SPIE Volume 5403

Ocular Scanning Instrumentation: Rapid Diagnosis of Chemical Threat Agent Exposure

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ABSTRACT

Military personnel and first responders are in critical need of a sensitive technology for the rapid evaluation and diagnosis of exposure to adverse chemical agents. Ideally such a technology would be automated, easily portable, possess a high degree of sensitivity and specificity, and provide non-invasive assessment of health status. A potential method for meeting these requirements is via monitoring of ocular characteristics. Due to the interconnection between the eyes and the various physiological systems of the body, insults to the body may create a unique "thumbprint" upon the eyes based upon how these various physiological systems are differentially affected. In turn, these thumbprints (biomarkers) may be used to perform diagnostic evaluations of an individual's health status. Based upon this principle, the Ocular Scanning Instrumentation (OSI) technology is being developed as an automated device for non-invasive monitoring of optically apparent characteristics and attributes of the eyes for in-the-field diagnosis of battlefield traumas, insults, and threat agent exposures. The current manuscript presents comparative data for two of the agents which we have evaluated, carbon monoxide and cyanide. The defined methods provide the required specificity and sensitivity needed for detecting exposures at time points which provide an ample therapeutic window for medical intervention.

Keywords: Ocular Scanning Instrumentation (OSI), chemical warfare agent, cyanide, carbon monoxide, biomarker.

1. INTRODUCTION

Exposure to noxious chemicals or agents pose a pertinent and serious health hazard which often requires expeditious evaluation, diagnosis, and medical intervention. Such exposures may occur intentionally (e.g., warfare or terrorist attack) or accidentally (e.g., spill site or handling exposure). Regardless of the setting, there often exist a number of difficult hurdles to be navigated in order to minimize casualties. One such hurdle is the circumstances under which clinical presentation often occurs. For example, the agent of exposure may be relatively unique or uncommon (i.e., chemical agent attack on civilians) making diagnosis potentially arduous even for trained professionals. Also, there may be large numbers of individuals exposed, thus overwhelming available trained resources and heightening the need for rapid diagnostic triage capabilities. Perhaps the best solution to address this developing need is through technology. In order to significantly advance the current capabilities in responding to noxious agent exposures, any such technology will need to meet or exceed the speed, specificity, and accuracy which presently exist with respect to diagnosis while minimizing the need for user training. With these objectives in mind, we are currently performing animal and simulation research to develop algorithms for a rapid, non-invasive diagnostic technology – the Ocular Scanning Instrumentation (OSI).

In order to reliably and noninvasively detect exposure to a potentially wide variety of agents, it is important to employ a target organ, tissue, or source that may signal deleterious effects to diverse physiological systems. One such source is the eye. Over the past century, numerous scientific studies and reports have identified the eye as a prime indicator for a vast array of diseases, syndromes, abnormalities and exposures – from cranial nerve dysfunction to mental illness. The ability of the eyes to provide pertinent information regarding such a wide variety of ailments is due to their tight interconnection with the cardiovascular, musculoskeletal, lymphatic, ectodermal, and nervous systems of the body. As such, specific abnormalities may create a unique "thumbprint" upon the eye based upon how they differentially affect the various physiological systems to which the eyes are interconnected. Consequently, once properly defined, characterized, and quantified, such ocular characteristics may be employable for diagnostic purposes.

As an example of the approach we have adopted for the development of the diagnostic capabilities of the OSI technology, the present manuscript presents the results from experiments performed with two agents, cyanide and carbon monoxide. Acute carbon monoxide (CO) poisoning casualties are typically the result of engine exhaust inhalation or gas/oil-fueled heat and appliances, causing thousands of deaths per year[1]. Additionally, home or industrial fires also often result in acute CO poisoning for those unable to quickly evacuate from the site. On the other hand, cyanide poisoning is more rare and is also a more likely intentional threat, either during warfare or via a terrorist attack. Cyanide exposures, like CO, also occur as the result of home or industrial fires – victims inhale the cyanide gas which is the byproduct of common building materials. Initially, the clinical presentation of exposure to either toxin is similar (nausea, headaches, dizziness, labored breathing), as the body is unable to properly transport oxygen to the tissues and organs of the body. However, due to their varying mechanisms of action, treatment for the incorrect toxin can, in fact, exacerbate the deadly effects of these poisons. Hemoglobin (Hgb), the oxygen carrying molecule of the blood, has an affinity for CO which is approximately 200-250 times greater than that for oxygen. The absorption of CO by the blood (creating carboxyhemoglobin; COHgb) causes a leftward shift in the oxygen-hemoglobin dissociation curve, resulting in a decreased oxygen carrying capacity and impaired release of oxygen to the tissues. This, in turn, leads to cellular/tissue hypoxia[2,3]. Though other mechanism are involved which lead to delayed and/or prolonged symptoms, this oxygen displacement serves as the primary acute action of CO. Cyanide acts by combining with the ferric ion in mitochondrial cytochrome oxidase, preventing electron transport in the cytochrome system and, thus, bringing oxidative phosphorylation and ATP production to a halt. Though fully oxygenated, the cells cannot utilize the oxygen and thus increased demands are placed on anaerobic glycolysis. This results in lactic acid production/accumulation and eventual cell death due to histotoxic anoxia.

In order to develop a differential non-invasive diagnosis between CO- and cyanide-exposure via ocular characteristics, we have chosen to exploit the opposing way in which these agents affect the circulating blood, more precisely, the red blood pigment (Hgb). The quaternary structures of oxygen bound and CO bound Hgb are quite different, resulting in differential coloration for these distinct states of the molecule-complex. In essence, the more oxygenated the Hgb (and thus the blood), the brighter its appearance. Therefore, under normal circumstances, the blood is brighter in the arteries (compared to the veins) as it has yet to unload oxygen in the tissues, and this differential coloration may be quantitatively discriminated and analyzed. In the case of poisoning by either cyanide or carbon monoxide, this normal pattern is disturbed. For cyanide exposure, the blood does not unload oxygen since the tissues are already fully oxygenated (though they are not utilizing it), resulting in an equally bright red color of both the arteries and the veins. Carbon monoxide poisoning, however, actually decreases the oxygen carrying capacity of the blood (as much as 80-90% before death occurs), yielding a distinctly different appearance in the blood color (darker). It is these differential side effects of poisoning by cyanide and carbon monoxide that the present study utilizes as a means for rapid differential diagnosis of toxin exposure. Though methods exist to more accurately quantify the total oxygenation of the circulating blood, we have found that employing relatively simple methods (detailed below) can provide an accurate and sensitive differential diagnosis in a logistically and temporally efficient manner.

2. METHODOLOGY

2.1 Lethality experiments

Initial experiments were performed to examine the acute lethal effects (via a 48-hour LD_{50} analysis) for potassium cyanide (KCN; Sigma-Aldrich, St. Louis, MO) in a rodent model of exposure (Sprague-Dawley rat, Harlan). Studies using subcutaneous KCN indicated an LD_{50} of 6.7±0.6 mg/kg compared to literature values ranging from 6 to 9 mg/kg. All animal handling and KCN injection methods were identical to those described below. Additionally, studies were done with both anesthetized (with Ketamine/Xylazine, Sigma-Aldrich, St. Louis, MO) and unanesthetized animals. No differential effects were observed. The nature of CO, namely that it is a gas, places such lethality experiments more at the discretion of the researcher. Whereas with chemicals/toxins a standard single injection LD_{50} study may be employed, with inhalational agents there is the added variable of application time. A 48-hour administration of CO was not feasible (would require continual animal monitoring) and there is no standard protocol for such tests. Thus, reported values for lethal levels of various gases/gas mixtures vary with respect to both concentration of gas and length of application. Often these reported values will be for lethal effects ensuing after 4 or 8 hours of gas administration. However, since the protocol used for the subsequent studies described below employ a 60 minute observation window, the 1 hour time point was used for the lethality determination endpoint. The 60 minute exposure LD_{50} for Sprague-

Dawley rats determined in the present studies was 3123 ± 476 ppm (parts-per-million). Animal handling, data procurement, and CO application were identical to those described below.

2.2 Potassium cyanide exposures

For completion of these studies, a rat model (Sprague-Dawley) of subcutaneous (s.c.) exposure to KCN was employed. For all experiments, KCN was dissolved in normal saline such that a volume between 0.2 and 0.5 ml would be injected. Post-injection data was compared to control data obtained prior to injection of KCN for each animal. In addition, a group of animals were given 0.5 ml saline s.c. as an added control group. These studies were performed using a modified fundoscope for imaging the internal regions of the rat eye under non-mydriatic conditions. Due to the requirement of having the animals remain very still during recording, long term anesthesia with a ketamine/xylazine mixture (80 mg/kg ketamine hydrochloride; 12 mg/kg xylazine hydrochloride) was induced prior to recording. In short, experimental procedures were as follows: animals were weighed, anesthetized with ketamine/xylazine, and then placed on an adjustable platform in position for proper fundoscopic imaging. Approximately 10-15 minutes after anesthetic induction, a needle (23-gauge) attached to a 1ml syringe containing KCN was inserted under the skin and between the rear shoulder blades of the animal. Baseline fundoscopic imaging was then continually recorded. Five minutes after the beginning of recording, the plunger on the 1ml syringe was depressed, subcutaneously injecting the animal with KCN. Images were continually recorded for 60 minutes or until death (whichever came first).

2.3 Carbon monoxide exposures

In addition to changes in lethality testing analysis, the route of exposure for CO (inhalation) mandated changes to the dosing protocol. Typically, single injection agents are dosed based on the normal logarithmic concentration-response characteristics. Thus a dosing pattern of 1, 3, 10, 30, etc. is normally employed to give a symmetrical log-dose comparison. However, the compounding effect of continual gas exposure leads to a toxic load which makes such dosing patterns impractical for proper quantitative analysis, as they will not yield concentration-response curves which sensitively and accurately reflect the actions of the gas (i.e., the dose ranges are too broad/diffuse). For this reason, variable increments in carbon monoxide percentage were used in the present experiments, concentrating primarily around the region of initially observable significant effects.

For completion of the studies, a rat model (Sprague-Dawley) of inhalational exposure to CO was employed. As with the KCN experiments, post-exposure data was compared to control data which was obtained prior to CO administration for each animal. In addition, a group of animals were connected to the inhalational exposure apparatus and administered compressed normal air (79% nitrogen, 21% oxygen) as an added control group. Imaging studies have been performed with a modified fundoscope for imaging the internal regions of the rat eye under non-mydriatic conditions. Again, due to the requirement of having the animals remain very still during recording, long term anesthesia with a ketamine/xylazine mixture (80 mg/kg ketamine hydrochloride; 12 mg/kg xylazine hydrochloride) was induced prior to recording. In short, experimental procedures were as follows: animals were weighed, anesthetized with ketamine/xylazine, and then placed on an adjustable platform in position for proper fundoscopic imaging. The animals were then connected to the inhalation apparatus and allowed to breath normal air. Approximately 10 minutes after initial anesthetic induction, baseline fundoscopic images were continually recorded. Approximately 15 minutes after anesthetic induction, a valve was adjusted to switch the animal's breathing mixture from 79% N_2/21% O_2 to a predetermined mixture including CO (or, in control cases, a separate normal air mixture). Due to the logistics of preparing a large range of CO doses, pure CO was mixed with appropriate levels of compressed normal air, resulting in proportional decreases in both N_2 and O_2 partial pressures/composition percentages. Images were continually recorded for 60 minutes or until death, whichever came first.

2.4 Image acquisition and processing

Subsequent to obtaining streaming images, the next step in the experimental process was to isolate individual images from the streaming video (at specific time periods) and then quantitatively analyze vessel coloration. Arteries and veins were identified and subsequently isolated based on characteristic presentation (caliber, location, etc.). When possible, A-V pairs (adjacent artery and vein) were evaluated to minimize any illumination-induced measurement errors. In order to complete this analysis a number of different digital signal processing (DSP) methods were examined and tested for

optimizing the discrimination capabilities of the system while accounting for the expected variability between individuals (in this case animals) and minimizing the analysis time. The reported data employed analysis conducted using very simplistic processing algorithms. This involved converting the images to a gray scale, flattening illumination characteristics, and subsequently employing histogram equalization to improve contrast in the color range for which the vessels reside. The resulting color values were subsequently used to determine the means and standard errors for data analysis and comparison.

3. RESULTS

3.1 Potassium cyanide exposures

After exposure to KCN, the onset of altered blood coloration, generalized exposure signs/symptoms, and death (after large lethal doses) is quite rapid, even with the subcutaneous route of application used here. In response to 1 mg/kg KCN (s.c.), arterial vessel coloration did not change significantly (though there was a slight trend toward brighter coloration) whereas venous coloration brightened significantly within 60 seconds of cyanide injection ($p<0.05$). These alterations in venous coloration/oxygenation were often quickly followed by generalized symptoms such as rapid respiration with increased depth of breath. Maximal changes in venous coloration were typically noted within five minutes of KCN administration. Figure 1 graphs the time course of venous coloration change in response to various concentrations of KCN.

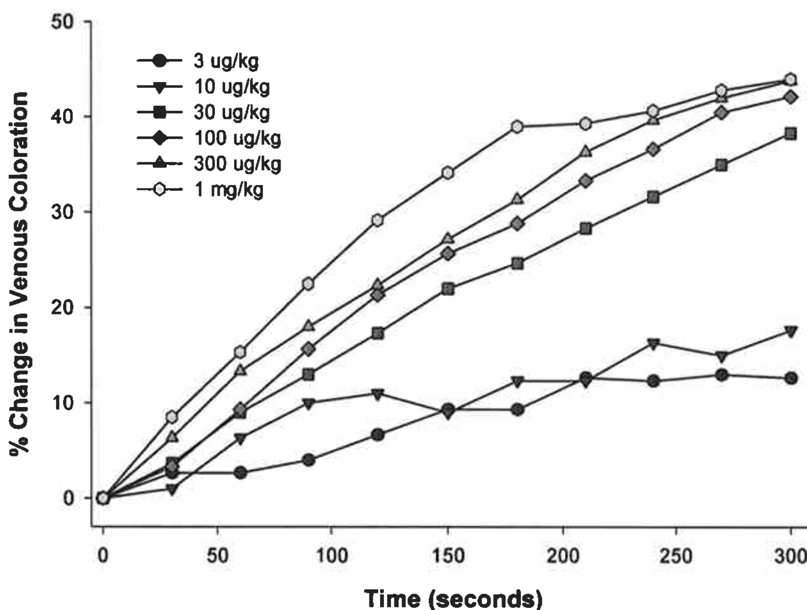

FIGURE 1. Percent change in venous vessel coloration in representative Sprague-Dawley rats in response to subcutaneous injection of various concentrations of KCN over time. Percent changes are compared to pre-dosing individual control levels (normalized to control). Error bars have been omitted for clarity.

For construction of the concentration-response relationship between KCN and venous coloration, the 5 minute post-administration time point was chosen for analysis. As noted above, all changes in venous coloration reached a maximum level by this time point and only animals which were administered supramaximal lethal doses of 30 mg/kg or greater expired within this time frame. Curve fitting analysis of the concentration-response curve (Figure 2) for venous coloration change 5 minutes after subcutaneous KCN injection estimated an EC_{50} for maximal detected venous color change of 24.6±3.1 μg/kg. However, statistically significant changes were not seen in every animal examined until the 30 μg/kg test group ($p<0.05$).

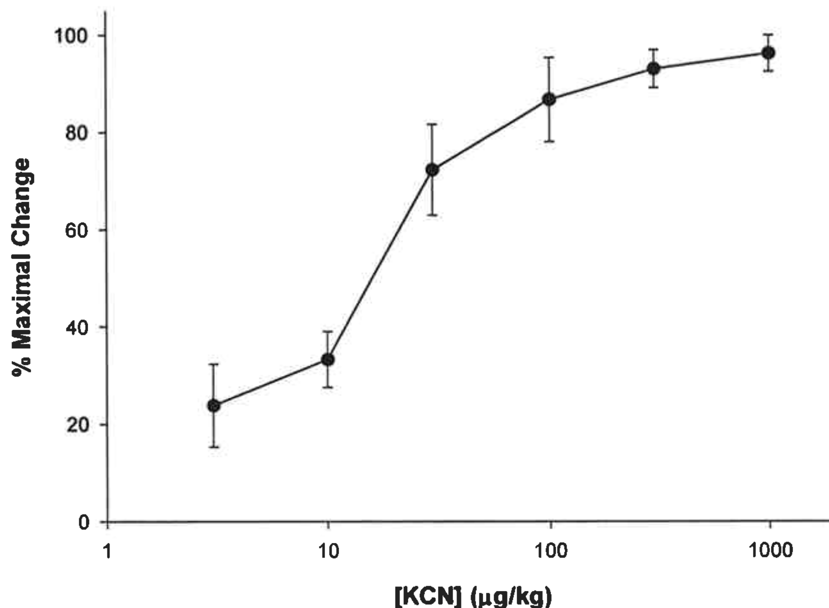

FIGURE 2. Concentration-response curve for venous coloration changes in Sprague-Dawley rats in response to subcutaneous injection of KCN. Data was obtained 5 minutes after KCN injection. Extrapolated EC_{50} = 24.6±3.1 μg/kg. Percent changes are compared to pre-dosing individual control levels. Means have been normalized to the maximal observed change in all animals (53%) and error bars represent ± S.E.M.

In addition to the approximate 200-fold diagnostic window (LD_{50} = 6.7 mg/kg; significant venous changes at 30 μg/kg) provided by venous alterations, it also became apparent that there may be some diagnostic utility in the analysis of arterial coloration. We found a slight but significant increase in the color value of the arteries at greater KCN dosage levels. This change becomes significant at the 300 μg/kg level ($p<0.05$). Since the arterial coloration does not appear to be as sensitive a diagnostic indicator for KCN exposure it may be employable as a secondary indicator for exposure level. Thus, an indication of significant coloration changes (to brighter, more oxygenated levels) in both arterial and venous systems indicates a greater level of exposure than venous changes alone. Furthermore, it should be noted that only animals which displayed changes in both systems (arteries and veins) later died as a result of their exposure. Thus, in mass casualty situations this may provide a valuable discriminating tool to identify individuals who have been exposed to sublethal levels of cyanide versus those who have been exposed to potentially lethal levels of the compound.

3.2 Carbon monoxide exposures

As one examines the time course of vessel coloration change in response to an approximate 60 minute LD_{50} dose (3000 ppm; Figure 3), two characteristics become evident. Firstly, even at this high level of CO administration, the alteration in blood coloration is relatively gradual and linear. This fact is likely due to the route of administration and mechanism of action of CO. The change in coloration is proportional to the toxic load of CO, which increases in an