An inhalational swine model for the characterization of physiological effects and toxicological profile associated with cyanide poisoning

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ABSTRACT
Cyanides are highly toxic compounds that have been used as weapons of terrorism throughout history. Cyanide (CN) is acutely toxic by all routes of administration; however, inhalation is the main exposure route. To adequately test effective countermeasures against inhalational CN threats, robust and well-characterized animal models are needed. This paper describes the initial development of a hydrogen cyanide (HCN) exposure swine model for documenting the physiological effects and toxicological profile during and after HCN inhalation exposure. Animals were implanted with telemetry transmitters for heart rate (HR), blood pressure, and electrocardiogram monitoring, and vascular access ports for serial blood collections. Nine female swine were exposed to HCN concentrations of 500 ± 6 ppm while breathing parameters were monitored real-time. Inhaled HCN doses ranged from 2.02 to 2.83 mg/kg. Clinical signs included vocalization, agitation, salivation, respiratory distress and apnea. After HCN exposure initiation, systemic arterial pressure fell dramatically with a concomitant increase in HR. Blood samples were collected to determine CN blood levels using LC-MS/MS and blood gas analysis. In summary, the developed HCN inhalation swine model permitted documentation of the physiological effects associated with CN poisoning. This model could be used to evaluate potential CN medical countermeasures in the event of a public health emergency stemming from inhalational CN threats.

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Introduction
Toxic chemicals have long been used as chemical weapons in war, including World War 1 and 2 (Chauhan et al., 2008; Fitzgerald, 2008; Riedel, 2004; Tucker, 2007; Vilches et al., 2016), and most recently, in the Middle East (Dolgin, 2013; Dons, 2013). The devastating consequences of chemical weapons have promoted the search for more effective medical countermeasures that can be used by the military as well as civilian populations.

There are three different primary routes by which a substance can enter the body: inhalation, dermal contact and ingestion (Figure 1). Inhalation is the most probable route of chemical exposure for chemicals in form of gases, vapors or particulates (Brenneman et al., 2000; Das & Blanc, 1993; Emad & Rezaian, 1997). Once these substances are retained within the respiratory system, they diffuse to the blood causing severe systemic health effects in the organs that are sensitive to these toxicants.

Although cyanide (CN) toxicity is generally considered a rare form of poisoning, it is still a threat for people who inhale smoke from industrial fires (Symington et al., 1978), or from consumption of CN-containing foods (Mouaffak et al., 2013), and especially for people who have been under a terrorist attack when used as a weapon (Eckstein, 2008; Keim, 2006). Cyanide is a fast-acting and potentially deadly compound found abundantly in nature (e.g. apricot pits and cassava) as well as in man-made materials such as plastics, petrochemicals, steel and aluminum. Its existence has created complex problems for society ranging from its role in normal metabolism to pollution and its rapid lethal action (Baumeister et al., 1975).

Cyanide is a natural inhibitor of complex IV (Cooper & Brown, 2008; Leavesley et al., 2008), also known as cytochrome C oxidase, a transmembrane protein found on the inner mitochondrial membrane and an integral part of the Electron Transport Chain (ETC). The ETC is essential for aerobic respiration and the production of the mammalian cellular based energy currency, Adenosine Triphosphate (ATP) (Hodnick et al., 1994). Cytochrome C oxidase receives an electron from each of four cytochrome C molecules, and transfers them to one oxygen molecule, thus reducing oxygen to convert molecular oxygen to two molecules of water. During this process, cytochrome C oxidase binds four protons from the inner aqueous phase to make water, and translocates four protons across the membrane, helping to establish a transmembrane difference of proton electrochemical potential that the ATP synthase then uses to synthesize ATP (Beasley & Glass, 1998). Without the production of ATP, cellular bioenergetics fail, the cell cannot use oxygen and dies (Brunelle & Chandel, 2002; Nelson, 2000).
Cells with the greatest oxygen and energy demand are the first cell types that are affected, particularly cells of the brain and heart. This makes CN extremely toxic and detrimental to the neurological and cardiovascular systems. Within minutes, the person exposed to CN will begin to have difficulties in breathing, and display signs of dizziness, weakness, and restlessness (Baud, 2007). Other symptoms include headache, nausea and vomiting, and rapid heart rate (HR) (Baud, 2007). When exposed to larger amounts, the symptoms escalate to convulsions, severe lowering of blood pressure and HR, loss of consciousness, lung injury, and ultimately respiratory failure which leads to death (Beasley & Glass, 1998; Cummings, 2004; Gracia & Shepherd, 2004).

Cyanide poisoning has been previously studied using rat and mouse models (Carpenter et al., 1974; Esposito & Alarie, 1988; Mattijak-Schaper & Alarie, 1982; Moore et al., 1991; Norris et al., 1986); however, these are poor predictors of human responses. For example, morphometric data for the human and rat tracheobronchial tree revealed significant differences in airway branching patterns (Hofmann et al., 1989). Also, Travillian et al., developed a structural difference model to correlate the anatomy of Homo sapiens and mice, and found significant differences in the anatomy of the lung between species (Travillian et al., 2005), leading to variability of the airway system in comparison to humans; therefore, effective testing of medical countermeasures would be best performed using a relevant animal model and route of exposure due to the similarities in size, anatomy, and physiology between pigs and humans (Judge et al., 2014).

The HCN median lethal inhaled dose in the swine model was determined to be 2.21 mg/kg with 95% confidence bounds of 1.94 and 2.51 mg/kg (Staugler et al., 2018). The objective of this study was to document the physiological response near this median lethal inhaled dose (Figure 1). The results found here support the use of a relevant large animal exposure model that could be used to evaluate potential CN exposure.

**Materials and methods**

**Materials**

Hydrogen cyanide cylinders with balance nitrogen, were purchased from Praxair Inc. (Des Moines, IA). The HCN was manufactured by SPECGAS, Inc. (Warminster, PA) with certified concentrations of 0.991% and 0.992% (9910 and 9920 ppm, respectively).

**Animals**

Female Yorkshire swine (*Sus scrofa domesticus*) were obtained from Oak Hill Genetics (Ewing, IL). The animals were maintained under an animal care and use program accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and procedures approved by Battelle’s institutional animal care and use committee. Animals were group housed with space meeting the requirements of the guide for the care and use of laboratory animals (Institute for Laboratory Animal Research, 2011). The light/dark cycle was 12 hour (hr) each day using fluorescent lighting with no twilight. Air temperature in animal rooms was maintained within 16–22 °C range, with relative humidity maintained between 30 and 70%. The animals were fed Swine Chow pellets at 2–3% of body weight daily (PMI Feeds, Inc., St. Louis, MO). Water was provided *ad libitum*. Animals weighed ∼20 ± 5 kg when placed on study. Animals were 8–11 weeks old when placed on study; however, age was not a requirement for placement on the study as it was not designed to evaluate the CN toxicity for different swine age groups.

**Telemetry and vascular access ports surgery**

Telemetry transmitters (Model’s L21, L11 & M11, Data Science International (DSI), St. Paul, MN) and vascular access ports (VAPs) were surgically implanted at Battelle following a
A 7-day quarantine period. Six animals had both telemetry transmitters and VAPs, while 6 other animals had only VAPs. Telemetry transmitters were implanted into the animal cavity to monitor electrocardiography (ECG), temperature and blood pressures. VAPs were placed into the carotid artery. Prior to surgery, animals were fasted overnight, then anesthetized with ketamine [20 mg/kg intramuscular (IM)] and isoflurane (3–5% induction, 0.5–3% maintenance). Buprenorphine SR [0.2 mg/kg, subcutaneous (SQ)] was used prior to surgery and followed by tablets for two days postsurgery for pain control. Enrofloxacin (5 mg/kg) was administered IM or orally once daily for 7 days. Post-surgical recovery time was ~2 weeks prior to placement on study.

Animal acclimation
After surgery and recovery, the animals were acclimated to a sling and a nose cone over a 2-week period to reduce stress on the animals during exposure. Animals were monitored, and vitals were taken periodically to ensure animals were not overstressed during training. Food enrichment was provided during acclimation and training period for adequate comfort and reward.

Inhalation system
The system has been described in detail by Staugler et al., briefly, a custom designed HCN exposure system (Figure 2) was constructed to deliver controlled inhalation exposures to the swine via the nose/muzzle-only route. Nine individual awake swine were restrained using a customized restraint sling with an exposure nose cone secured over the muzzle. Target HCN vapor exposures were generated by diluting certified HCN gas with dry air (<3% RH). Calibrated mass flow controllers (MFC) metered the flow rate of HCN and dilution air to achieve the target HCN exposure concentration of ~500 ppm (553 mg/m³). The exhaust system maintained flow through the nose cone at ~20 L/min.

Exposure delivery control
Twelve animals were placed on study, 3 controls and 9 HCN exposed. Real-time respiratory data and HCN concentration data were used to ensure animals received their target inhaled dose (Staugler et al., 2018).

Endpoints
Cardiovascular monitoring
Two of the 6 instrumented pigs were implanted with a telemetry device (L21) having a systemic arterial catheter introduced at the femoral artery and a left ventricular catheter implanted in the apex of the left ventricle. The other 4 instrumented animals were implanted with a telemetry device (L11, M11) having a systemic arterial catheter. All 6 telemetry devices also incorporated ECG biopotential leads
placed in a modified Lead II configuration. Each animal’s home cages and restraints slings were equipped with a DSI telemetry receiver. The transmitters, receivers, data matrices, ambient pressure monitors, cabling and computers were all components of the Physio Tel Telemetry System. Operation of the Physio Tel Telemetry System was used to collect ECG, aortic blood pressure (AoP), and left ventricular pressure (LVP) waveforms. From these recordings, the following measurements were collected: HR in beats/min (bpm); diastolic, systolic and developed LVP pressure in millimeters of mercury (mmHg); systolic aortic blood pressure (mmHg), mean aortic blood pressure (mmHg), diastolic aortic blood pressure (mmHg), and pulse pressure (mmHg). Also, R-R ECG interval (RR), P-R ECG interval (PR), Q-R-S ECG interval (QRS), Q-T ECG interval (QT), Q-T Fridericia corrected ECG interval (QTCf) (Hanson et al., 2006), and ST-segment elevation (STE) were analyzed.

All ECG intervals were measured in milliseconds (msec) and the STE in millivolts (mV). Physiological recordings were continuously obtained at least 24 hr before each dosing exposure to a target of 24 hr after exposure (for surviving animals).

Blood sampling
Arterial blood samples were collected from the VAP prior to HCN exposure and at 2.5, 5.0, 7.5, 10, 12.5, 15 and 30 min after the start of HCN exposure. Approximately 1 mL of blood was collected in SafePICO Aspirator syringes with safe TIPCAP (Radiometer America Inc., Brea, CA) for subsequent blood gas analysis (See Section 2.6.2.1 and 2.6.2.2, respectively). Additional blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainers (BD Biosciences, San Jose, CA) at the same time points stated above and at 24 hr post-exposure, flash frozen in liquid nitrogen, and stored at −70 °C until analyzed for CN levels in whole blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (See section 2.6.3.2).

Blood gas analysis. The arterial whole blood samples were immediately analyzed using an ABL90 Flex Blood Gas Analyzer (Radiometer America Inc., Brea, CA) to measure arterial lactate, glucose, methemoglobin (FMetHb%), pH, potassium (K+), calcium (Ca²⁺), oxygen tension (PO₂) and carbon dioxide tension (PCO₂). Note: the detection system reached saturation at a lactate concentration of 24 mmol/L.

Cyanide blood analysis. Whole blood samples were analyzed by LC-MS/MS to quantify the circulating CN levels. [13C⁸]N labeled potassium cyanide (K⁵[13C⁵]N) was used as internal standard (IS). Stock solutions of potassium cyanide (KCN) and K⁵[13C⁵]N were prepared in 0.1 M sodium hydroxide (NaOH) to concentrations of 4.00 mg/mL and 500 µg/mL, respectively. Calibration standards were made by diluting the KCN stock solution with 0.1 M NaOH to concentrations of 20.0, 100, 200, 300, 400, 500 and 600 µg/mL. Quality control (QC) samples were prepared from the KCN stock solution by diluting with 0.1 M NaOH to low, mid and high concentrations [Low Quality Control (LQC): 60.0 µg/mL; Middle Quality Control (MQC): 120 µg/mL; High Quality Control (HQC): 520 µg/mL]. A working internal standard (WIS) solution was prepared by diluting the K⁵[13C⁵]N stock solution with water to a final concentration of 5.00 µg/mL. Calibration standards and QC samples were stored frozen at −10 to −30 °C and the WIS was stored refrigerated at 2–8 °C in amber glass until ready to use.

Matrix calibration standards and matrix QC samples were prepared fresh daily by spiking 2 mL of swine naïve blood with 100 µL of calibration standards and QC samples prepared in 0.1 M NaOH. All matrix calibration standards and matrix QC samples were immediately flash frozen to replicate the storage condition of study samples. After removing standards and QCs from liquid nitrogen and study samples from −70 °C (2 mL), they were all thawed unassisted at room temperature. Two milliliter of WIS solution was added to each sample (except double blanks), thoroughly mixed, and transferred to individual Conway cells. Hydrogen cyanide was released from Conway cells (depth center: 0.15 in; diameter outer: 3.26 in; material: polypropylene) collected into NaOH as CN. The micro diffusion Conway cells were set up by adding: (1) 3 mL of 0.01 M NaOH in the center ring; (2) 2 mL of 0.01 M sulfuric acid in the outermost ring and dispensed dropwise around the entire ring; (3) 3 mL of sample on one side of outer ring; and (4) 2 mL of concentrated acid on another side of the outer ring. The Conway cell was immediately closed with a lid and the blood and sulfuric acid mixed together by titling and swirling for 1 min. After 4 hr incubation at room temperature, dilution was completed by transferring 50 µL of the sample from the NaOH center ring to a microcentrifuge tube containing 200 µL of water and 700 µL of methanol. Two hundred fifty microliters of the diluted sample was transferred into a 96-well plate, and 50 µL of 5 mM taurine and 50 µL of 1 mM 2,3-naphthalenedicarboxaldehyde (NDA) solutions made in 1:1 50 mM potassium tetraborate tetrahydrate: 50 mM potassium phosphate dibasic trihydrate were added and vortexed for 1 min. The 96-well plate containing all samples was sealed with a lid and placed in the dark at 2–8 °C for 10 min. Samples were then submitted for LC-MS/MS analysis using multiple reaction monitoring (MRM).

Results
Hydrogen cyanide exposures and mortality
Hydrogen cyanide exposures were delivered to 9 female swine (Staugler et al., 2018). Table 1 summarizes the data obtained during HCN exposures. Target inhaled doses ranged from 2.0 to 3.0 mg/kg; this concentration range was selected for this study because the concentrations were near the previously defined LD50. This range was chosen to increase the likelihood that the monitored physiological effects would be significantly altered from baseline. Hydrogen cyanide concentrations ranged from 549 to 559 mg/m³ (497–506 ppm); inhaled doses ranged from 2.02
to 2.83 mg/kg; 3 animals survived the HCN exposures and 6 died. From the 9 animals that were exposed to HCN, 3 animals with DSI devices were chosen as representative animals corresponding to low (L, Animal 64357 at 2.02 mg/kg), mid (M, Animal 64362 at 2.25 mg/kg), and high (H, Animal 64359 at 2.40 mg/kg) inhaled doses for purposes of discussing the results obtained. Animals 64360 and 64354 would have been a better representation for the high exposure dose but these were not surgically implanted with telemetry devices, and therefore no cardiovascular data was obtained.

**Endpoints**

**Cardiovascular monitoring**

Cardiovascular monitoring for the selected representative animals is discussed below. The data corresponding to the other animals equipped with telemetry devices and/or LV catheter implant, but not selected for discussion, are presented in Supplementary Figures 1–4.

**Animal 64357 (L).** Heart rate and mean arterial blood pressure was stable with minor fluctuations for Animal 64357 during baseline. Following the start of exposure ($T = 0$), the animal exhibited tachycardia reaching a maximum of 254 bpm followed by a decline. Heart rate remained elevated with some fluctuations throughout the 30 min monitoring period. Following the start of exposure, the animal became hypertensive with mean aortic blood pressure reaching a maximum of 157 mmHg at ~6 min. Systolic aortic blood pressure increased by a greater extent than diastolic blood pressure. Systolic and diastolic aortic blood pressure decreased and then stabilized by ~16 min (Figure 3(A)).

The RR and PR intervals were slightly variable during baseline then decreased in response to the increased HR (Supplementary Figure 5(A,B)). The PR interval recovered then increased as HR declined through the 30 min monitoring period. The QRS interval was stable prior to HCN exposure. Following the start of exposure, the QRS interval increased from 3 to 4 min, then declined from ~5–10 min followed by a recovery to baseline levels and stabilization by ~20 min (Supplementary Figure 5(C)).

The QT and QTcF intervals were fairly stable prior to dose administration. Following the start of exposure, the QT interval initially decreased, followed by an increase and continued variability. The QTcF interval increased at ~7 min to 440 msec followed by continued variability and finally stabilization towards the end of monitoring (Supplementary Figure 5(D,E)). The prolongation was not HR dependent as indicated by the HR corrected value and the corresponding tachycardia at the time of prolongation. The STE was stable for this animal over the course of the experimental monitoring period (Supplementary Figure 5(F)).

**Animal 64362 (M).** This animal had a stable HR and mean aortic blood pressure during baseline. The animal exhibited tachycardia reaching a maximum HR of 242 bpm ~5 min after exposure initiation (Figure 3(B)). Mean aortic blood pressure increased to a maximum of 174 mmHg at ~6 min. Mean aortic blood pressure then declined to 110 mmHg at ~9 min and then slowly increased to a hypertensive but stable level (max of 183 mmHg) by 20 min after exposure initiation throughout the remaining 30 min of monitoring (Figure 3(B)).

The RR and PR intervals were stable during baseline and decreased as influenced by tachycardia and increased with reduced HR (Supplementary Figure 6(A,B)). The QRS interval was stable during baseline. Following the start of exposure, the QRS interval decreased slightly from 3 to 7 min followed by stabilization that continued throughout the 30 min monitoring period (Supplementary Figure 6(C)).

The QT and QTcF intervals were stable during baseline. Following exposure initiation, the QT interval declined as HR elevated, followed by a recovery and then another decline at ~13 min. The HR corrected QTcF interval prolonged (rather than decreased as seen in the uncorrected QT interval) within the first 5 min, then declined at 13 min (similar to the QT interval) demonstrating an initial increase in repolarization time followed by a reduction in repolarization time at 15 min (Supplementary Figure 6(D,E)). The STE was stable during baseline followed by some minor instability within the first 10 min and then recovery to stable through the 30 min monitoring period (Supplementary Figure 6(F)).

**Animal 64359 (H).** This animal had considerable blood pressure signal inconsistencies due to implant catheter issues, making interpretation of the HR and blood pressure changes difficult from the blood pressure waveforms. Therefore, HR was determined from the RR interval of the ECG signal; however, the HR values appear to be

| Animal ID | Weight (kg) | Target inhaled dose (mg/kg) | Average HCN Conc. (mg/m³) | Inhaled Vol (m³) | Inhaled dose (mg/kg) | Exposure duration (min) | Mortality | Time of death (min) |
|-----------|-------------|---------------------------|--------------------------|-----------------|---------------------|------------------------|-----------|-------------------|
| 64357     | 22.6        | 2.0                       | 559                      | 0.0817          | 2.02                | 5.75                   | Survive   | NA                |
| 64358     | 16.3        | 2.5                       | 549                      | 0.0613          | 2.07                | 17.00                  | Dead      | 25                |
| 64361     | 20.3        | 2.5                       | 549                      | 0.0806          | 2.18                | 21.00                  | Dead      | 29                |
| 64362     | 20.0        | 2.3                       | 549                      | 0.0821          | 2.25                | 11.92                  | Survive   | NA                |
| 64364     | 22.9        | 2.3                       | 559                      | 0.0947          | 2.31                | 7.25                   | Survive   | NA                |
| 64355     | 15.5        | 2.3                       | 549                      | 0.0654          | 2.32                | 10.00                  | Dead      | 21                |
| 64359     | 19.4        | 2.4                       | 559                      | 0.0833          | 2.40                | 13.72                  | Dead      | 20                |
| 64360     | 20.3        | 2.5                       | 559                      | 0.0909          | 2.50                | 11.25                  | Dead      | 19                |
| 64354     | 18.6        | 3.0                       | 559                      | 0.0942          | 2.83                | 16.00                  | Dead      | 25                |

*aTime of death confirmed by a veterinarian.
*bCardiovascular data was recorded until 21 min but death was not confirmed by a veterinarian until 29 min.
*cTechnical difficulties were encountered with this animal. Cardiovascular, gas blood and blood cyanide data are not available.

Table 1. Inhalation parameters obtained during HCN exposures and mortality ($n = 9$).

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overestimated due to noise artifacts in the ECG waveforms. During baseline, the HR was periodically elevated and decreased in a similar manner following exposure. There was a decline to ~55 bpm at 16 min (Figure 3(C)).

The RR and PR intervals were variable during baseline and following exposure initiation until ~15 min. At this time, the RR and PR interval increased corresponding to a period of bradycardia (Supplementary Figure 7(A,B)). The QRS interval was also slightly variable during baseline. Following the start of exposure, the QRS interval declined slightly within the first 7 min. At ~18 min, the QRS interval increased to a maximum of 48 msec (Supplementary Figure 7(C)).

The QT and QTcF intervals were extremely variable with changes in HR, indicating that the HR correction factor was not effective under extreme HR changes (Supplementary Figure 7(D,E)). The STE was stable during baseline. Following the start of exposure, the STE increased at ~3 min followed by stabilization until ~17 min when the ST segment largely elevated to ~0.579 mV at 19 min (Supplementary Figure 7(F)).

Blood gas analysis
Table 2 summarizes the arterial lactate, arterial glucose, FMetHB%, pH, K+, Ca2+, PO2 and PCO2 obtained from arterial blood samples collected at different time-points for the 3 selected representative animals. Supplemental Table 1 summarizes the data for the remaining dosed animals.

Lactate level in blood is a sensitive marker for CN toxicity (Salkowski & Penney, 1995). The normal range of lactate in unstressed patients is from 0.5 to 1.9 mmol/L. A lactate concentration of greater than this strongly suggests significant CN exposure. Sodium nitrite and sodium thiosulfate have been used extensively as antidotes to treat CN poisoning (Baskin et al., 1992). The calculation of the percentage of methemoglobin in whole blood provides a guide for continued therapy after the use of methemoglobin-inducing antidotes. Elevated levels of methemoglobin (>10%) indicate that further sodium nitrite or sodium thiosulfate therapy is not indicated.

Lactate and methemoglobin values were plotted against time in min (Time 0 = start of exposure) for the 3 representative animals (Figure 4). Lactate data for Animals 64357 (L) and 64362 (M) indicate that there is significant CN exposure 5 min after exposure initiation (Figure 4(A,B)) while at higher doses of HCN, Animal 64659 (H) shows high CN exposure as soon as the exposure started (Figure 4(C)). This animal was agitated with elevated ventilation prior to HCN exposure (Staugler et al., 2018). This may have led to hyperventilation which is associated with elevated lactate levels. Although lactate concentration for Animal 64357 (L) was high at 5 min, lactate concentration reached a maximum value of 21 mmol/L and started
decreasing after 10 min, whereas with Animals 64362 (M) and 64359 (H), the concentration of lactate reached a maximum of 24 mmol/L which is the highest value the equipment was capable to read causing a saturation due to the high levels of CN in the blood. On the other hand, the methemoglobin data shows that for all the animals (low, mid and high doses) the values were below 10%. The low percentages of methemoglobin suggest that a proper antidote would have to be administered in response to the CN poisoning. This interpretation is based on the thought that sodium nitrite exerts its therapeutic effect by reacting with hemoglobin to form methemoglobin.

Cyanide blood analysis

Calculated concentrations were obtained for each time point using tandem mass spectrometry and the method described in Section 2.6.3.2. These concentrations were plotted against time in min (Time 0 = start of exposure) (Figure 5). Mild toxicity is observed at calculated concentrations of 0.5–1.0 μg/mL.

**Animal 64357 (L).** Animal 64357 (L) shows a CN concentration as high as 5.40 μg/mL at 12.5 min and reaches 6.65 μg/mL in 5 min. After this time-point, the concentration, which is indicative of acute CN poisoning, starts dropping and over 24 hr the animal survived, and the concentration of CN was below the limit of quantitation (LOQ) of the instrument (Figure 5 Red line).

**Animal 64362 (M).** Animal 64362 (M) was exposed to a higher inhaled dose and at 12.5 min the maximum CN concentration was calculated as 16.3 μg/mL. Although the exposure dose was higher for this animal, survival after 24 hr was achieved, and the concentration of CN was dropped to below the LOQ which means that the exposure was not acute poisoning at this time-point (Figure 5 Blue line)

**Animal 64359 (H).** Animal 64359 (H) which was exposed to a higher inhaled dose (2.40 mg/kg), reached a maximum CN concentration of 10.7 μg/mL at 10 min and died after 15 min from acute poisoning (Figure 5 Green line). This maximum CN concentration is lower than the one obtained for Animal 64362 (M). We believe this high dosed animal was dying from CN poisoning and several body systems were shutting down. This could have greatly altered CN distribution.

Discussion

The objective of this effort was to use an HCN inhalation exposure system capable of administering accurate inhaled doses to a large sized animal that mimics a real-life scenario of human exposure. Human disease may best be recapitulated in a large mammal such as the pig. The porcine is often the primary biomedical model for several diseases, for surgical research, and for organ transplantation owing to the similarity in size, anatomy, and physiology between pigs and humans (Swindle et al., 2012) such as the lung structure and the cardiovascular, urinary, integumentary, digestive physiology, feeding patterns, dietary habits, kidney structure and function, pulmonary vascular bed structure, propensity to obesity, respiratory rates, and social behaviors (Lunney, 2007); therefore, used here to study the physiological effects caused by HCN exposure.

Nine animals were successfully administered HCN inhaled doses between 2.02 and 2.83 mg/kg body weight.
The selected range was the result from previous characterization of the swine model that could represent significant changes in physiological effects compared to the background and is the best range that could be used to monitor physiological effects that would be significantly altered from baseline (Staugler et al., 2018). Also, this range correlates with CN doses used in swine and other animal studies (rat and goat) published in literature: Bhandari et al. evaluated the behavior of CN in 11 swine females that were infused intravenously with 1.7 mg/kg KCN (Bhandari et al., 2014). Sousa et al. evaluated the toxicokinetic of blood CN in rats and pigs following oral KCN exposure at 3.0 mg KCN/kg body weight (Sousa et al., 2003).

All pigs exhibited cardiovascular effects immediately following the start of HCN exposure. All pigs developed dramatic but brief increases in HR from the onset of exposure (0 min) to 5 min into HCN exposure. This could be attributable to a psychological response to odor. Because of the variability in HR responses among the pigs, and although all pigs responded to exposures identically in the first 5 min and 3 pigs (Animals 64355, 64359 (H) and 64361) died with bradycardias after 21, 20 and 29 min, respectively; it is difficult to ascribe a common response in HR to HCN. For the terminal pigs, rate of discharge of the sinoatrial (SA) node was clearly depressed as indicated by distinct bradycardia; such depression could result from hypoxemia or from direct suppression of SA nodal pacemaker cells or of cells in exit pathways to the right atrium. From the onset of exposure, aortic pressures, systolic more than diastolic, thus pulse pressure increased in all pigs. This is consistent with the increase in HR.

Among the parameters obtained from the blood gas analysis, we focused mainly on the lactate levels and the FMetHB%, due to their effectiveness as markers towards CN poisoning. In humans, a lactate concentration of 8 mM or higher (Baud et al., 2002), which is associated with reduced levels of consciousness among other symptoms, is an indication to initiation of poisoning antidote. The levels of lactate increased rapidly to over 10 mM within 10 min of exposure initiation for the low and mid dosed animals, whereas the lactate level for the high dosed animal reached higher than 10 mM as fast as in 2.5 min. The lactate levels detected in the blood of Animal 64357 (L) increased to 20 mM in about 7.5–10 min but decreased significantly over the time of observation balancing to a level where almost no poisoning was present. On the other hand, for the other two representative Animals (64362 (M) and
64359 (H)), the lactate levels increased rapidly and reached instrument saturation at 24 mM. These clinical results are of extreme value to monitor cases where a fast CN blood analysis is not available, tracking the levels of lactate in plasma (e.g. using portable lactate analyzers) could be indicative of a fast response and proper administration of antidotes.

The fraction of methemoglobin relative to total hemoglobin was evaluated to demonstrate its potential use for continuing therapy after the use of methemoglobin-inducing antidotes. Methemoglobin is an abnormal hemoglobin in which the iron moiety of unoxgenated hemoglobin is in ferric state rather than ferrous state due to an ongoing process of oxidative process. The result of this is the inability to carry oxygen to the cell. It was observed in all three cases of low, mid and high HCN inhaled dosed animals, that the methemoglobin levels started at 0.75% (normal baseline) and continuously increased over time to a maximum of 1.5% during the 30 min monitoring period. One antidotal strategy used to treat CN poisoning is the induction of methemoglobinemia with amyl nitrite or sodium nitrite (Kirk et al., 1993). When the concentration of methemoglobin increases from its normal levels (0.5–2%), the source of circulating ferric ion increases and competes with cytochrome oxidase for binding by CN, causing CN to dissociate from cytochrome oxidase in tissue and to move into blood. Elevated methemoglobin percentages obtained in blood after using methemoglobin-inducing antidotes (>9%) indicate that further nitrite therapy is not needed (Johnson et al., 1989).

Finally, an accurate CN concentration present in whole blood was calculated for all animals at different time-points after exposure initiation using an LC-MS/MS method. The analytical method employed using K13C15N as IS and Conway micro diffusion cells, demonstrated to be accurate, sensitive, and reproducible for detecting blood CN at levels above and below the toxic ranges for CN in whole blood, thus confirming the potential applicability of this method in clinical and forensic toxicology.

In conclusion, the inhalation system proved to be useful in the determination of the physiological effects caused by cyanide poisoning in a female swine model. The characterization of this system provides a full spectrum for the proper implementation of CN medical countermeasures in response to high threat caused by potential exposures.

Disclosure statement
No potential conflict of interest was reported by the authors.

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