KETAMINE AS A NEUROPROTECTIVE AGENT FOLLOWING SOMAN EXPOSURE

by

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To my heroes. Those who walk through hell, knowing that if something were to happen to you, I would be there. You trust me to know how to take care of you. I will never stop learning. I will never stop fighting to bring you home when you come through my doors. You are the reason I stay. You are the reason I fight. I am humbled by your dedication and love for one another.

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ABSTRACT

Ketamine as a Neuroprotective Agent Following Soman Exposure

Geoffrey W. Duncklee, Doctor of Philosophy, 2016

Thesis directed by: Dr. Joseph McCabe, Professor, Anatomy, Physiology and Genetics

Current medical countermeasures to chemical warfare nerve agents (CWNA) do not always adequately control CWNA-induced seizure activity. NMDA antagonists have shown promise in controlling CWNA-induced seizures even when given at substantial delays. The combinations of atropine and ketamine administration were examined when given in the traditional treatment scenario almost immediately after intoxication with the nerve agent, soman. Three dose levels of atropine sulfate (AS) (0.5, 1 and 3mg/kg) were tested to increase survivability along with four dose levels of ketamine (0, 7.5, 15, and 30 mg/kg) to enhance control of seizure activity. On the day of the experiment, guinea pigs were pretreated with pyridostigmine and challenged 30 minutes later with 2xLD_{50} soman. One minute after soman exposure, AS and 2-PAM were injected. One minute after the onset of seizure activity, midazolam and either one of the four doses of ketamine or saline were injected. In the group that did not receive ketamine, there was a significant difference in the survival at 24-hours between the 0.5mg/kg and the 1 or 3mg/kg AS groups. Administration of ketamine significantly decreased the duration of seizure activity.

However, as the dose of ketamine increased, the survivability at 24-hours decreased. We also determined the pharmacokinetic properties of ketamine in the guinea pig model by developing and validating a liquid chromatographic-mass spectrometric (LC-MS/MS) method to determine ketamine concentration in guinea pig plasma. Calibration curves were prepared by spiking ketamine into Dunkin-Hartley guinea pig plasma at concentrations of 195 pg/mL-200 ng/mL using deuterated ketamine (ketamine-d4) as the internal standard (3ng/mL). This method was employed to determine the pharmacokinetics of ketamine intramuscularly administered to guinea pigs at three IM doses (7.5, 15 and 30 mg/kg) and was validated according to the guidelines set by the FDA (Food and Drug Administration, 2001). Plasma concentration time-course employed samples taken at 0, 2.5, 5, 10, 20, 40, 80, 160, and 320 minutes. In an attempt to evaluate ketamine further, we examined brain tissue samples from the animals in the first aim and stained the tissue with DAB using rabbit anti-activated caspase-3 antibodies to determine if ketamine alone would increase neuropathology though apoptosis. The cerebral cortex, piriform cortex, thalamus, hippocampus and amygdala were examined and there was no difference in pathology noted in ketamine control groups.

In summary, ketamine was able to terminate seizure activity quicker than medications in the current standard protocol. However, only AS was able to increase survival after soman exposure. This study developed a fully validated method for the detection of ketamine in guinea pig plasma and successfully applied it to the pharmacokinetic analysis of ketamine-dosed guinea pigs. Although there was no evidence that ketamine increased neuropathology, more data is needed to definitively answer this question.

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CHAPTER 1: Introduction

Statement of the Problem

Chemical Warfare Nerve Agents's (CWNA) are highly toxic chemicals taken into the body through absorption, inhalation and ingestion. Traditional CWNAs include soman (GD), sarin (GB), tabun (GA), cyclosarin (GF), VR and VX. All of these agents bind to acetylcholinesterase and thereby prohibit the hydrolysis of acetylcholine (ACh) into choline and acetate (Bajgar, 1997, Hajek et al., 2004, Bajgar et al., 2008). This inhibition results in increased amounts of ACh at the synapse of cholinergic neurons throughout the body, initially in peripheral organs and eventually in the central nervous system. A "cholinergic crisis" includes physical symptoms of overactive cholinergic receptor activity, manifested with myosis, salivation, lacrimation, urination and defecation, and gastrointestinal symptoms starting with stomach cramping then leading to nausea and vomiting (Newmark, 2007). As the symptoms progress to the skeletal muscles, subjects exhibit fasciculations and then involuntary twitching. Seizures soon follow these peripheral signs and symptoms.

Atropine sulfate (AS) is the medication currently fielded to combat the cholinergic effects of CWNA exposure. For NATO forces and emergency workers, up to 6mg of AS can be administered through three separate autoinjectors if exposure is suspected (2mg per autoinjector). According the World Health Organization, AS is one of the essential medications (World Health Organization, 2014). It can be purchased for between $0.06 and $0.44 per mg, making it a reasonable medication to stock in third-

world and developing countries. This is why, when CWNA exposures occur in countries with few resources, medical providers have given large amounts of atropine sulfate to keep those exposed alive (Newmark, 2007). Iranian medical providers reported administering 50 to 100 mg of AS to severe casualties after Iraq exposed them to sarin (Rodgers, 1998).

The current problem is ensuring quality of life for those who survive CWNA exposure. With the limited resources Iran had during the 1980's, we do not have linear data concerning long-term sequelae in Iranian soldiers exposed to sarin. However, individuals who were exposed to sarin in the attack on the Japanese subway trains in 1995 have been followed closely and report cognitive, motor and behavioral changes at a greater rate than the rest of the population (Murata et al., 1997). Two decades of data show PTSD-like symptoms including depression, anxiety, memory and attention deficits, sleep problems and emotional disturbances in this population (Kawana et al., 2001, Okumura et al., 2005). If the dose is large enough to cause seizures, disorganized electrical activity will result in neuronal death (Collombet, 2011, Holmes, 2002, Fujikawa, 2005). There also appears to be a dose dependent difference in performance on neurobehavioral testing found at seven years post-exposure (Miyaki, et al., 2005). Pre-clinical studies have confirmed clinical observations that there are long-term behavioral impairments from CWNA exposure (Collombet et al., 2011). Therefore, finding a substance that diminishes the brain damage by decreasing the seizure activity can decrease the long-term adverse effects of CWNA exposure.

Benzodiazepines were already used to increase the seizure threshold for other disorders. Therefore, they were first tested, and then placed into the Convulsive Antidote, Nerve Agent (CANA) kits that soldiers now carry with them into areas where CWNA exposure is deemed likely. However, over the last decade, research has discovered that benzodiazepines not only fail to stop seizures, they actually increase the total time of seizure activity over a 24-hour period (McDonough et al., 2000, McDonough et al., 2010). This information highlights the need for the development of medications that decrease seizure activity by actions unrelated to the benzodiazepine family drugs.

As CWNA induced seizures progress, increased activity from cholinergic neurons disrupts the balance between the glutamatergic and GABAergic systems (Lallement et al., 1992, McDonough and Shih, 1997, Raveh et al., 2008). Microdialysis experiments on freely moving, male, Hartley guinea pigs determined there are neurotransmitter changes after sarin exposure (O'Donnell et al., 2011). As seizures progressed, extracellular acetylcholine levels increased to a plateau at ~30 minutes. Likewise, in animals that exhibited seizure activity, glutamate release was also elevated. GABA research has been ruled out as an effective treatment of seizure activity due to studies that show that GABA either increases well after seizure activity, or remains at a constant level (Lallement et al., 1993).

Since glutamate is the most abundant excitatory neurotransmitter in the brain, that was tested next. Microdialysis studies showed increase after CWNA exposure

(Lallement et al., 1991). N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are the main receptors for glutamate in the central nervous system. Research has identified the NMDA receptor as contributing the most to calcium influx after activation of neuronal cells in vitro; leading researchers to believe that the NMDA receptor has the greatest contribution to seizure activity once cholinergic neurons activate the glutamatergic system (Sattler et al., 1998). Additionally, there was a significant decrease in neuronal death in the cultures where NMDA receptors were blocked compared to the other glutamate receptors. This suggests that, not only is calcium responsible for signaling one of the pathways that leads to the eventual death of the cell, but that activation of specific signaling pathways is as important as the amount of calcium that enters the cell. Whether the signaling pathway leads to proteolysis, or death through other processes such as the release of free radicals and nitric oxide, preventing the entry of calcium though the NMDA receptor is an important step for the reduction of seizure activity and neuronal death (Sattler et al., 1998).

The two main types of cell death implicated after CWNA exposure are necrosis and apoptosis. These are different processes, and have different outcomes for the surrounding tissue. Necrosis is a process marked by cell and organelle swelling. When the cell ruptures, the intracellular contents change the extracellular milieu, and lead to an inflammatory process. This inflammatory process, in turn, leads to vascular permeability, edema, and scarring. Inversely, apoptosis is an organized process where

the cell shrinks, the organelles become condensed and the DNA is packed into small vesicles before being phagocytosed. The cellular contents do not leak into the extracellular space, and the death of that one cell does not necessarily detrimentally affect the surrounding cells (Kerr et al., 1972, Wyllie, 1980, Arends and Wyllie, 1991).

MK-801 and ketamine are NMDA antagonists and have proven useful after the onset of seizures. Ketamine has already been approved by the FDA as an anesthetic and analgesic agent in humans and animals and is therefore a worthy agent to test. Ketamine also has a good safety profile and has been used safely in children (Reich and Silvay, 1989, Green et al., 1998). Earlier studies with ketamine already show that it acts as a neuroprotective agent for animals 30 minutes after seizures induced by nerve agents (Dhote et al., 2012).

To investigate the possible efficacy of ketamine as an adjuvant to the administration of atropine, an oxime, and anticonvulsant now employed in standard medical countermeasures, male Hartley guinea pigs were chosen as the animal model because they have historically been used in nerve agent research to evaluate responses against CWNA exposure after similar medical countermeasure administration. Unlike rats, guinea pigs have low levels of carboxylesterase, which makes their susceptibility and responses to the toxic effects of the G-type nerve agents similar to that of nonhuman primates and, presumably, man (Maxwell et al., 1988). The pretreatment, pyridostigmine that is used for protection against oxime-resistant nerve agents also resembles that of the nonhuman primate and man more than any other rodent species (Berry and Davies, 1970,

Dirnhuber et al., 1979). Extensive amounts of data indicating the responsiveness of guinea pigs to standard medical countermeasures (atropine, oxime, anticonvulsant) have been generated using this animal model; these data closely agree with data on the effectiveness of these drugs in nonhuman primates (Gordon and Leadbeater, 1977, Inns and Leadbeater, 1983, Koplovitz et al., 1992, Shih et al., 2009, 2010). Male guinea pigs will be used to minimize potential interactions between drug treatments and stages of the estrous cycle, which can occur with the use of female guinea pigs.

The goal of this work will be to evaluate the value of ketamine as a neuroprotective agent for soman exposure. It will be given in combination with changing doses of AS to determine the effect that both substances contribute to survival, seizure control and neuropathology. To address the utility of atropine and ketamine, the following aims are planned:

Specific Aims

Aim 1: Determine whether the addition of ketamine to standard countermeasures, reduces seizures, and reduces neuron cell death in guinea pigs after soman exposure.

Guinea pigs were used in this study because this model most closely resembles the non-human primate in response to CWNA (Pereira et al., 2014), and the long-term goal of this research is to investigate a medication that will eventually be self-administered by military personnel after CWNA exposure. Screws were placed in the guinea pig skull one week before exposure to CWNA to monitor seizure activity via EEG. A standardized protocol was employed where, after pretreatment with pyridostigmine, guinea pigs were challenged with 2 x LD_{50} of soman. Atropine sulfate, 2-PAM, and either ketamine or saline were administered one minute after the nerve agent exposure. EEG data was gathered for four hours and then animals were returned to their home cages. The following morning, 30 minutes of EEG data was collected before euthanasia. After tissue was collected, the brains were prepared for staining using hematoxylin and eosin (H&E), and quantification of neuronal damage was determined using a board certified veterinary pathologist.

Aim 2: Determine if administration of ketamine reduces necrosis and apoptosis after soman exposure.

The primary mechanism of neuronal death following CWNA exposure is necrosis, followed by apoptosis (Kan, 2006). However, at least one report found that in immature rats, ketamine could cause neuronal death through apoptosis (Zou et al., 2009). The exposure protocol is the same as above. These animals were sacrificed 24 hours after exposure to determine the nature of cell death. Caspase-3 was used to determine if the cell was going through apoptosis.

Aim 3: Determine the pharmacokinetics of an intramuscular injection of ketamine.

No studies, to this point, have studied the pharmacokinetics of ketamine after intramuscular injection in a guinea pig model. Since the mode of administration for medical countermeasures against nerve agent intoxication is by intramuscular injection, and since timing of the injection is very important to the questions in the rest of this research, determining the onset, peak concentration and duration of ketamine in the plasma was important to determine. A baseline blood draw was conducted before ketamine administration. After an intramuscular injection of 7.5, 15 or 30 mg/kg of ketamine, blood was taken at 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 minutes and used to analyze the amount of ketamine in the plasma using a liquid chromatography/tandem mass-spectrometry method.

The long-range goal of this study was to assess ketamine as an adjunct treatment to standard CWNA countermeasures. The results of this study may provide additional evidence of the efficacy of ketamine, and form the groundwork for the development of a new medication administration model for nerve agent exposure.

CHAPTER 2: Evaluation of Ketamine as an Adjunct Treatment to Standard Medical Countermeasures Following Soman Exposure in Guinea Pigs

Introduction

CWNAs are highly toxic organophosphate chemicals that can be taken into the body through skin contact, inhalation or ingestion. All of these agents bind to the active site of the enzyme acetylcholinesterase and thereby prohibit the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetate (Bajgar 1997; Bajgar et al. 2008; Hajek et al. 2004; Shih et al. 2005). After some time has passed, this binding to acetylcholinesterase becomes irreversible (Sirin et al. 2012). The consequence of decreased breakdown of ACh is a rapid increase in ACh at neuromuscular junctions, glands and the synapses of cholinergic neurons. Seizures soon follow the initial peripheral signs of nerve agent exposure. Without the aggressive use of an anticholinergic (AS), oxime (2-pyridine aldoxime methyl chloride) and anticonvulsant (diazepam/midazolam) medications, and potentially supportive care by intubation and mechanical ventilation, the toxic effects of a large dose of CWNA can be rapidly fatal.

In the 1970-1980's nerve agent-induced seizures were recognized to be similar, if not identical, to the clinical condition known as *status epilepticus* (SE). Also, much like SE, early control of nerve agent-induced seizures could prevent brain damage and enhance survival (Baze 1993; Browne and Penry 1973; Lipp 1968, 1972, 1973; Murphy et al. 1993; Neligan and Shorvon 2009). Based in part on this research, diazepam was

fielded in the early 1990's as the Convulsive Antidote for Nerve Agent (CANA) kit. However, diazepam will be replaced in the near future by midazolam, which has a more potent and rapid effect when given IM (McDonough et al. 1999). Most noteworthy, as has been demonstrated in other animal models of SE, is that benzodiazepine anticonvulsant treatment is less effective when its administration is delayed, and seizures can potentially reoccur (Goodkin and Kapur 2003; McDonough et al. 2010; Walton and Treiman 1988). Data show that increased seizure durations lead to a greater possibility of brain pathology (Lallement et al. 1994; McDonough et al. 1995). The delay in treating the Japanese victims of the 1995 terrorist nerve agent attacks may be the reason for subsequent reports of long-term neurological issues (Nakajima et al. 1997; Yanagisawa et al. 2006). Because of this, more rapid and permanent control of nerve agent-induced seizures is desirable.

Glutamate is the most abundant excitatory neurotransmitter in the brain, and microdialysis studies demonstrate increased levels after CWNA exposure (Lallement et al. 1991a; Lallement et al. 1991b). This rise in brain glutamate concentrations has been hypothesized to directly contribute to the neuropathology seen following nerve agent-induced seizures (McDonough and Shih 1997; Solberg and Belkin 1997). Thus, inhibition of glutamate action could reduce CWNA-related brain injury. Studies continue to target the two main receptors for glutamate in the central nervous system, the N-methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. There has been work aimed at determining

the brain areas and the specific glutamatergic receptor subtypes responsible for the onset of seizure activity produced by CWNA exposure (Apland et al. 2009; Apland et al. 2013; Myhrer et al. 2010, 2013; Skovira et al. 2010; Skovira et al. 2012). Research has identified the NMDA receptor as the major contributor to neuronal death in cultures where NMDA receptors were blocked as compared to when other glutamate receptor subtypes were blocked (Sattler et al., 1998). Earlier studies have shown that ketamine, a well-known NMDA antagonist, as well as other NMDA antagonists (e.g., MK-801, GK11) were neuroprotective (resulted in reduced brain pathology) when given before or even as late as 40 minutes after the onset of seizures induced by nerve agents (Braitman and Sparenborg 1989; Dorandeu et al. 2007; Dorandeu et al. 2005; Dorandeu et al. 2013; Lallement et al. 1998; Shih et al. 1999). With these previous studies in mind, ketamine may be a useful adjunctive treatment to standard medical countermeasures to prevent nerve agent-induced lethality and to preserve neurological function following exposure. In the present study, we investigated the therapeutic effectiveness of ketamine as an adjunctive treatment to standard medical countermeasures (AS, oxime, benzodiazepine) in a guinea pig model of seizures induced by the CWNA soman.

Materials and Methods

Animals

A total of 144 male Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) weighing between 250-300 g at the time of surgery were used as subjects. The

animals were individually housed in filtered, shoebox cages and were maintained on a 12-hour light-dark cycle (lights on at 6:00 a.m.). Ambient temperature was kept between 23 ± 3°C, and humidity was maintained at 50+20%. Standard rodent laboratory chow and water were available *ad libitum*.

Chemicals

Soman (O-pinacolyl methylphosphonofluoridate) was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). The oxime 2-pyridine aldoxime chloride (2-PAM), isoflurane, ketamine hydrochloride, meloxicam, midazolam, bupivacaine and saline were obtained from Animal Health International (Mount Joy, PA), pyridostigmine bromide came from Henry Schein Medical (Melville, NY), and AS was obtained from Wedgewood Pharmacy (Swedesboro, NJ). The sodium pentobarbital was purchased through West-Ward Pharmaceuticals (Eatontown, NJ). Drugs were diluted using sterile saline whenever necessary immediately prior to their use.

Placement of EEG Monitoring Device

Approximately one week prior to the experiment, cortical screws were placed to measure EEG activity. Subjects were injected with meloxicam (0.5mg/kg, subcutaneous [SC]) and 15 minutes later anesthesia was induced using isoflurane. The surgery was performed on a heating pad under sterile conditions. Heart rate, oxygen saturations and temperature were monitored throughout the procedure. After the animal was secured in a

stereotaxic frame, bupivacaine was infiltrated intradermally down the midline of the scalp and a midline incision was made. Three burr holes were drilled using a hand drill equipped with a stop and stainless steel screws with attached wires were threaded in the holes. Two screws were placed bilaterally equidistant between lambda and bregma and 3 mm lateral to midline. The third screw was placed in the posterior cavarium. A miniature connector plug was attached to the wires on the screws and secured to the animal using a dental adhesive. The incision was sutured using non-absorbable sutures. The animals was then placed in a warming chamber until sternal and then given 0.9% saline or lactated ringers 20-50 ml/kg, SC, for additional hydration before being returned to the animal quarters.

Agent Challenge

On the day of the experiment, animals were taken from their home cages and placed in individual recording chambers. EEGs were recorded using CDE 1902 amplifiers and displayed on a computer running Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). The baseline EEG was recorded for at least 30 minutes. After the baseline data were collected the subjects were injected with pyridostigmine (0.026 mg/kg intramuscular [IM]). This pretreatment dose of pyridostigmine is sufficient to produce 20-40% inhibition in red blood cell acetylcholinesterase (Lennox et al. 1985). Thirty minutes after the pyridostigmine injection, soman (56 ug/kg; 2LD50) was injected SC. One minute later AS (0.5, 1 or 3 mg/kg) and 2-PAM (25 mg/kg) were injected IM.

One minute after the onset of seizure activity, midazolam (0.66 mg/kg) and either one of the four doses of ketamine (0 [saline], 7.5, 15, or 30mg/kg) were injected IM. There were 12 animals/treatment group. Subjects were monitored for four hours, and then those that survived were returned to their home cages. During the four hours, some animals did not survive. Once an isoelectric EEG was noted, the animal was observed for spontaneous respiration, and palpation was used to identify a heartbeat. Animals were considered dead once the observer could no longer palpate a heartbeat, and the time was noted. Twenty-four hours later, surviving animals were returned to the experiment cages, and an EEG was recorded for at least 30 minutes to determine their seizure status.

Histology

After the 24-hour EEG recordings were completed, animals were anesthetized with >75mg/kg sodium pentobarbital by intraperitoneal (IP) injection. After pain reflexes were absent, the animals were transcardially perfused with saline followed by 9% formalin. Brain tissue was extracted and placed in formalin until processing. The brain was subsequently blocked, embedded in paraffin, and cut at 10 µm thickness. The sections were then stained using Hematoxylin and Eosin (H&E) and sent to a board-certified veterinary pathologist, who was blinded to the treatment group. The pathologist scored 5 areas of interest (cerebral cortex, piriform cortex, amygdala, hippocampus and thalamus) from 0 to 4 (0= No lesion; 1= Minimal, 1-10%; 2= Mild, 11-25%; 3= Moderate, 26-45%; 4= Severe, >45%). These scores were added to create the total

pathology score (maximum total score = 20) (McDonough et al. 1995; McDonough et al. 1989; Shih et al. 2003; Shih et al. 2007). The criteria used to characterize the pathology were neuronal necrosis often with accompanying neuropil effacement in grades 2 through 4.

Data Analysis

The EEG record of each animal was reviewed to determine when seizure activity started and if and when it stopped following treatment. Each animal was categorized as having the seizure controlled (seizure off) or not controlled (seizure on) by a given treatment. To be considered as having the seizure controlled, all epileptiform spiking had to stop and the EEG had to be normal at all subsequent observation times (4-hour and 24-hour). In some cases, spiking or short spike trains were noted after what appeared to be an initial termination. While this was noted, the time for final termination of seizures was defined as the time of the final seizure termination during the initial four-hour observation period. A Cox regression was used for analysis of seizure onset latency after the different AS treatments alone and for the analysis of survival data across treatment groups. A log-rank Mantel-Cox test was performed, if needed, to determine if there was significance between groups. A Chi-square test followed by Fisher's exact test were used for analysis of the incidence of neuropathology data. For non-continuous data sets with small group sizes, a Yate's Correction Chi-square test was used to determine initial significance. Statistical significance was defined as $p<0.05$ for all tests.

Results: Standard Countermeasures

Standard Countermeasures

These groups of animals represent the outcomes when standard nerve agent medical countermeasures (pyridostigmine pretreatment, AS plus 2-PAM post-exposure and midazolam after seizure onset) were administered. The groups differed by the amount of AS (0.5, 1.0 or 3.0 mg/kg) that was administered (n = 12, 12, 12, respectively). Early signs of soman exposure included continual chewing followed by occasional full body muscle fasciculation. As time progressed after agent exposure, EEG recordings indicated rhythmic seizure-like spiking activity. The physical signs progressed to bouts of fore and hind paw clonus before righting reflex was lost. Although the majority of the animals who seized displayed physical signs of seizure activity, these physical signs did not consistently correlate with the onset of EEG spiking. Some animals developed EEG spiking while staring and showing no signs of clonus. However, physical convulsive signs soon (<2 min) developed once spiking became evident in the EEG record. None of these animals received ketamine.

Seizure Onset Latency

AS was given one minute after soman exposure, but before the onset of seizures. A Cox regression determined there was a significant difference in seizure onset times between the AS doses compressed across all ketamine doses (χ^2=20.07, df=2, p=<0.001).

A Mantel-Cox test revealed that the 0.5 mg/kg AS treated animals had significantly shorter seizure onset latencies than the 1 mg/kg and the 3 mg/kg AS treated animals ($p=0.042$ and $p=0.001$, respectively). It took longer for animals that received the 3 mg/kg AS dose to display seizure activity (median = 8.21 $\pm$ 2.7 minutes) on the EEG than animals that received the 0.5 mg/kg AS dose (median = 5.50 $\pm$ 10.5 minutes). It also took longer for animals that received 1 mg/kg AS to display seizure activity (median = 7.12 $\pm$ 1.36 minutes) than the animals in the 0.5 mg/kg AS dose group. There was no significant difference between the 1 mg/kg and 3 mg/kg AS dose groups ($\chi^2=2.860$, df=1, $p=0.091$). These data are displayed in Figure 1.

Seizure Control

Seizure activity was evaluated at 4 and 24 hours after nerve agent exposure. Using the Chi square test, there were no significant differences in the proportion of animals in which seizures were controlled as a function of AS dose at the 4-hour time point ($\chi^2=3.229$, df=2, $p=0.199$). However, at the 24-hour time there was an increase in the ability for the high AS dose to maintain seizure control ($\chi^2=6.200$, df=2, $p=0.045$). At the 24-hour time, there were significantly fewer animals with seizure activity in the 3 mg/kg AS dose group compared to the 1 mg/kg AS dose group ($\chi^2=4.866$, df=1, $p=0.027$), while there was no difference between the 0.5 mg/kg AS dose and the 1 mg/kg AS dose groups ($\chi^2=2.400$, df=1, $p=0.121$) or the 0.5 mg/kg AS dose and the 3 mg/kg AS dose ($\chi^2=2.444$, df=1, $p=0.621$). These results may be skewed due to the low number of

survivors (N=2) at 24 hours in the 0.5 mg/kg AS group relative to the other two AS groups (Table 1).

Survival

Survival was measured at two times during this experiment. The first time point was after the initial 4-hour recording on the day of the experiment. The second time point was taken at the 24-hour mark. If an animal died after being returned to its home cage, it was counted as alive at the 4-hour time and dead for the 24- hour time.

There were no significant differences in survival rate between AS dose groups at the 4-hour time (χ^2=2.971, df=2, p=0.226), but by the 24-hour time there were significant differences (χ^2=8.529, df=2, p=0.014). Fisher's exact tests showed that significantly fewer guinea pigs survived that received the 0.5 mg/kg AS dose than those that received the 1 mg/kg AS dose (p=0.014) or the 3 mg/kg AS dose (p=0.038). However, there was no difference between the 1 mg/kg AS and 3 mg/kg AS dose groups (p=0.666). Increasing the AS dose from 0.5 mg/kg to 1 mg/kg significantly increased the number of animals that survived for 24 hours. However, increasing the AS dose to 3 mg/kg did not have any significant advantages over the 1 mg/kg dose of AS on 24-hour survival.

Results: Standard Countermeasures plus ketamine

All groups were divided by AS doses to look at the effect that AS dose had on the different doses of ketamine. Where appropriate, the observations made at various AS doses were collapsed to evaluate the overall effect that ketamine had on a specific

dynamic of seizure activity. The no ketamine groups of guinea pigs were administered medications that are the current standard of care for nerve agent exposure and saline instead of ketamine, and were used for comparison to the treatment groups where guinea pigs received ketamine.

Seizure Control

When the data across all ketamine doses were compared by AS dose, a Cox regression determined there was a significant difference in the duration of seizure activity between the groups that did not receive ketamine (median = 4.02 $\pm$ 1.23 minutes) and the groups that received ketamine (median = 2.25 $\pm$ 11.31 minutes) (χ^2=17.23, df=1, $p=<0.001$). We evaluated the effects that ketamine had within each AS dose condition separately (Figure 2). In the 0.5 mg/kg AS dose condition there was a significant effect of ketamine dose on seizure duration (χ^2=11.39, df=3, p=0.001); seizure activity in the 7.5 mg/kg (median = 2.22 $\pm$ 0.42 minutes), 15 mg/kg (median = 2.19 $\pm$ 0.57 minutes) and 30 mg/kg (median = 2.1 $\pm$ 0.10 minutes) ketamine groups was significantly shorter in duration than in the no ketamine group (median = 3.44 $\pm$ 1.24 minutes; p=0.002, p=0.012 and p =0.001, respectively). There was no effect of ketamine at any dose on seizure duration in the 1 mg/kg (χ^2=7.550, df=3, p=0.056) or 3 mg/kg AS conditions (χ^2=5.531, df=3, p=0.137).

Survival

Survival curves for the different AS doses are plotted as a function of ketamine doses in Figure 3. In animal groups that received no ketamine, a Cox regression determined there was a significant reduction in 24-hour survival in animals that had received 0.5 mg/kg AS compared to those that received 1 mg/kg AS (χ^2 =4.633, df=1, p=0.031) or 3 mg/kg AS (χ^2 =5.662, df=1, p=0.017) (Figure 3A). There was no difference between the 1 mg/kg and 3 mg/kg AS doses (χ^2=0.009, df=1, p=0.923). It can be seen that at the no ketamine or 7.5 mg/kg dose of ketamine (Figure 3A and 3B, respectively) there were no differences in survival as a function of AS dose at the 4-hour observation time. In the 7.5 mg/kg ketamine group, a Cox regression found that the only significant difference was between the 0.5 mg/kg and 3 mg/kg AS doses (χ^2=8.633, df=1,p=0.003), with the 3 mg/kg AS dose group having greater survival (75%) at 24-hours than the 0.5 mg/kg AS group (27%). At the 15 mg/kg dose of ketamine, again animals that received the 3 mg/kg AS dose had higher levels of survival (92%) than those that received either the 0.5 mg/kg (25%) or the 1 mg/kg AS doses (25%) (p=0.0010 and p=0.002, respectively; Figure 3C). There was a difference in the survival of the 3mg/kg AS dose group (83%) over the 1mg/kg AS dose group (33%) when the ketamine dose reached 30 mg/kg (p=0.022) (Figure 3D). One other noteworthy aspect of these data can be seen clearly in Figure 4. As the ketamine treatment dose was increased, early survival ($\leq$4 hr) decreased in a dose dependent fashion in the 0.5 mg/kg and 1 mg/kg AS dose groups. In animals not receiving ketamine, early survival ranged from 80-100% in the

0.5 mg/kg and 1 mg/kg AS groups. As the ketamine dose increased, this early survival progressively drops such that at the 30 mg/kg ketamine dose, survival in the 0.5 mg/kg and 1 mg/kg AS groups is <50%. In summary, as the ketamine dose increased, early survival progressively decreased, and this was most prominent in animals that received the two lower doses of AS. Conversely, Figure 4 displays these same data showing the survival of the different ketamine treatment dose groups as a function of AS dose. When animals received the low 0.5 mg/kg AS dose, 24-hour survival was <50% for the two highest ketamine dose groups and they dropped to this level in <4 hours (Figure 4A). When animals received the intermediate 1 mg/kg AS dose, the groups that received the two highest ketamine doses, 15 and 30mg/kg, had significantly lower levels of survival (25% and 33%, respectively) than the group that just received 1 mg/kg AS and no ketamine (75%) (χ^2=6.948, df=1, p=0.008 and χ^2=6.41, df=1, p=0.011, respectively). Again, as with the low 0.5 mg/kg AS dose above, the two higher ketamine dose groups dropped to ≤50% survival within 4 hr, with the 1 mg/kg AS dose. In this case, the 7.5 mg/kg ketamine group had higher rates of survival that did not differ from the group that just received the 1 mg/kg AS dose alone (Figure 4B). Finally, when the AS dose was increased to 3 mg/kg, 24-hour survival of all ketamine treatment groups increased to >75% with no distinguishable differences between groups (Figure 4C).

Pathology

Only 77 animals across all treatment conditions survived to be perfused and have their brain assessed for neuropathology. Of these 77 animals, only 14 (18%) were judged to display any neural damage. The incidence of animals displaying neural damage across the various treatment groups is displayed in Figure 5. Collapsing across ketamine doses, a Chi-square analysis showed that AS dose had a significant (χ^2=6.883, df=2, p=0.032) effect on the incidence of neuropathology (Figure 5A). Further evaluation with Fisher's exact tests showed that animals treated with AS 1 mg/kg had a higher incidence of neuropathology than animals treated with AS 3 mg/kg (p=0.020). There were no other group differences as a function of AS dose. When a similar analysis was performed by collapsing across AS doses, a Chi-square showed that ketamine dose had a significant (χ^2=13.37, df=3, p=0.004) effect on the incidence of neuropathology (Figure 5B). Fisher's exact tests showed that the incidence of neuropathology was significantly higher in the groups that received no ketamine when compared to the 7.5 mg/kg and 30 mg/kg ketamine groups (p=0.013 and p=0.008, respectively) while the comparison between the group that did not receive ketamine and those that received 15 mg/kg ketamine just missed conventional levels of significance (p=0.067). There were no significant differences between any of the groups that received ketamine. Another measure of neuropathology was severity of damage. Of the 57 surviving animals that received any combination dose of AS and ketamine, only 5 (9%) displayed neuropathology, and 4 of these 5 (80%) showed the lowest total neuropathology score of 1 (indicates minimal [1-

10%] damage in a single brain region), while the other animal had a score of 4. In contrast, of the survivors that had just received AS and no ketamine, 9 of 20 (45%) displayed pathology. Of these 9 animals, 5 had total pathology scores ranging from 2 to 10 (indicating more severe and widespread damage in multiple brain areas), while the other 4 animals had scores of 1. It is noteworthy that 4 of the 5 animals with the highest neuropathology scores also continued to show epileptiform activity on their EEGs at the 24-hour recording session.

Discussion

By manipulating the doses of both ketamine and AS, this study was able to help determine the beneficial properties that can be ascribed to each of these substances for the treatment of nerve agent intoxication. Previous studies have given these treatments together and reported that ketamine alone was able to increase survival, decrease seizure time and therefore reduce neurological damage. In contrast, the present results show that the dose of AS is critical to observe a beneficial effect from ketamine and that AS contributes to survival while ketamine affects seizure duration.

Traditional medical countermeasures to nerve agents, either anticholinergics or benzodiazepines, display a decreased ability to stop seizure activity the longer seizures are allowed to progress before treatment is given (McDonough et al., 1999; McDonough et al. 2010; McDonough and Shih 1993; McDonough et al. 2000). Because of this phenomenon, many researchers have investigated different anticonvulsant treatments that

may be given at substantial delays following seizure onset. In particular, Dorandeu and colleagues investigated the ability of ketamine to control nerve agent-induced seizures under delayed conditions (Dorandeu et al. 2005; Dorandeu et al. 2007; Dorandeu et al. 2013). In their studies, they found that administration of ketamine or the active S(+) isomer of ketamine could terminate nerve agent-induced seizures when given up to an hour after seizure onset. Animals that were thus protected had excellent 24-hour survival, displayed minimal or no brain pathology and had significantly reduced markers of neuroinflammation. However, in all these studies, repeated high doses of AS (2-10 mg/kg) had to be administered to prevent ketamine-induced respiratory depression. This agrees with the current results that show that survival is highly dependent upon the dose of AS even for low doses of ketamine and even when treatment is given shortly after agent exposure.

The doses of ketamine used in this study were meant to span the range of doses used by Dorandeu et al. (2005). The current results show that a dose of 7.5 mg/kg ketamine was just as effective in stopping seizure activity as the higher doses as long as it was administered shortly after seizure onset. In fact, even lower doses may have been effective given how robust the effect was with the 7.5 mg/kg dose. The fact that ketamine was administered in conjunction with midazolam may also explain why such low doses of ketamine were effective. Dorandeu et al. (2005) also found a synergistic anticonvulsant effect against nerve agent-induced seizures when midazolam was combined with ketamine.

There are two major differences between this study and those of Dorandeu et al. (2005, 2007, 2013): the timing of therapy delivery and the doses of AS used. In the military, nerve agent antidote countermeasures are carried by each individual service member. In the U.S. military, service members are issued 3 autoinjectors, each of which contains 2 mg of AS and 600 mg of the oxime 2-PAM, as well as a separate autoinjector containing 10 mg of diazepam (soon to be replaced by autoinjectors containing 10 mg of midazolam) as an anticonvulsant. All three autoinjectors of AS and oxime as well as the anticonvulsant autoinjector are to be administered at the signs of severe nerve agent poisoning. Delivery of therapy is designed to be as soon as possible to minimize seizures and other toxic signs. The Dorandeu et al. studies concentrated on delayed treatment, 30 or 60 min after soman exposure, where seizures were already well established and refractory to standard anticholinergic or benzodiazepine treatment. The second difference between the studies is the doses of AS. The starting AS dose in this study, 0.5 mg/kg, was based on Food and Drug Administration (FDA) recommended body surface area scaling of doses between animals and humans (Food and Drug Administration, 2005). So the 0.5 mg/kg dose of AS in guinea pigs is the human equivalent dose based on body surface area for 6 mg of AS in a 60 kg human. In turn, the 1.0 mg/kg dose used here would scale to 12 mg total dose AS in humans. This is a dose that is close to those used to treat severe accidental human intoxications with the nerve agents soman and sarin (Sidell 1974). The 1.0 mg/kg AS dose is also where survival was statistically improved in this study over that provided by the 0.5 mg/kg AS dose. These dosing levels (0.5 – 1.0

mg/kg) of AS used in guinea pigs are thus realistic in the treatment of severe human casualties. In contrast, the 3.0 mg/kg AS dose represents a six-fold increase over the immediate treatment dose for AS, a situation that is highly unlikely to be practical in field treatment of severe nerve agent intoxication.

The findings of Dorandeu et al. (2005, 2007) reinforce other work that shows a progressive need for higher amounts of anticonvulsant drug, be it an NMDA antagonist like ketamine or a benzodiazepine (McDonough et al., 2000, 2010), to stop nerve agent-induced SE seizures the longer treatment is delayed. This is why most military forces have incorporated immediate anticonvulsant treatment of a nerve agent casualty with a benzodiazepine (diazepam, midazolam) into their treatment doctrine. It would seem that compounds like ketamine, which require co-administration of high amounts of AS, would be better suited to the infrequent situations where seizures are not controlled by the initial benzodiazepine treatments and higher levels of care can also be provided than are available to the field medic.

Ketamine, AS, Survival and Neuropathology

We found that ketamine did not affect 24 hr survival when given at a time of one minute after seizures. We found that only AS dose-dependently improved 24 hr survival in our model. While AS dose had a greater effect on 24 hr survival, the results clearly showed a complex relationship between AS and ketamine doses on early, $\leq$4 hr, survival.

The higher ketamine doses (15 and 30 mg/kg) resulted in greater numbers of early deaths especially when combined with the two lower AS doses (0.5 and 1 mg/kg).

In the Dorandeu et al. (2005, 2007) studies, AS at high doses was given along with every dose of ketamine. This suggests that it may have been the AS that provided the increase in survivability by preventing the respiratory depressant effects of ketamine and thus allowing ketamine to control the seizures. Both drugs no doubt have a synergistic effect in counteracting the effects of nerve agent intoxication. Shih et al. (2003, 2007) have shown that control of nerve agent-induced seizures is essential for survival and prevention of neuropathology and that seemingly small changes in AS dose can modulate the effect of anticonvulsants under these conditions. The present results provide additional data to support this contention. The neuropathology results show that failure to control seizure activity was associated with the more severe and widespread cases of brain damage, and that any ketamine dose could improve this outcome, presumably by more rapidly stopping the seizures.

Glutamateric Circuits and Seizure Propagation

McDonough and Shih (1997) and Solberg and Belkin (1997) had hypothetized that nerve agent-induced seizures are initially triggered and driven by central cholinergic overstimulation and that after some time the seizure activity per se activated the glutamatergic system to maintain seizure activity independent of this initial cholinergic drive. Various neurochemical studies that have used microdialysis support the fact that

brain glutamate levels rise dramatically following the onset of nerve agent-induced seizures and that seizure activity is essential for this rise to occur (Lallement et al. 1991a; O'Donnell et al. 2011; Wade et al. 1987). The fact that seizures were stopped in such a very short time after they began and after ketamine administration shows that the glutamateric circuits play a larger role than previously theorized. In fact, based on these results the glutamatergic system is clearly almost immediately engaged in parallel with the initial cholinergic overstimulation.

Summary

Ketamine was evaluated as an adjunct treatment to standard medical countermeasures (AS, 2-PAM, midazolam) in a guinea pig model of lethal nerve agent poisoning. While ketamine was capable of rapidly controlling nerve agent seizures and providing substantial neuroprotection, the amount of AS available for immediate treatment of nerve agent intoxication may not be sufficient to counter potential respiratory effects of ketamine. Ketamine should be considered for control of seizures that have become refractory to immediate benzodiazepine treatment, but only within a medical treatment facility equipped to provide additional AS and supportive care.

CHAPTER 3: A Liquid Chromatographic-Tandem Mass Spectrometric (LC-MS/MS) Method for the Determination of Ketamine in Guinea Pig Plasma

Introduction

Organophosphorus nerve agents are highly toxic chemicals that can be absorbed through skin contact, inhaled or ingested. This class of agents binds to acetylcholinesterase (AChE) and thereby prohibits the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetate (Bajgar, 1997, Hajek et al., 2004, Shih et al., 2005, Bajgar et al., 2008). As the level of acetylcholine increases, seizures develop. In the 1970-1980's it was recognized that nerve agent-induced seizures were similar, if not identical to the clinical condition known as *status epilepticus* (SE). In both cases, early termination of seizure activity decreases the probability of developing brain damage and enhances survival (Lipp, 1968, 1972, Browne and Penry, 1973, Lipp, 1973, Clements et al., 1982, Baze, 1993, Murphy et al., 1993). Conversely, increased seizure durations lead to a greater possibility of brain pathology (Lallement et al., 1994, Neligan and Shorvon, 2009). Glutamate is the most abundant excitatory neurotransmitter in the brain and studies demonstrate levels are increased, along with acetylcholine, after nerve agent exposure (Lallement et al., 1991a, McDonough et al., 1995). Ketamine, a well-known N-methyl-D-aspartate (NMDA) antagonist, was shown to rapidly control seizure activity and provide neuroprotection (reduced brain pathology) when given 40 minutes after the

onset of nerve agent induced seizures (Braitman and Sparenborg, 1989, Lallement et al., 1991b, Lallement et al., 1998, Dorandeu et al., 2005, Dorandeu et al., 2013). Interest in utilizing ketamine as an adjunctive treatment to standard medical countermeasures to prevent nerve-agent induced lethality and preserve neurological function following exposure continues to increase as studies show its usefulness in treating refractory status epilepticus without the traditional drawbacks of intubation (Gaspard et al., 2013, Bayrlee et al., 2015, Ilvento et al., 2015). By developing a technique to determine ketamine plasma levels in a guinea pig model, further studies can provide a better understanding of the pharmacodynamics of ketamine in the guinea pig model. Additionally, this study will assist future researchers in the selection of single and multiple dose ketamine regimens.

Methods and Materials

Chemicals

Ketamine hydrochloride (50mg/mL stock solution) was obtained from Animal Health International (Mount Joy, PA). Ammonia hydroxide, methanol, water 0.1% with formic acid and acetic acid were all obtained from Fisher Scientific Company (Pittsburg, PA). All chemicals were LCMS grade.

Analytical Instrumentation

Liquid chromatography (LC)

Liquid chromatography was performed using an Agilent 1290 Infinity Liquid Chromatograph (Agilent Technologies, Santa Clara, CA). Separation was performed on

a Halo C18 Column (2.7µm, 2.1mm x 50mm) (Advanced Materials Technology, Wilmington, DE) with mobile phase A being methanol with 0.1% Formic Acid and mobile phase B being 0.1 Formic Acid in water. A chromatographic ramp was employed consisting of: 0-3min = 95% mobile phase B → 95% mobile phase A; 3min-3.1min = 95% mobile phase A → 95% mobile phase B; 3.1min-6.1min = 95% mobile phase B. The chromatograph flow rate was 500µL/min. The autosampler compartment was held at 10° C.

Mass Spectrometry (MS)

A Sciex 6500 QTrap Triple Quadpole Mass Spectrometer (Sciex, Ottawa, CA) was used for tandem mass spectrometry. It was operated in electrospray mode using multiple reaction monitoring (MRM). The ion source temperature was 650 degrees. Capillary voltage was +5500V. The curtain gas was set at 30. The collision assisted dissociation (CAD) gas was medium. Ion source gas 1 and 2 were 50 and 75. Declustering potential was 30V. Entrance potential was 10V. The quantifier ion transition was 238.3Da to 125Da with collision energy of 37eV and collision exit potential of 16V were monitored. The qualifier ion transition was 238.3Da to 179.2Da with collision energy of 24eV and collision exit potential of 6V was used. The deuterated ketamine ion transition was 242.3Da to 129Da with a collision energy of 24eV and collision exit potential of 23V were monitored. Peak areas were integrated using Analyst software (Sciex, Ottawa, Ontario).

Procedures

Calibration Curves and Quality Controls

Commercially purchased guinea pig plasma in sodium heparin (BioreclamationIVT, Chestertown, MD) was used to prepare the calibration curves and quality control samples. The plasma was spiked at 200 ng/mL with ketamine and serial diluted to yield final concentration of 0.19, 0.78, 3.13, 12.50, 50.00, and 200.00 ng/mL. Calibration curves were prepared in duplicate and analyzed in triplicate over a 1-month period. A total of eight calibration curves were used for this study.

Quality control (QC) samples were prepared with the commercially purchased guinea pig plasma in sodium heparin and spiked with ketamine-d4 at 3ng/mL. The final concentration of the QC samples was 0.4, 2.0, 20.0 and 100.0 ng/mL. The QC samples were used to determine intra- and inter-day variability. Intra-day variability was assessed by analysis of six (n=6) identical determinations of QC samples made on the same day. The QC samples were quantified with a single calibration curve run concurrently with the samples. Inter-day variability was assessed by analysis of QC samples over the course of six non-consecutive days during a one-month period. Quantification of the QC samples was accomplished by running a calibration on each day. A linear least squares analysis with a 1/y weighting scheme was used to calculate the values for the calibration curve and QC samples. The precision (%CV) was calculated using the formula: %CV = (SD/mean) x 100% and the accuracy (%error) was calculated using the formula: % error = ((calculated concentration – actual concentration)/actual concentration) x 100%.

Sample Preparation

Plasma, for preparation of calibrators and QCs, and animal samples were thawed from the -80 degree storage freezer before use. Plasma (200μL) was removed from all animal samples, calibrators and quality control (QC)-containing vials, transferred to new microcentrifuge tubes followed by the addition of 60μL ketamine-d4 solution (13 ng/mL), as an internal standard for a final concentration of 3.0ng/mL ketamine-d4. Acetic acid (800 μL, 1.0 M) was added to each of the sample tubes in preparation for solid phase extraction (SPE).

Solid Phase Extraction

Oasis 1cc MCX cartridges with 30mg sorbent (Waters Corporation, Milford, MA) were prepared for the samples by adding 2.0 mL of methanol followed by 2.0 mL acetic acid. Solid-phase extraction was performed in duplicate and the replicates were analyzed via LC-MS/MS in triplicate. 450 μL of animal samples, calibrator and QC's were loaded into the cartridges. This was followed by a wash with 1.0mL of 1.0 M acetic acid. Waste tubes were replaced by 15 mL BD Falcon™ tubes (BD Biosciences, Bedford, MA) and the samples were extracted with 2.0 mL of ammonia hydroxide and methanol. The analyte was collected in polystyrene tubes and evaporated under a dry nitrogen stream at 40 degrees. Samples were reconstituted in 90 μL of 30% methanol in 0.1% formic acid in water.

Animal Samples

This study used 36 Male Hartley Guinea Pigs (Charles River Laboratories, Wilmington, MA) weighing between 250-300 g at the time the samples were drawn. The animals were individually housed in filtered, shoebox cages and were maintained on a 12-hour light-dark cycle (lights on at 6:00 a.m.). Ambient temperature was kept between 21 ± 2°C and humidity was maintained at 50 ± 10%. Standard rodent laboratory chow and water were available *ad libitum.*

The animals were divided into three treatment groups. Each group received one of three doses of ketamine (7.5, 15.0 or 30.0 mg/kg) administered intramuscularly (IM). Samples were obtained through the toenail clip method at 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 minutes after ketamine injection (Vallejo-Freire, 1951). Each ketamine group was then divided into two groups so only 5 samples were taken from any one animal. The first group had samples drawn at 0, 2.5, 10, 40 and 160 minutes after ketamine administration. The second group had samples taken at 0, 5, 20, 80 and 320 minutes. Each dose had 12 animals divided between the groups leading to 6 samples at each time point at each dose. Approximately 500µL of whole blood was collected and placed into microfuge tubes that already contained heparin as previously described (Shih et al., 2009). Samples were centrifuged at 1320 rpm for 5 minutes and plasma was collected, placed into a new microfuge tube and immediately stored at -80°C until analysis. Animal samples were prepared by first diluting into blank guinea pig plasma by adding 3uL

sample to 297uL plasma. Samples were treated the same as all calibrators, blanks and QCs after this dilution.

Pharmacokinetics

Mean plasma time-concentration data were fit to standard pharmacokinetic models using WinNonlin (Version 1.5, Scientific Consulting, Inc. Cary, NC) non-linear regression software. The analysis of mean plasma concentration-time data generated observed versus predicted concentrations as a function of time as well as pharmacokinetic parameter estimates. Parameter estimates generated were apparent volume of distribution (V_d), area under the time-concentration curve (AUC), time to maximum plasma concentration (T_{max}), maximum plasma concentration (C_{max}), and elimination half-life ($T_{1/2}$-elim).

Results

LC-MS/MS analysis and Calibration curves

A representative LC-MS/MS chromatogram of ketamine using the developed method is presented in Figure 6A. The chemical structure for ketamine is presented in Figure 6B. There were no observed interferants at the retention time of ketamine with the chosen transitions. For this study, calibration curves were prepared and analyzed each day that samples were run. Total area under the curve (AUC) was used to calculate the analyte to internal standard ratio for the quantification of animal and QC samples. Mean (n=8) calculated data for the calibration curves and intra- (n= 6) and inter-day (n= 6)

QC's are presented in Figure 7. The average correlation coefficient for the curves was 0.9980. The LOD was calculated to be 60 pg/mL and LLOQ was 195 pg/mL. LLOD was determined using the formula LLOD = 3σ where σ is the standard deviated of the blank. LLOQ was determined using the formula LLOD = 10σ where σ is the standard deviated of the blank.

Precision and accuracy

Inter-day variability

Inter-day variability was assessed by analysis of QC samples over the course of six non-consecutive days. Quantification of the QC samples was accomplished by running a calibration curve each day. Concentrations for all samples (calibration curve concentrations and QC samples) were calculated from each day's respective calibration curve. Data obtained from the inter-day samples is displayed in Table 3. For the QC's, the %error was between -12.1 and 7.43 and %CV ranged from 8.94 and 12.5. The correlation coefficient (weighted r^2) for the curve used to calculate QC values was 0.9993.

Intra-day variability

Intra-day variability was assessed by analysis of six (n=6) identical determinations of QC samples made on the same day. The QC samples were quantified with a single calibration curve run along with the samples. The %error ranged from -10.7

to 8.9 and the %CV ranged from 8.29 to 13.6. Correlation coefficients (weighted r^2) for the curves used to quantify the QC samples ranged between 0.9969 and 0.9997.

Ketamine study

The pharmacokinetics of ketamine after IM administration to guinea pigs were best described by a one-compartment model with first-order absorption and elimination described by Equation 1 (below).

Equation 1:

$$C(t) = \left(\frac{D}{V_d}\right)\left(\frac{K_{01}}{K_{01} - K_{10}}\right)\left(e^{-k_{10}t} - e^{-k_{01}t}\right)$$

Where C = plasma concentration (µg/ml), t = time (min), D = dose (µg/kg), V_d = volume distribution (L/kg), k_{01} = rate of absorption (min^{-1}), and k_{10} = rate of elimination (min^{-1}).

Pharmacokinetic parameter estimates for volume distribution (V_d) were 3.19, 2.65, and 2.51 L/kg, area under the curve was 35, 100, and 246, µg·min/mL. Elimination half-life was 10.3, 11.7, and 15.1 min. And C_{max} was determined to be 1.93, 5.05 and 9.26µg/mL for the three doses (7.5, 15.0, and 30.0 mg/kg), respectively. These parameters are summarized in Table 4. Figure 8 displays the experimental mean concentrations and the model-predicted curve for each dose.

Discussion

The method described herein has been shown to be useful for determining the concentration of ketamine in guinea pig plasma. The use of the tandem mass-spectrometry offers a highly sensitive and specific technique for the plasma matrix. Calibration curves demonstrated acceptable linearity ($r^2 = 0.9980$) across the entire concentration range. In addition, intra- and inter-day variability precision and accuracy determinations of QC samples demonstrated values within acceptable limits (±15%).

The plasma-time course for IM administered ketamine (7.5, 15.0 and 30.0 mg/kg) is presented in Figure 8 (A-C). Raw data (± SD) are overlaid with model predicted curves. Ketamine demonstrated a rapid absorption and elimination profile with T_{max} ranging from 2.8 to 4.3 minutes and the half-life of elimination ranging from 10.34 to 15.0 minutes. After five half-lives, approximately 97% of a compound would be expected to be distributed from the blood or eliminated from the body. The half-life of the largest dose of ketamine utilized in this study (30 mg/kg) was determined to be approximately 15.0 minutes. Therefore, it is estimated that at 75 minutes following administration, the compound would be to be mostly eliminated from the blood. It follows that the smallest dose of ketamine (half-life = 10.3 min) be essentially gone in less than an hour. This rapid absorption and elimination observed in the guinea pig is consistent with results found in previous studies that looked at the pharmacokinetics in humans. An IM injection of 0.5 mg/kg ketamine in humans resulted in a rapid increase in plasma levels (less than 4 minutes), a Cmax of 243 ng/mL, half-life of 155 min and an AUC of 23.6

min • ng/mL (Clements et al., 1982). Ketamine demonstrated linear pharmacokinetics in that peak plasma concentration (C_{max}) and area under the curve (AUC) were both linearly related to dose, demonstrating correlation coefficients of $R^2 = 0.9891$ and 0.9993 respectively. In the guinea pig larger doses would be predicted to produce proportionally greater AUC and C_{max} values.

In summary, a precise, accurate and sensitive LC-MS/MS method for determining ketamine concentrations in guinea pig plasma has been developed. Plasma samples assayed by this procedure yielded mass chromatograms that were free from interference. The method was used to determine pharmacokinetics in guinea pigs following IM administration of three doses (7.5, 15.0 and 30.0 mg/kg) of ketamine. The pharmacokinetic data obtained in these experiments are similar to data from the literature in terms of rapid absorption and elimination. The linear pharmacokinetics suggest one could estimate AUC and C_{max} with greater doses. With this technique, further studies may explore pharmacokinetics in conjunction with pharmacodynamics of ketamine in a guinea pig model. The results suggest that IM administration of ketamine should be considered when studying early administration of seizure treatments or when the treatment with anticonvulsant must be administered quickly.

CHAPTER 4: No Increase in Apoptosis after Ketamine Administration in Guinea Pigs Exposed to Soman

Introduction

This section explores the question of whether ketamine administration causes increased neuropathology. Based upon a previous report (Olney, 1991), that ketamine administration can cause neuron cell death, this section explores whether this phenomenon is observed with guinea pigs in our experimental model. Radiographic evidence showed that lesions occur in a receptor affinity dependent manner when NMDA receptor antagonists are administered where MK-801 displayed the greatest likelihood of creating lesions and ketamine showed the least likelihood of creating a lesion. Although chemical weapon nerve agents (CWNA) also lead to brain pathology if nerve agent-induced seizure activity is not controlled, there is a potential to further increase the amount of damage seen when using ketamine.

CWNA's bind to cholinesterases and prevent the hydrolysis of acetylcholine (ACh) to acetate and choline. As the level of ACh increases in the synapses of cholinergic neurons, blurred vision, muscle fasciculations, increased secretions and stomach upset leading to nausea and vomiting occur. After the peripheral symptoms start, seizures will develop. As the cholinergic neurons start the seizure activity, glutamatergic neurons act as an additional pathway to continue the seizure activity. Seizures resulting from exposure to CWNA cause irreversible brain damage. However, decreasing the duration of seizures mitigates neuronal damage (McDonough & Shih,

1997, McDonough et al., 1995, Shih et al., 1998). In previous studies, ketamine has been shown to stop seizures and decrease the pathology associated with seizure activity (Dhote et al., 2012). The majority of this damage is caused by necrosis, due in part to over-activation of the excitatory neurotransmitter, glutamate (Ankarcrona et al., 1995). Ketamine is an antagonist of NMDA glutamate receptors, and earlier studies show that ketamine as well as other NMDA antagonists act as neuroprotective agents by reducing seizure activity induced by nerve agents (Braitman & Sparenborg, 1989, Lallement et al., 1998, Dorandue et al., 2005,2007,2013).

The difference between this study and previous studies is the time that we allowed the animals to seizure before administering the treatment. This timing was based, in part, on previous work performed with primary cultures (Ankarcrona et al., 1995). The cells in this study died within 3 hours of necrosis when exposed to glutamate. The remaining cells in culture were observed going through apoptotic death approximately 24 hours later.

Methods

Histology

Animals were attached to an EEG monitor for thirty minutes before any medication administration to acquire the baseline EEG. Thirty minutes after the pyridostigmine injection, soman (56 ug/kg; 2LD50) was injected SC. One minute later AS (0.5, 1 or 3 mg/kg) and 2-PAM (25 mg/kg) were injected IM. One minute after the

onset of seizure activity, midazolam (0.66 mg/kg) and either one of the four doses of ketamine (0 [saline], 7.5, 15, or 30mg/kg) were injected IM. There were 12 animals in each treatment treatment group and 3 animals in each control group. 24 hours after the soman exposure, EEG recordings were completed, the animals were anesthetized with >75mg/kg sodium pentobarbital by intraperitoneal injection (IP). After pain reflexes were absent, the animals were transcardially perfused with saline followed by 9% formalin. Brain tissue was extracted and placed in formalin until processing. The brain was subsequently blocked, embedded in paraffin, and cut at 10μm thickness. The sections were then stained using Hematoxylin and Eosin (H&E) and sent to a board certified veterinary pathologist, who was blinded to the treatment group. The pathologist scored five areas of interest (cerebral cortex, piriform cortex, amygdala, hippocampus and thalamus) from 0 to 4 (0= No lesion; 1= Minimal 1-10%; 2= Mild 11-25%; 3= Moderate 26-45%; 4= Severe >45%). These scores were added to create the total pathology score (maximum total score = 20) (McDonough et al., 1989, 1995; Shih et al., 2003, 2007). The criteria used to characterize the pathology was neuronal necrosis often with accompanying neuropil effacement in grades 2 through 4.

Slices adjacent to the H&E stained were used for activated caspase-3 staining. Previously paraffin-embedded slices were stained using rabbit anti-activated caspase-3 polyclonal antibodies that were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). Simply, the paraffin was removed from the slides using alcohol and xylene. Endogenous peroxidase activity was blocked using hydrogen peroxide. A Pelco

microwave was used to induce antibody retrieval. Slides were prepared with ABC solution from Sigma Aldrich (St. Louis, MO) and then stained using a DAB solution from Sigma Aldrich. We then counterstained the slides using hematoxylin to better see the cell structures when looking for DNA fragmentation and compaction in the nucleus. Rat tonsil glands were used as controls for this study (Figure 10).

Results

Activated Caspase-3

Adjacent slices from the brains of animals in aim 1 were used to determine if there was apoptosis. The antibody manufacturer recommended the positive control for activated caspase-3 with hematoxylin counterstain was rat tonsillar tissue. This was successfully performed before slides were stained (Figure 10). The control tissue displayed typical signs of apoptosis, cell shrinkage and pyknosis that leads to a tightly packed nucleus typically found near the edge of the cell. There are also cells that display both the activated caspase-3 and hematoxylin stains but had no characteristic signs of apoptosis. A representative slide from an animal that received a 60mg/kg of ketamine and no agent is displayed in Figure 11. The animals that received the highest pathology score is represented in Figure 12. The same method to score the H&E slides was used to score the activated caspase-3 slides although the cells must have had both colocalization of stain and displayed signs morphological signs of apoptosis to be included in the count (cerebral cortex, piriform cortex, amygdala, hippocampus and thalamus scored from 0 to

4 (0= No lesion; 1= Minimal 1-10%; 2= Mild 11-25%; 3= Moderate 26-45%; 4= Severe >45%) for a total possible score of 20) (McDonough et al., 1989, 1995, Shih et al., 2003, 2007).

Discussion

There was no difference in the expression of activated caspace-3 between the ketamine doses in the groups of animals that did not receive soman. Since the same brains were used for both aims 1 and 2, we looked to see if there was a difference between doses when the animals received soman. We found that activated caspase-3 activity increased as pathology scores from the H&E scores increased suggesting that apoptosis correlated with signs of observed tissue lesions and obvious cellular damage. However, there were a greater number of cells that showed co-localization without the signs of apoptosis. This suggests that early stages of cell injury are evident by H & E and caspase-3 immunohistochemistry, but that morphological signs of apoptosis are slower to appear. In a previously unpublished study (Kan et al., personal communication), necrotic brain regions were evident within 24 hours after nerve agent exposure, while it took 48 hours to observe apoptotic cells. Studies that increase the time before study termination would help to differentiate whether the cell will terminate through necrosis or apoptotic mechanism.

CHAPTER 5: Discussion

Traditional medical countermeasures to nerve agents, either anticholinergics or benzodiazepines, are unable to decrease seizure activity the longer seizures are allowed to progress (McDonough et al., 1999; McDonough et al. 2010; McDonough and Shih 1993; McDonough et al. 2000). Many researchers have investigated different anticonvulsant treatments that may be given at the times when these medications cease to be effective. Dorandeu and colleagues investigated the ability of ketamine to control nerve agent-induced seizures under delayed conditions (Dorandeu et al. 2005; Dorandeu et al. 2007; Dorandeu et al. 2013). In all these studies, repeated high doses of AS (2-10 mg/kg) accompanied ketamine administration to prevent ketamine-induced respiratory depression. In this study we administered multiple doses of ketamine and compared the results with multiple doses of ketamine when given after soman exposure. We developed a pharmacokinetic curve after IM injection of ketamine in guinea pigs to better understand the dosing time and plasma levels of our treatment and we explored possible adverse neuronal side effects of ketamine.

The current results show that a dose of 7.5 mg/kg ketamine was just as effective in stopping seizure activity as the higher doses as long as it was administered shortly after seizure onset. In fact, even lower doses than were given in this study may have been effective.

There are two major differences between this study and those of Dorandeu et al. (2005, 2007, 2013): the timing of therapy delivery and the doses of AS used. Delivery of therapy is designed to be as soon as possible to minimize seizures and other toxic signs. The Dorandeu et al. studies concentrated on delayed treatment, 30 or 60 min after soman exposure, where seizures were already well established and refractory to standard anticholinergic or benzodiazepine treatment. The second difference between the studies is the doses of AS. The starting AS dose in this study, 0.5 mg/kg, was based on Food and Drug Administration (FDA) recommended body surface area scaling of doses between animals and humans (Food and Drug Administration, 2005). So the 0.5 mg/kg dose of AS in guinea pigs is the human equivalent dose based on body surface area for 6 mg of AS in a 60 kg human. In turn, the 1.0 mg/kg dose used here would scale to 12 mg total dose AS in humans. This is a dose that is close to those used to treat severe accidental human intoxications with the nerve agents soman and sarin (Sidell 1974).

The findings of Dorandeu et al. (2005, 2007) reinforce other work that shows a progressive need for higher amounts of anticonvulsant drug, be it an NMDA antagonist like ketamine or a benzodiazepine (McDonough et al., 2000, 2010), to stop nerve agent-induced SE seizures the longer treatment is delayed. This is why most military forces have incorporated immediate anticonvulsant treatment of a nerve agent casualty with a benzodiazepine (diazepam, midazolam) into their treatment doctrine. It would seem that compounds like ketamine, which require co-administration of high amounts of AS, would be better suited to the infrequent situations where seizures are not controlled by the initial

benzodiazepine treatments and higher levels of care can also be provided than are available to the field medic.

McDonough and Shih (1997) and Solberg and Belkin (1997) had hypothesized that nerve agent-induced seizures are initially triggered and driven by central cholinergic overstimulation and that after some time the seizure activity per se activated the glutamatergic system to maintain seizure activity independent of this initial cholinergic drive. Various neurochemical studies that have used microdialysis support the fact that brain glutamate levels rise dramatically following the onset of nerve agent-induced seizures and that seizure activity is essential for this rise to occur (Lallement et al. 1991a; O'Donnell et al. 2011; Wade et al. 1987). The fact that seizures were stopped in such a very short time after they began and after ketamine administration shows that the glutamateric circuits play a larger role than previously theorized. In fact, based on these results the glutamatergic system is clearly almost immediately engaged in parallel with the initial cholinergic overstimulation, making a medication that is lipophilic, like ketamine, a likely candidate to stop seizure activity shortly after seizure onset.

The use of the tandem mass-spectrometry offers a highly sensitive and specific technique for determining the concentration of ketamine in the guinea pig plasma. The plasma-time course for IM administered ketamine (7.5, 15.0 and 30.0 mg/kg) demonstrated a rapid absorption and elimination profile with T_{max} ranging from 2.8 to 4.3 minutes and the half-life of elimination ranging from 10.34 to 15.0 minutes. After five half-lives, approximately 97% of a compound would be expected to be distributed from

the blood or eliminated from the body. The half-life of the largest dose of ketamine utilized in this study (30 mg/kg) was determined to be approximately 15.0 minutes.

The pharmacokinetic data obtained in these experiments are similar to data from the literature in terms of rapid absorption and elimination. The linear pharmacokinetics suggests one could estimate AUC and C_{max} with greater doses. With this technique, further studies may explore pharmacokinetics in conjunction with pharmacodynamics of ketamine in a guinea pig model. The results suggest that IM administration of ketamine should be considered when studying early administration of seizure treatments or when the treatment with anticonvulsant must be administered quickly.

Finally, there was no difference in the expression of activated caspace-3 between the ketamine doses and the no ketamine group in the groups of animals that did not receive soman. While there was evidence of DAB staining, there were no cells that displayed the morphological signs of either apoptosis. Since there was no apparent difference between any ketamine group and the no ketamine group, it appears that ketamine alone does not increase or decrease neuropathology.

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Figure 1

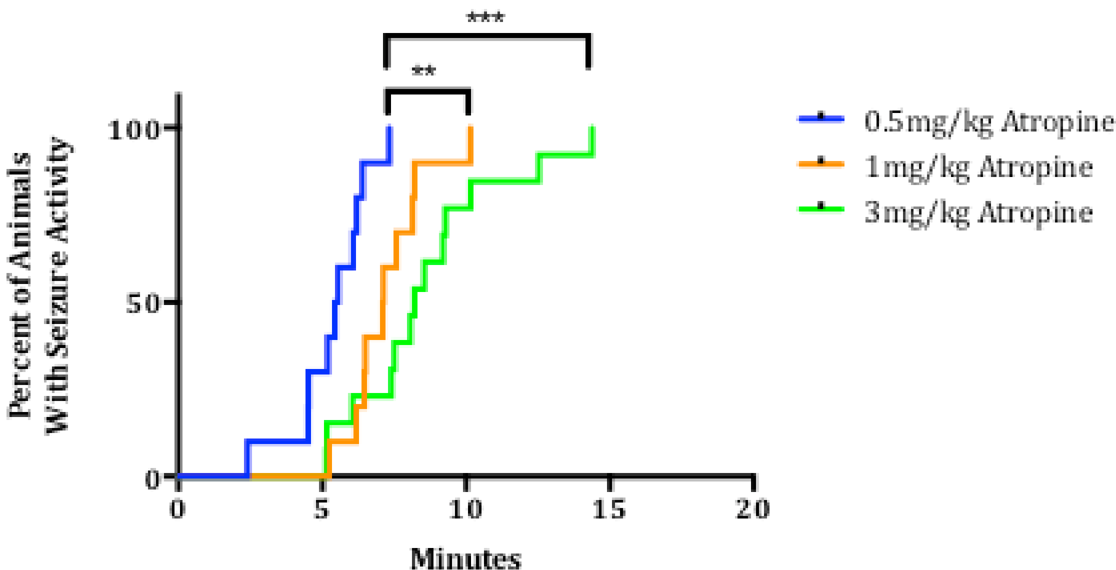
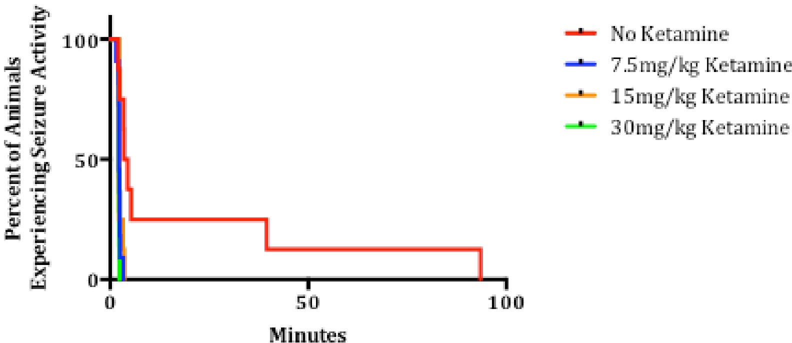
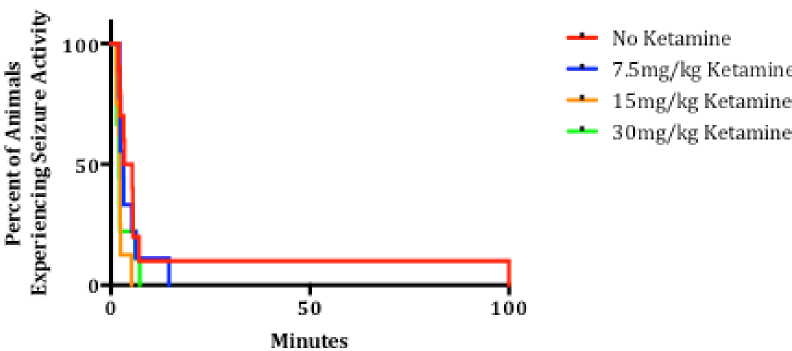


Figure 1. A Kaplan-Meyer plot of seizure onset latencies as a function of AS treatment dose. Administration of 1 mg/kg or 3 mg/kg AS delayed seizure onset significantly longer than when 0.5 mg/kg AS was given.

Figure 2





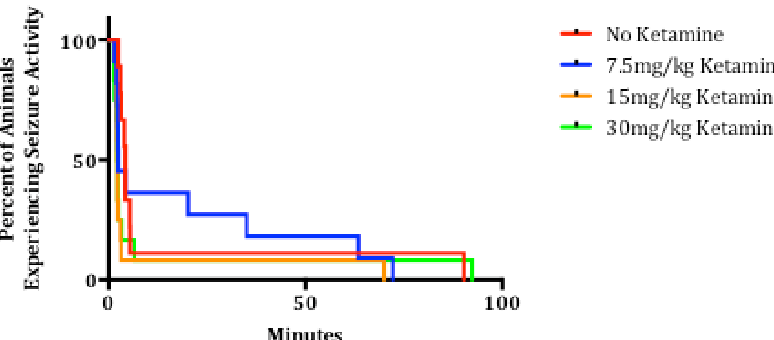
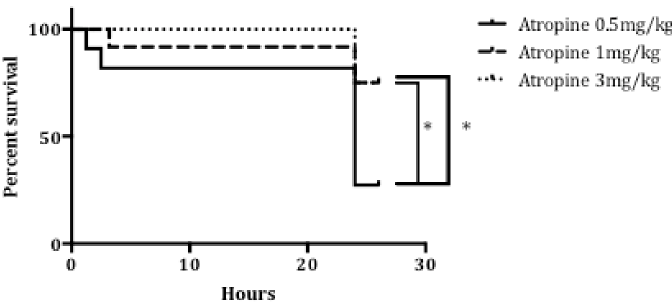
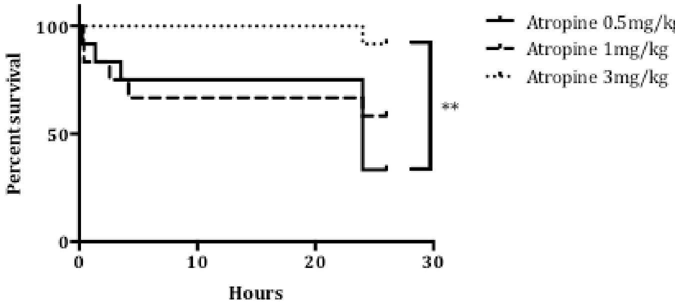
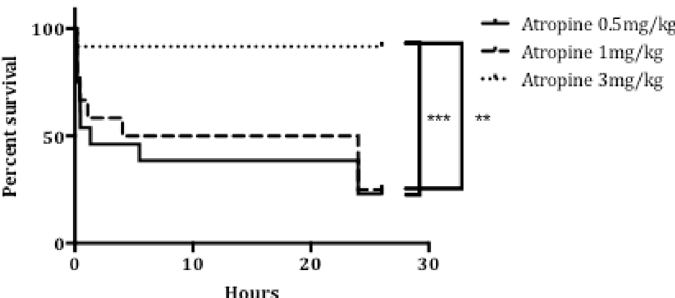


Figure 2. Seizure termination presented by AS dose condition and analyzed by ketamine dose. In the 0.5 mg/kg AS dose condition, seizures terminated faster in the 7.5 mg/kg, 15 mg/kg and 30 mg/kg ketamine groups than in the no ketamine group. There were no significant effects of ketamine on seizure termination in the 1 mg/kg or 3 mg/kg AS dose conditions.

Figure 3







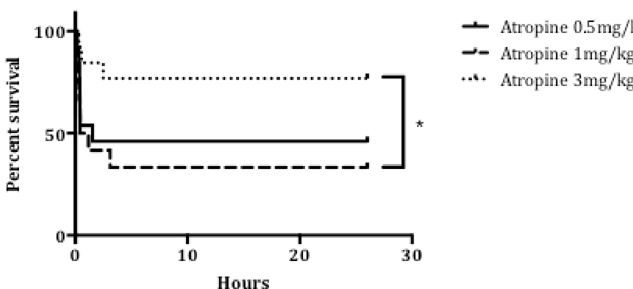
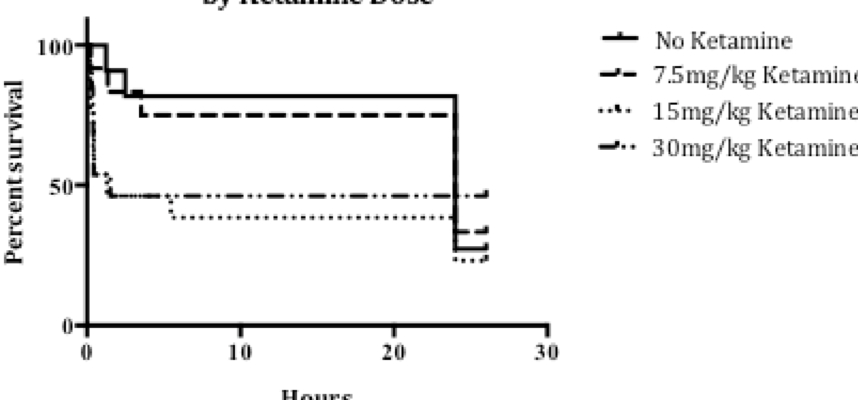
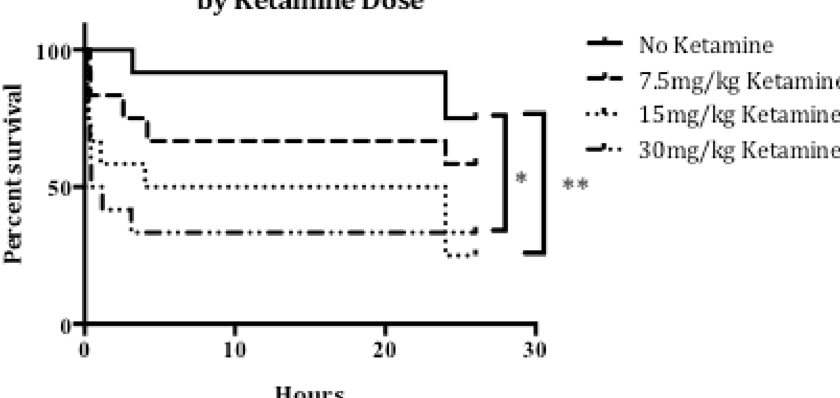


Figure 3. The 24 hour survival percentage of the different treatment groups as a function of ketamine dose. A: These groups received no ketamine; significantly fewer animals survived at 24 hours in the 0.5 mg/kg AS dose condition compared to the 1 mg/kg or 3 mg/kg AS dose conditions. B: These groups received 7.5 mg/kg of ketamine; significantly fewer animals survived at 24 hours in the 0.5 mg/kg AS dose condition compared to the 3 mg/kg AS dose condition. C: Animals is these groups received 15 mg/kg ketamine; significantly fewer animals died in the 3 mg/kg AS dose condition than those in either the 0.5 or 1 mg/kg AS dose conditions. D: Animals is these groups received 30 mg/kg ketamine; fewer animals survived in the 1 mg/kg AS dose condition than in the 3 mg/kg AS dose condition.

Figure 4





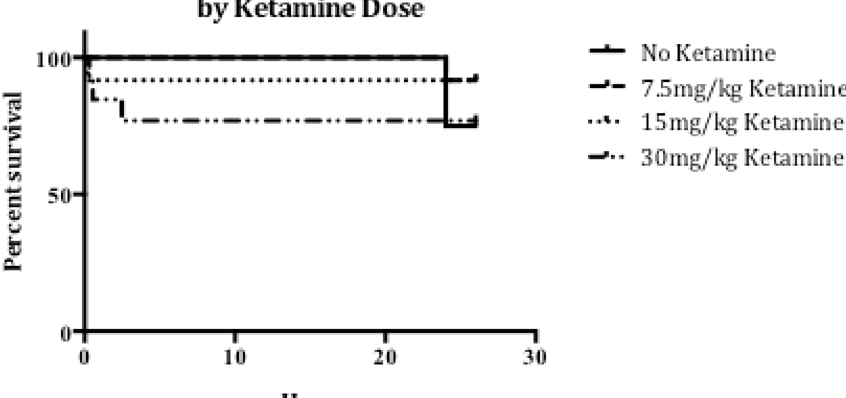
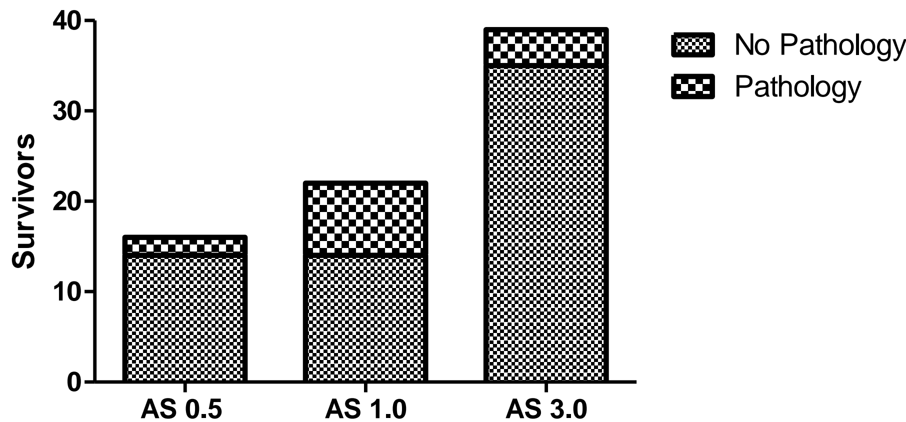


Figure 4. Survival percentage of the different treatment groups as a function of AS dose condition at 24 hours. There was no difference between survival between ketamine treatment groups for both the 0.5 mg/kg and 3 mg/kg AS dose conditions. In the 1 mg/kg AS dose condition the group that received no ketamine had significantly higher survival rates than the 15 mg/kg or 30 mg/kg ketamine groups.

Figure 5

A **Incidence of Neuropathology as a Function of AS Dose**



B **Incidence of Neuropathology as a Function of Ketamine Dose**

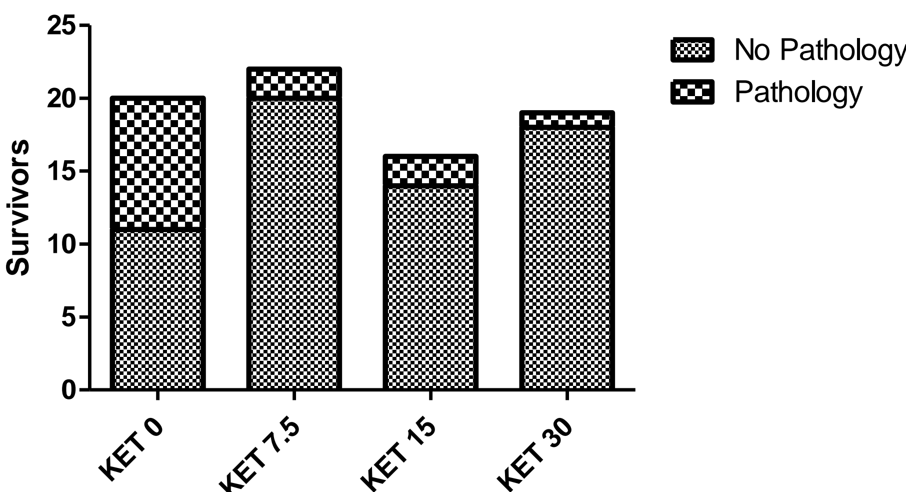


Figure 5. A: Incidence of neuropathology as a function of AS dose. A significantly greater proportion of animals in the 1 mg/kg AS dose condition displayed neuropathology than in the 3 mg/kg AS dose condition. B: There were a significantly greater proportion of animals in the no ketamine group that displayed neuropathology than in the 7.5 mg/kg or 30 mg/kg ketamine groups.

Figure 6

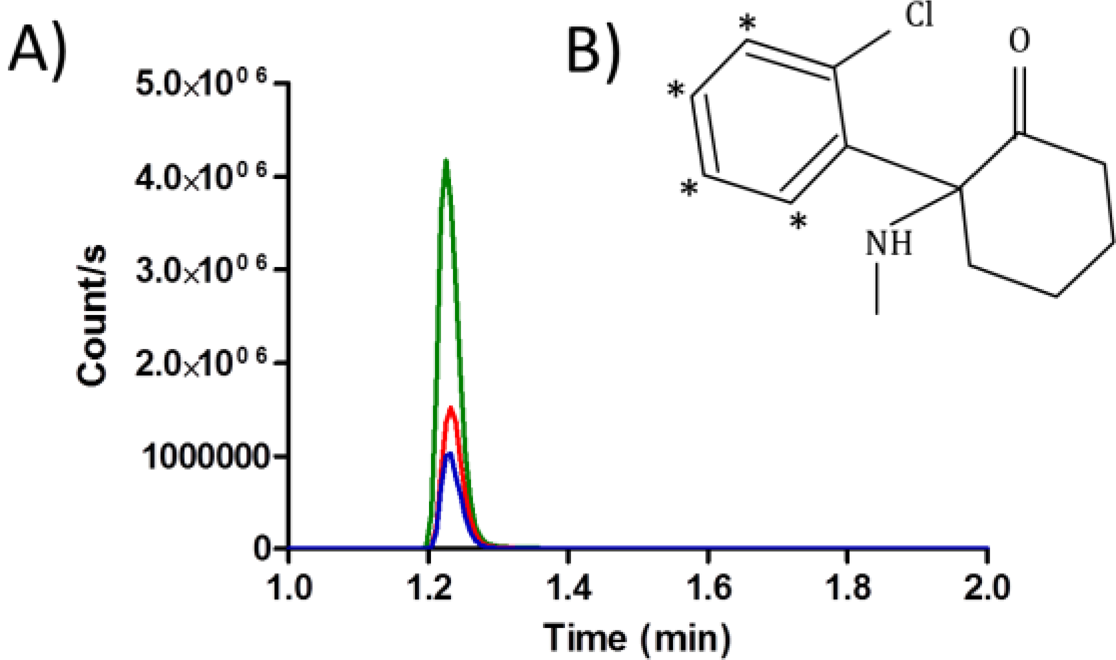

A)
B)
5.0×10⁰ ⁶
4.0×10⁰ ⁶
3.0×10⁰ ⁶
2.0×10⁰ ⁶
1000000
0
Count/s
1.0
1.2
1.4
1.6
1.8
2.0
Time (min)
Cl
O
NH
*
*
*
*

Figure 6. A) LC-MS/MS graphic representation of the ketamine transitions: 238.3Da to 125Da (green), 238.3Da to 179.2Da (red) and 242.3Da to 129Da (blue) with B) Structure of ketamine. Asterisks indicate deuterium labeled sites.

Figure 7

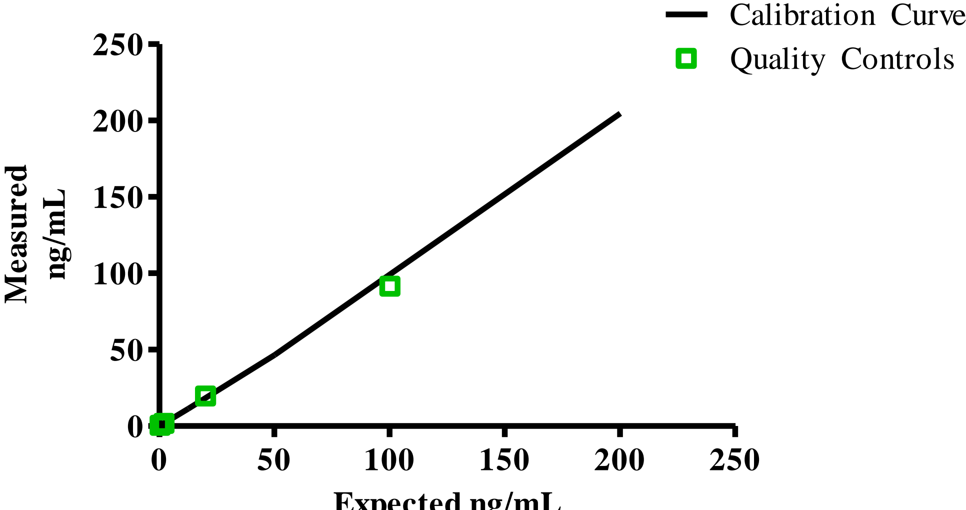


Figure 7. Calibration curves and QCs were prepared in duplicate and analyzed in triplicate over a 1-month period. A total of eight calibration curves were generated. These data represent mean calculated concentrations for all calibrators (line) and QCs (boxes).

Figure 8.

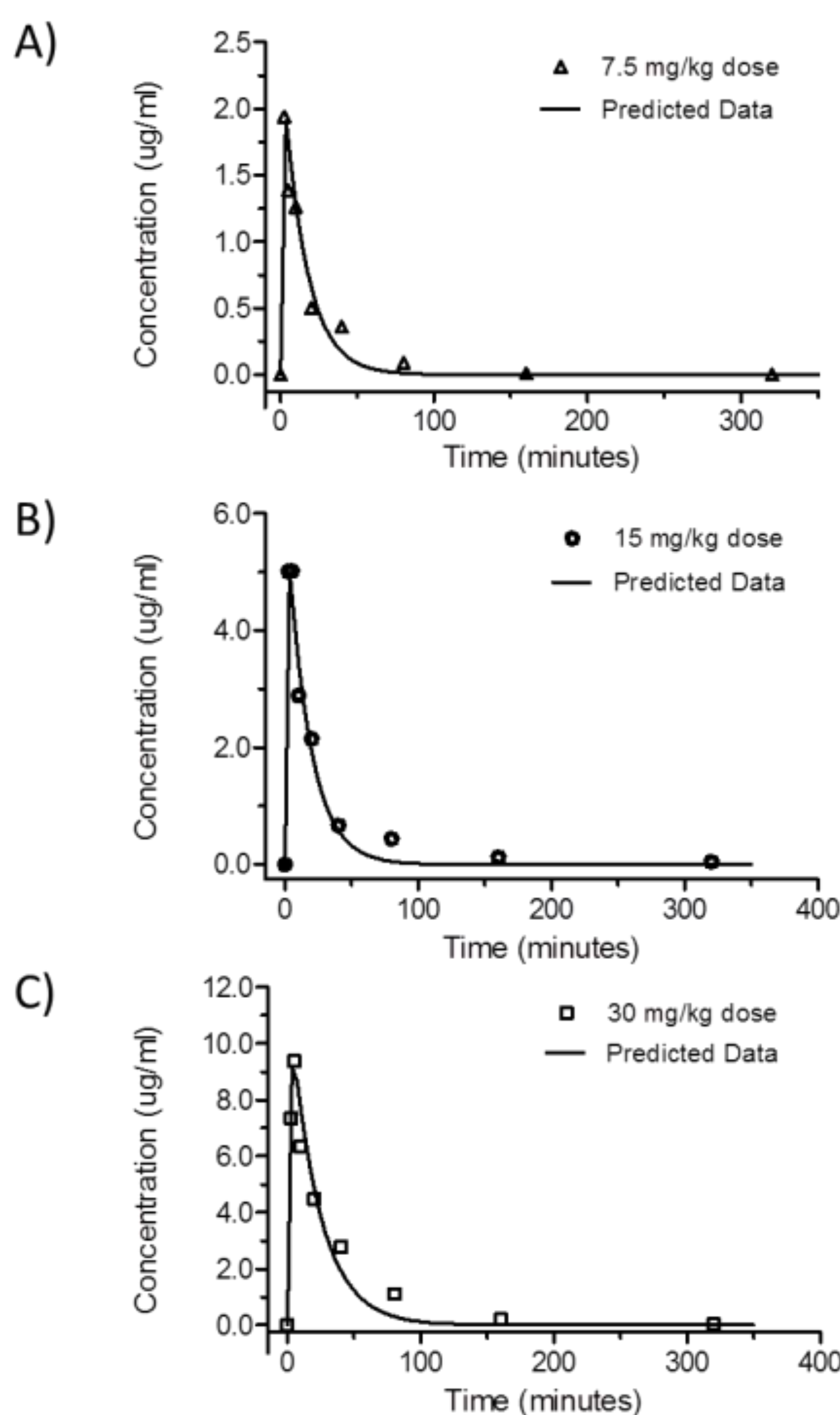
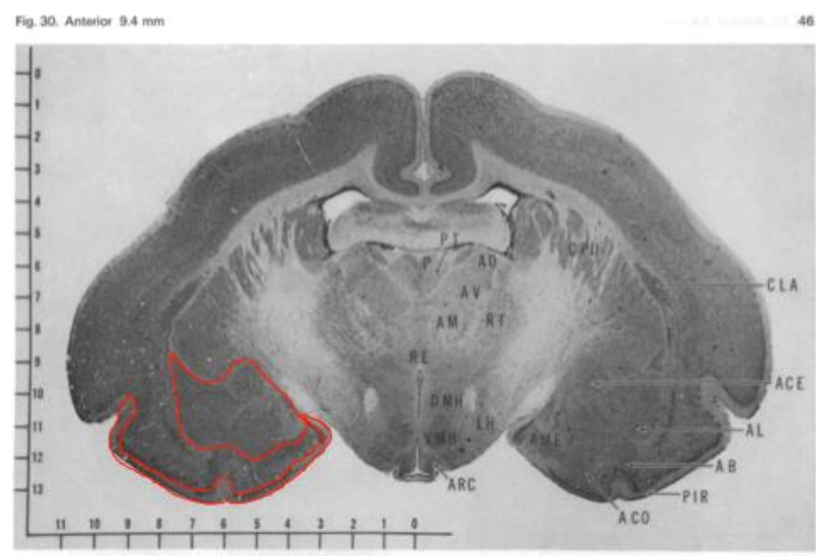


Figure 8. Mean concentration of ketamine at A) 7.5mg/kg B) 15mg/kg and C) 30mg/kg in guinea pig plasma after intramuscular injection compared to model-predicted curves.

Figure 9

A



B

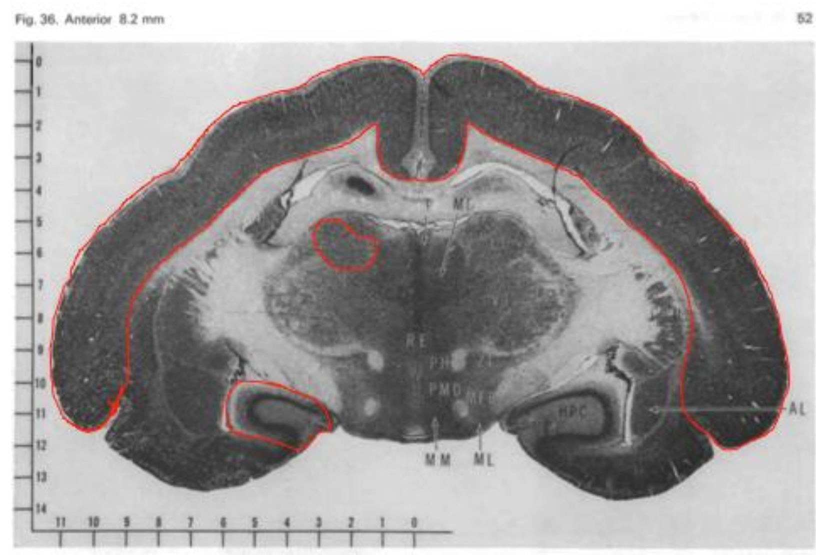


Figure 9. Regions of interest are outlined with red lines. Photographs from an atlas of the guinea pig brain stained with Thionin and Weil technique.

(A) Amygdala (ACE, AL, A CO) and Piriform Cortex (PIR) surrounded with red lines on the contra lateral side of the labels from the authors of the atlas.

(B) Thalamus (LT), Hippocampus (HPC) and cerebral cortex surrounded with red lines on the contra lateral side of the labels from the authors of the atlas.

Figure 10

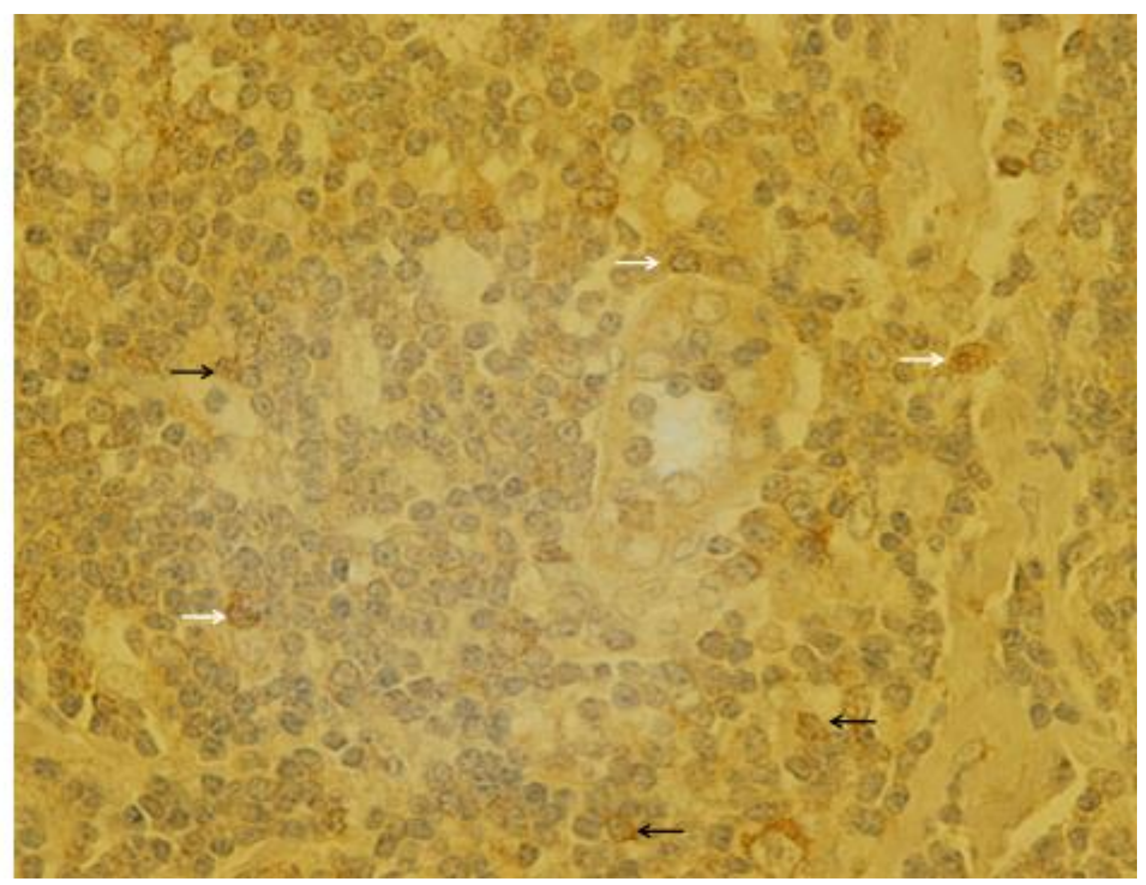


Figure 10. Rat tonsillar tissue used as a positive control for the DAB/Hematoxylin staining technique. Black arrows point to representative cells displaying counter stain and characteristic signs of apoptosis. White arrows represent cell that display counterstain yet do not display the characteristic signs of apoptosis.

Figure 11

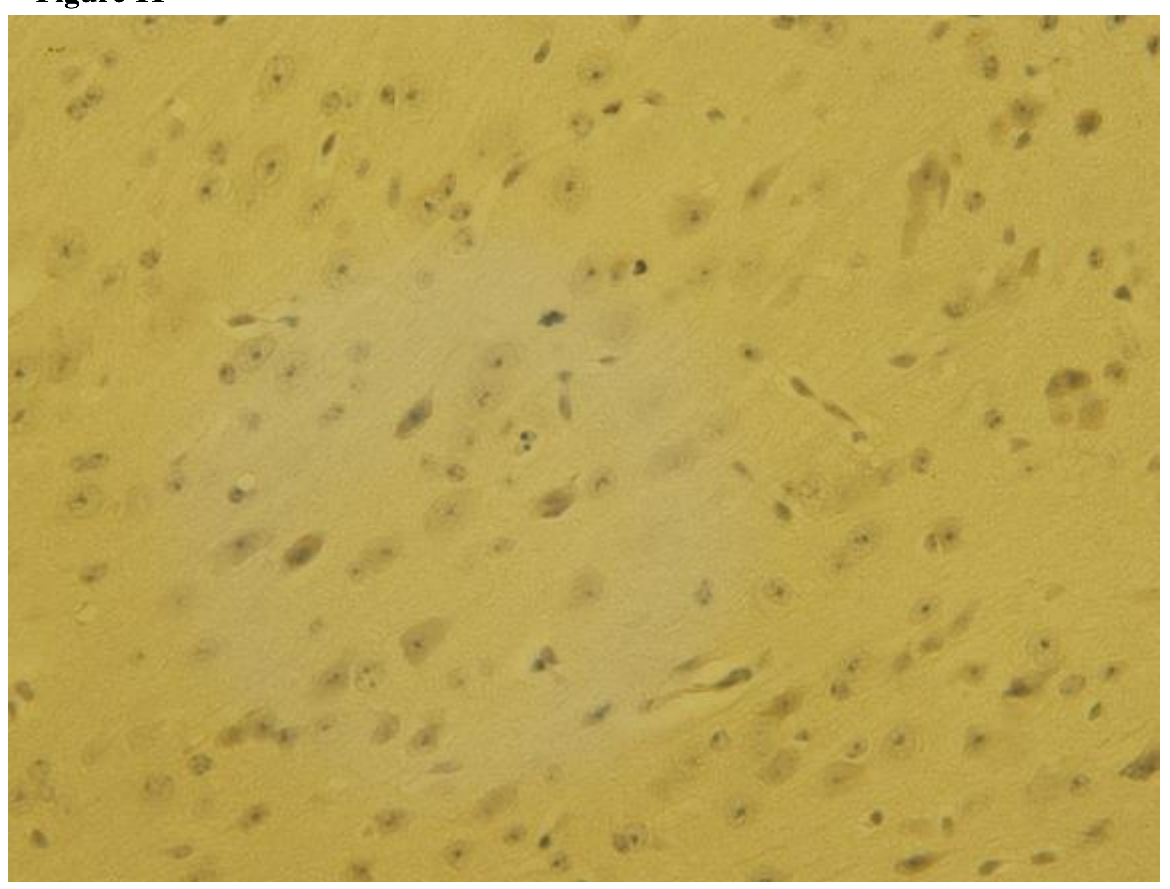


Figure 11. 20x view of the amygdala from an animal that received 60mg ketamine and no agent. DAB counterstained with hematoxylin. The pathology score for this animal was 0.

Figure 12

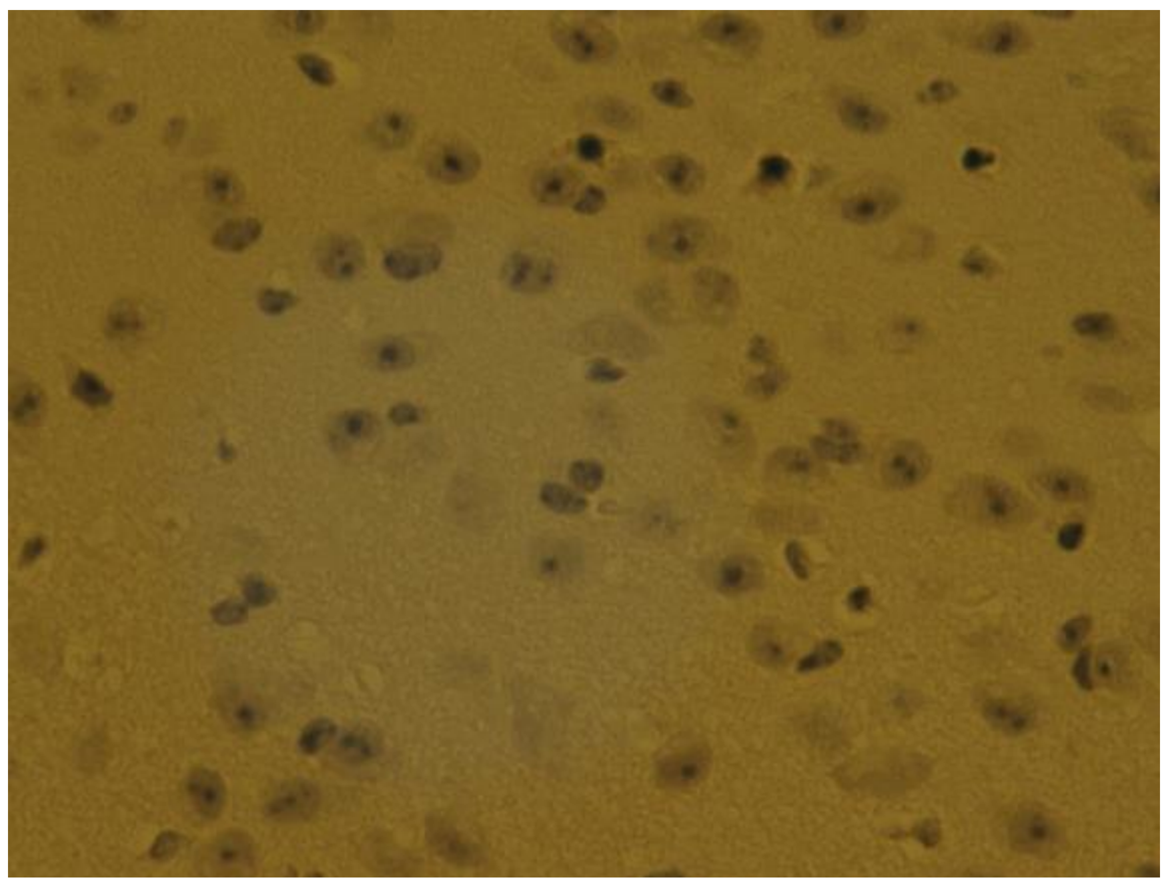


Figure 12. 40x view of the amygdala from an animal that received no ketamine yet received agent. This was the animal with the most pathology noted by the pathologist (10/20) and the amygdala scored the highest on this animal (3/5). Activated caspase-3 and hematoxylin were used to stain.

Table 1

	4-Hour Time			24-Hour Time		
AS Dose (mg/kg)	0.5	1.0	3.0	0.5	1.0	3.0
Alive	8	12	12	2	10	9
N OFF	4	4	9	2	4	8
Percentage OFF	50	33	75	100	40	89

Table 1. Survival and seizure control at the 4-hour and 24-hour time by AS dose group.

Table 2

Actual Concentration (ng/mL)	Mean Calculated Concentration (ng/mL)	Precision (Percent)	Accuracy (Percent)
100	98.2	10.1	-1.82
20	20.5	8.94	2.41
2.0	1.76	12.5	-12.1
0.4	0.43	9.98	7.43

Table 2. Inter-day quality control samples for ketamine-spiked guinea pig plasma were prepared in duplicate and analyzed in triplicate.
Six replicates (calibrators and QCs) were prepared on six non-consecutive days. Each sample was run on the same day it was prepared. Mean calculated concentration (n=6); %CV = percent coefficient of variation (SD/Mean x 100); % error = ((calculated concentration – actual concentration) / actual concentration) x 100.

Table 3

Actual Concentration (ng/mL)	Mean Calculated Concentration (ng/mL)	Precision (Percent)	Accuracy (Percent)
100	**91.5**	**13.6**	**-8.52**
20	**19.6**	**8.29**	**-1.82**
2	**1.79**	**10.4**	**-10.7**
0.4	**0.44**	**11.0**	**8.90**

Table 3. Intra-day quality control samples for ketamine-spiked guinea pig plasma were prepared in duplicate and analyzed in triplicate.
Six replicates were prepared and analyzed on the same day. The concentrations used were the same as the inter-day samples. Mean calculated concentration (n=6); %CV = percent coefficient of variation (SD/Mean x 100); % error = ((calculated concentration – actual concentration) / actual concentration) x 100.

Table 4

Pharmacokinetic Parameter Estimate	30.0 mg/kg	15.0 mg/kg	7.5 mg/kg
Volume of Distribution (l/kg)	2.65	2.51	3.19
Area Under the Curve (ug · min/ml)	246.11	100.32	34.98
T ½ - Elimination (min)	15.07	11.65	10.34
T_{max} (min)	4.33	2.8	2.9
C_{max} (ug/ml)	9.26	5.05	1.93

Table 4: Pharmacokinetic parameter estimates for the three doses of ketamine used in this study. Mean plasma time-concentration data were fit to a one-compartment model with first-order absorption and elimination using WinNonlin (Version 1.5, Scientific Consulting, Inc. Cary, NC) non-linear regression software.

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