are both antidotal to cyanide and both antagonize the antidotal action of nitrite are very surprising. Possibly some general physiological explanation exists for this observation, such as lowered heart rate and blood pressure under anesthesia leading to slower systemic distribution of cyanide – but this argument does not account for how Avertin can be so much more protective than Nembutal (Figure 3A). Like Nembutal, Avertin may alter the action of membrane ion channels, but perhaps this has as much to do with Avertin dissolving in the membrane and changing its fluidity as binding to specific receptors. Consequently, the channel perturbation and associated change of membrane polarity may act to prevent the uptake of both cyanide and nitrite under either type of anesthesia, but it is reasonable to expect the magnitudes of the effect in the two cases to be dissimilar.

Of course, since Avertin is structurally related to ethanol, the question naturally arises as to whether there is any evidence that alcoholic beverages might be antidotal toward cyanide. Regarding this question, there is at least one case history on record of a drunk individual surviving a potentially fatal cyanide dose⁴³. Then there is the historical anecdote concerning the death of Rasputin, whose murderers seem to have made the mistake of getting him drunk before attempting (and failing) to poison him with cyanide, then almost farcically, resorting to shooting and drowning him⁴⁴. Ethanol is thought to dissolve in and generally increase membrane fluidity and thus, would also be expected to perhaps alter the function of ion

channels⁴⁵. Curiously, while most authors would not select a single ion channel as being a target of ethanol, at least some have some evidence for the inhibition of GABA receptor gated structures^{46,47} more in keeping with the proposed action of Nembutal rather than Avertin.

The reported inhibitory effect of isoflurane on mitochondrial electron-transport chain (ETC) complex I (NADH dehydrogenase) and, possibly, also complexes II (succinate dehydrogenase) and III (cytochrome *c* reductase)⁴⁰ provides a clue as to a plausible mechanism for the interaction between anesthesia, cyanide and nitrite (Figure 3B). NO displaces cyanide anion from the active site of ETC complex IV (cytochrome *c* oxidase)^{3,4} and nitrite-derived NO is the active antidotal agent in the amelioration of cyanide toxicity by sodium nitrite². Note that the NO left bound to complex IV after the cyanide has been displaced must then itself be removed for the enzyme to continue functioning. This can be achieved by oxygen-dependent conversion of the bound NO to nitrite anion by a reaction ($\text{NO} + \text{O}_2 + 2\text{H}^+ + 3\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$) sustaining ETC activity^{3,4}. We thus envisage the functioning mitochondrial ETC as a sink for NO and a source of nitrite, the reduction of nitrite to NO must occur largely outside the mitochondrion – we know much of this takes place in the bloodstream². However, NO is also vasodilatory and too much NO will result in hypotension. Therefore, if nitrite is administered and begins to be converted to NO while the ETC is inhibited by anesthetic (or anything else), the normal process by which the mitochondrion converts NO to nitrite will be slowed down and NO must accumulate, leading to hypotension. This is a plausible explanation for the longer righting recovery time for mice given sodium nitrite in addition to Avertin, compared to those given Avertin only (Figure 3B, columns 3 and 4). When cyanide is also present, the situation is even worse (Figure 3B, column 5) because the ETC is now further inhibited at complex IV itself. There may also be an additional effect where NO is normally pooled (preferentially partitioned) into membranes, but an inhalation anesthetic partitioning into the membrane displaces some of the NO releasing higher than normal levels of NO into the extramembrane environment.

Verifying these ideas concerning the nature of the interaction between anesthesia, cyanide and nitrite will take a great deal more work and is beyond the scope of the present study. There are two more practical toxicological consequences of these observations (Figures 3A and 3B) that need to be communicated. First, one cannot assess the effectiveness of nitrites as cyanide antidotes in anesthetized animals – sodium nitrite is clearly very effective in unanesthetized mice² but simply does not work in the anesthetized animals (Figure 3B). In the past, experimental HCN vapor exposures of mice and rats have been conducted with/without anesthesia and, sometimes, an effect of anesthesia observed⁴⁸. However, it is becoming increasingly difficult to obtain institutional approval to work with unanesthetized subjects and anesthesia has started to become the norm in inhalation toxicology, particularly where larger species are concerned⁴⁹. It is now clear that such methodologies test the effect of whatever putative antidote is being studied plus that of the particular anesthetic being used (in fact, a combination therapy). Thus, one should minimally anticipate equivocal dose-response results concerning the putative antidote being tested, but more seriously, a completely false impression of the test compound's usefulness might be obtained.

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Abbreviations

EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance
Hb	hemoglobin
ETC	electron transport chain
CNS	central nervous system

Figure 1. Isoamyl nitrite as a cyanide antidote

A: EPR spectra (x-band, 20 K) of mouse blood obtained comparing NO formation secondary to NaNO₂ or isoamyl nitrite. Swiss-Webster (males, 16-20 weeks of age) mice were sacrificed (CO₂) 10 min after *i.p.* doses (0.17 mmol/kg NaNO₂ or isoamyl nitrite) then blood drawn by cardiac puncture and cryopreserved in EPR tubes. **B:** Mice (Swiss-Webster males, 16-20 weeks of age) given NaCN in saline (5 mg/kg, *i.p.*). Following the administration of toxin (t = 0) mice were then given (t = 2 min) aqueous NaNO₂ (0.17 mmol/kg, see columns 1,4,7), isoamyl nitrite in propylene glycol (0.17 mmol/kg, see columns 2,5,8), 3-methyl-1-butanol in propylene glycol (0.17 mmol/kg, see insert), or aqueous NaNO₂ + 3-methyl-1-butanol (0.17 mmol/kg each, see columns 3,6,9) and, subsequently, placed on their backs. Each time the mice righted they were turned on their backs again immediately and the times at which they righted (3 times per mouse) were recorded. The data are plotted as durations of righting recovery timed from injections of toxin (not durations between successive rightings). Values represent means ± standard error. An average of 4 animals per point was used. Statistically significant differences from the controls (*) represent P < 0.05 unless otherwise indicated.

Figure 2. Dose-response showing the efficacy of inhaled NaNO₂ by monitoring the righting recovery of mice intoxicated with NaCN and EPR spectra (x-band, 20 K) of mouse blood demonstrating the rapid formation of NO following administration of sodium nitrite

A: Mice (Swiss-Webster males, 16-20 weeks of age) given NaCN in saline (5 mg/kg *i.p.*).

Following the administration of toxicant ($t = 0$) animals were placed on their backs in a nebulizer chamber and antidote delivery was started (NaNO₂ at $t = 2$ min). The times at which the mice righted were subsequently recorded. Values represent means $\pm$ standard error. An average of 4 animals per point were used. Statistically significant differences from the controls (*) represent $P < 0.05$ unless otherwise indicated. **B:** Mice were initially given NaCN in saline ($t = 0$, 5 mg/kg *i.p.*) sacrificed (CO₂) 5 min after *i.p.* dose (12 mg/kg NaNO₂ given at $t = 2$ min, upper trace), or 5 min after starting inhalation (480 mg/mL nebulized NaNO₂ solution given at $t = 2$ min, lower trace), then blood drawn by cardiac puncture and cryopreserved in EPR tubes. EPR spectra, at 20 K, demonstrate the presence of HbNO (~3400 gauss) and metHb (~1100 gauss).

Figure 3. Anesthesia antagonizes cyanide toxicity and interferes with the ameliorative effect of sodium nitrite

A: Mice (Swiss-Webster males, 16-20 weeks of age) were given NaCN in saline (6.4 mg/kg, *i.p.*) and times until death recorded (1st column). The duration of survival (oximetric determination of heartbeat cessation) was measured from the time of the cyanide injection ($t = 0$). A second group was given Avertin (306 mg/kg, *i.p.*) 2 min before the same NaCN dose (2nd column). A third group was given NaNO₂ (12 mg/kg, *i.p.*) 2 min after the same Avertin and NaCN doses (3rd column). A fourth group was given Nembutal (70 mg/kg) 2 minutes before the 6.4 mg/kg NaCN dose (4th column). A fifth group was given NaNO₂ (12 mg/kg, *i.p.*) 2 min after the same Nembutal and NaCN doses (5th column). [**Note:** Mice given 10 mg/kg NaNO₂ (only) 1 min before the same dose of NaCN all survived (5 out of 5) until experimental euthanasia 2 hr after administration of the toxicant]. **B:** In nonlethal studies, mice (Swiss-Webster males, 16-20 weeks of age) were given NaCN in saline (5.0 mg/kg, *i.p.*) and the times until righting recovery were determined (1st column). A second group was given NaNO₂ (12 mg/kg, *i.p.*) 2 min after the same NaCN dose (2nd column). A third group was given Avertin (306 mg/kg, *i.p.*) (3rd column). A fourth group was given NaNO₂ (12

mg/kg i.p.) 2 min after the same Avertin dose (4th column). A fifth group was given NaCN in saline (5.0 mg/kg, *i.p.*) a further two minutes following the same Avertin and NaNO₂ doses (5th column). **C:** Real time percent O₂ saturation of hemoglobin in the arterial blood reported after every heartbeat; **D:** Breath Rate (breaths per minute BRPM) reported after every heartbeat; **E:** Real time cardiac pulse rate (beats per minute, BPM) reported every 1.7 seconds. Physiological assessments were measured non-invasively using a pulse oximeter with Swiss-Webster mice (males, 16-20 weeks of age) following administration of one of the following solutions (*i.p.*): (i) 306 mg/kg Avertin; (ii) 306 mg/kg Avertin + 12 mg/kg NaNO₂ (2 min after Avertin); (iii) 12 mg/kg NaNO₂; (iv) saline. Data were collected for 5 minutes prior to injections and 45-60 minutes post injections with the un-anesthetized animals allowed to roam freely in individual cages (approximately 20 × 10 cm). Anesthetized mice were kept on a warming pad throughout the duration of the experiment. Data collected from 3 to 5 animals for each measurement (O₂ saturation, heart and breath rates) was averaged as follows. The baseline data was determined by averaging the final 30 seconds of measurements collected before the initial injection. Two minutes following the injection of Avertin, NaNO₂, or saline, measurements were averaged for 2 minutes. Values represent means ± standard error. An average of 4 animals per point was used. Statistically significant differences from the controls (*) represent P < 0.05 unless otherwise indicated.