5-3-2019

Efficacy of Novel Pyridinium Oximes against Two Organophosphates in Female Sprague Dawley Rats

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By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Environmental Toxicology
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2019

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Anticholinesterase organophosphate (OP) compounds were developed as insecticides and are also used as nerve agents in chemical warfare. Treatment against acute OP toxicity includes oximes which reactivate phosphorylated acetylcholinesterase (AChE) restoring enzymatic activity. The oxime currently approved for use in the U.S., pralidoxime (2-PAM), has limited efficacy penetrating the blood-brain barrier. Our laboratory has developed novel substituted phenoxyalkyl pyridinium oximes (US Patent 9,227,937) designed to more effectively penetrate the central nervous system. This research investigated any differences in oxime reactivation among four age/sex groups and also survivability in adult female Sprague Dawley rats challenged with a lethal dose of OP. Initially in *in vitro* experiments, paraoxon (PXN) and a nerve agent (sarin) surrogate, 4-nitrophenyl isopropyl methylphosphonate (NIMP), were incubated with pooled rat brain homogenate from four sex/age groups: adult male or female, and 12-day old male or female rats. Reactivation was performed utilizing 2-PAM or one of three novel oximes (15, 20, or 55), alone or in combination, and AChE activity was measured in a spectrophotometric assay. Overall, the oximes were more effective reactivating

inhibition from PXN than from NIMP. Out of all the oximes tested, 2-PAM showed the greatest reactivation percentages. Of the novel oximes, 15 and 20 displayed the highest reactivation against PXN and NIMP, respectively. No statistical difference was detected in reactivation for any oxime among the age/sex groups. For the *in vivo* study, female adult Sprague-Dawley rats were treated with LD_{99} dosages of NIMP or PXN. After development of seizure-like behavior, atropine and one of four oximes, 2-PAM, novel oxime 15, 20, or 55 or Multisol vehicle was administered. Animals were closely monitored for signs of cholinergic toxicity and 24-hour survivability. Against PXN, novel oximes 15 and 55 demonstrated an improved odds ratio of 6.5 and 3.1, respectively, over 2-PAM. The most effective oxime against NIMP was novel oxime 20 demonstrating an odds ratio of 3.2 over treatment with 2-PAM. These data indicate that the novel pyridinium oximes are equally efficacious reactivators in adult and juvenile rats of both sexes and enhance survivability against lethal-level OP toxicity as compared to 2-PAM in adult female rats.

DEDICATION

This work is dedicated to my family. To my wife, Tiffany, the love and support you have shown has helped me persevere in the face of adversity. Thank you for always being there for me, especially during times of stress and uncertainty. To my son, Dylan, you are the inspiration which drives me to achieve. Regardless of the path you choose in life, I hope that you may look upon this dissertation as a testament that hard work will pay back dividends towards your future. I love you both very much.

ACKNOWLEDGMENTS

First, I would like to acknowledge my mentor and major professor, Dr. Janice Chambers. She accepted me into her program despite the unique requirements which come with an active-duty member of the military and proceeded to provide unwavering support in the completion of my goals. Additionally, I would like to acknowledge the other members of my committee: Dr. Russell Carr, Dr. Allen Crow, Dr. Jonas King, and Dr. Matthew Ross. Always available for consultation despite demanding schedules, their advice and encouragement has been greatly appreciated. It has been a privilege to train in the Center of Environmental Health Sciences alongside such a great group of faculty, staff, and students. Among them, Dr. Edward Meek and Shane Bennett, whom I relied upon for completion of major portions of this research. Finally, I would like to thank the College of Veterinary Medicine and Mississippi State University which provide great opportunities to veterans and current members of the military.

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2-PAM	*Pralidoxime*
AChE	*Acetylcholinesterase*
ACh	*Acetylcholine*
ATCh	*Acetylthiocholine*
AMPA	*α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid*
BChE	*Butyrylcholinesterase*
BTCh	*Butyrylthiocholine*
DMSO	*Dimethyl sulfoxide*
DTNB	*5,5'-dithio bis(2-nitrobenzoic acid)*
EC_{25}	*Effective concentration at which twenty-five percent response is achieved*
EtOH	*Ethanol*
FC	*Final Concentration*
HCl	*Hydrogen chloride*
i.m.	*Intramuscular*
LD_{50}	*Lethal dose at which fifty percent of organisms expire*
LD_{99}	*Lethal dose at which ninety-nine percent of organisms expire*
NIH	*National Institutes of Health*
NIMP	*4-nitrophenyl isopropyl methylphosphonate*

OP	*Organophosphate*
P450	*Cytochrome P450*
PBPK	*Physiologically Based Pharmacokinetic*
PND	*Postnatal Day*
PXN	*Paraoxon*
s.c.	*Subcutaneous*
SDS	*Sodium dodecyl sulfate*
TV	*Total Volume*
WHO	*World Health Organization*

CHAPTER I

INTRODUCTION

Significance of Organophosphates

Organophosphate (OP) chemicals encompass a large group of compounds with diverse properties and molecular structures. Although OPs are most commonly associated with insecticides, they can also be found in oils, lubricants, plasticizers and flame retardants (Winder and Balouet, 2002). Common to all OPs is the pentavalent phosphorus atom which can form three, single covalent bonds to a variety of constituents, and a fourth, double bond typically linked to either a sulfur or oxygen atom. It is this phosphorus atom, often forming a phosphate or phosphorothionate group within the center of the molecule, which gives these compounds their name (Mileson *et al*., 1998).

Infamously, nerve agents are OP compounds specifically designed for chemical warfare. Nerve agents were initially developed in Germany derived from insecticides in the 1930s; however, because they can be easily absorbed through the skin and/or inhaled, when combined with their potent anticholinesterase characteristics, it made them too dangerous for agricultural use. Almost immediately after their discovery, these same characteristics made them ideal for weaponization by the Nazi party. The first group of nerve agents developed were the G-series agents. Designated "G" representing the Germans who developed them, and a second letter identifies the specific compound: GA (tabun), GB (sarin), GD (soman), and GF (cyclosarin). Additional series of nerve agents

have been developed since; the V-series of agents include the compounds VE, VG, VM and VX (Chambers and Carr, 2002; Rotenberg and Newmark, 2003; Johnson *et al.*, 2015). Although never used during World War II, history is replete with examples of OP utilization as weapons in military operations or terroristic acts. The government of Iraq used nerve agents in a conflict against the Iranian military from 1981 to 1988. During the same time period, Iraqi forces dropped bombs containing sarin and tabun on the Kurdish village of Halabja killing thousands of civilians (BBC, 2013; Johnson *et al.*, 2015). A cult in Japan, Aum Shinrikyo, deployed sarin gas in 2 separate attacks in 1994 and 1995 (Rotenberg and Newmark, 2003; Johnson *et al.*, 2015). More recently, the Syrian regime was implicated in the usage of sarin nerve agent in 2013 and 2017 during its civil war affecting civilian populations in rebel-held territory (Associated Press, 2017).

Since their discovery, the threat of chemical attack with weaponized OP compounds has not diminished with the passage of time; however, their potent neurotoxicity alone is not the only characteristic which drives their utilization. Chemical precursors used in the creation of nerve agents are easily procured, and the procedure for their synthesis can be accomplished through relatively simple means. Often colorless and odorless, small quantities of nerve agent are easily concealed and can have devastating effects if deployed properly (Johnson *et al.*, 2015; Musser, 2001; Rotenberg and Newmark, 2003). These are characteristics which may appeal to a terrorist organization with limited resources who are looking to make a large impact without regard for international retaliation. Even if access to the necessary precursors for production of a nerve agent such as sarin are unavailable or restricted, other organophosphorus

insecticides could be repurposed to such ends (Bajgar *et al.*, 2015). While the usage of organophosphorus insecticides in the United States has been decreasing since the passage of the Food Quality Protection Act in 1996, they still account for approximately 33% of all insecticides used (Atwood and Paisley-Jones, 2017). Worldwide usage is prevalent, especially in underdeveloped nations which rely on the inexpensive insecticides in agriculture (Soltaninejad and Shadnia, 2014). These organophosphorus insecticides, originally intended for agricultural use, represent sources from which terrorist groups could obtain compounds with relatively high toxicity for malevolent purposes (Bajgar *et al.*, 2015). They also represent a source of occupational hazard from accidental exposure and a disturbing trend is occurring in agricultural, socioeconomically depressed areas across Africa and South Asia where OP insecticides are utilized by individuals who wish to commit suicide (Adinew *et al*. 2017; Eddleston *et al*., 2009; Güllü *et al*., 2013; Pawar *et al*., 2006; Singh *et al*., 1995).

Mechanism of Action and Toxic Effects

Many OP compounds are not inherently potent anticholinesterase agents, but rather, the parent molecule must be bioactivated into their more active 'oxon' species in order to exert their toxic effects (Neal, 1980). It is widely known, that the cytochrome P450 monooxygenase (P450) system is responsible for this bioactivation (Chambers *et al*., 2010). For instance, in 1951, the OP insecticide parathion was known to be a potent cholinesterase inhibitor *in vivo*, but it had very minimal effects when *in vitro* inhibition tests were attempted. It was later discovered that in the presence of liver tissue, anticholinesterase activity was demonstrated. The postulation was made that an enzyme

in the liver tissue, which we now know is P450 and to a lesser extent, flavin-containing monooxygenase, transform parathion into a capable inhibitor of cholinesterase. This was later confirmed in 1952 when paraoxon was isolated from liver tissue in an in vivo test and matched with the results of the previous in vitro study (Gage, 1952).

Phosphorothionate insecticides which have a pentavalent phosphorus atom with one coordinate covalent bond attached to a sulfur atom must be bioactivated by P450. The P450-mediated desulfuration reaction facilitates an attack of the phosphorus atom by an oxygen atom, ultimately releasing sulfur (Neal, 1980). It is this substitution which forms the more potent oxon species which is then reactive towards non-target, endogenous serine esterases as well as acetylcholinesterase (AChE). Some phosphorothionate insecticides requiring bioactivation include parathion, malathion, chlorpyrifos, and phorate, while those OP compounds which do not require bioactivation from their parent molecule include dichlorvos and the nerve agents soman, sarin, tabun, and VX (Chambers *et al*., 2010).

As mentioned previously, OP compounds are best known for their acute toxicological effects as cholinesterase enzyme inhibitors. The mechanism of action of OPs lies in their ability to phosphorylate the serine hydroxyl moiety within the active site of esterases. Acute toxicity of an OP compound is often characterized by measuring the dosage at which 50% of the experimental subjects have a lethal response or outcome, otherwise known as LD_{50}. The LD_{50} of an OP compound depends upon the cholinesterase inhibiting potency, rate of aging, and lipophilicity of the OP molecule.

Higher lipophilicity means that an OP can penetrate cellular barriers easier and achieve a greater volume of distribution (Misik *et al*., 2015).

In a normal functioning nervous system, the action and degradation of the neurotransmitter acetylcholine (ACh) is extremely quick, occurring within a few milliseconds. Excitation of a neuron causes vesicles storing ACh to fuse with the pre-synaptic membrane. Upon releasing into the synaptic cleft, the hydrophilic ACh quickly diffuses the short 20 nm span to the post-synaptic membrane where binding occurs to its corresponding receptor. Almost instantly after binding, ACh is hydrolyzed by AChE into acetic acid and choline, thereby terminating the excitatory stimulus. Unlike other neurotransmitters which have a variety of mechanisms to mitigate their signal, ACh depends almost entirely upon the quick action of AChE which underscores why inhibitors of this enzyme are so toxicologically potent. Disruption of AChE by an inhibitor such as an OP or carbamate allows the neurotransmitter to accumulate and excessively prolong the stimulatory signal with no immediate remedy for termination (Kobayashi and Suzuki, 2010).

By inhibiting AChE, the target enzyme for OPs, ACh can remain in cholinergic synapses generating persistent excitatory stimuli to an organism's nervous system. A range of sympathetic and parasympathetic nervous system symptoms in mammals can occur including salivation, lacrimation, urination, and defecation, in a syndrome termed 'SLUD', as well as muscle spasms and tremors (Chambers *et al.*, 2001; Chambers and Carr, 2002). Overstimulation of the central nervous system due to increased levels of ACh within the hippocampus of the brain activate muscarinic and nicotinic receptors

which stimulate the release of the excitatory neurotransmitter glutamate inducing convulsions or seizure activity (Kozhemyakin *et al*., 2010; Todorovic *et al*., 2012). Ultimately, the cause of death in cases of acute OP toxicity is respiratory failure due to paralysis of muscles important for inspiration (Costa, 2008).

Oxime Therapy

There are an estimated three million cases of OP poisoning worldwide each year with approximately 200,000 occurring in developing countries (Adinew *et al*., 2017). Treatment for OP poisoning includes a regimen of different drugs. Sedatives such as a benzodiazepine like diazepam are employed for treatment of seizures associated with central nervous system poisoning. A muscarinic antagonist, such as atropine, can block or dampen some of the peripheral nervous system symptoms (Kassa, 2002). Atropine is considered a staple of OP poisoning treatment and given at high dosages, titrated by monitoring secretions and pupil size (Karakus *et al*., 2014; Singh *et al*., 1995). Finally, an oxime, such as pralidoxime (2-PAM), can be utilized to reactivate AChE phosphorylated by an OP compound; however, while treatment with atropine is well accepted, oxime therapy has been somewhat more controversial (Eddleston *et al*., 2006; Thiermann *et al*., 2011).

Since the discovery of oximes in 1955, only a handful have seen clinical use: 2-PAM, trimedoxime, obidoxime, and HI-6 (Childs *et al*., 1955). These early oximes represent the entire current standard of oxime therapy out of thousands created in the laboratory since the 1950s and 1960s (Worek *et al*., 2016). Oximes all contain similarities within their molecular structure which are responsible for their mechanism of

action. A quaternary nitrogen pyridinium ring serves to position the molecule within the active site of AChE allowing the oxime moiety (RCH = NOH) to conduct a nucleophilic attack on the serine hydroxyl group phosphorylated by the OP thereby transferring the phosphoryl group to the oxime. This transfer restores the serine hydroxyl group within the AChE active site, reactivating its enzymatic potential.

When an OP compound phosphorylates a serine esterase, such as AChE or butyrylcholinesterase, the reaction is often referred to as irreversible inhibition of the enzyme. Esterases however, can undergo spontaneous reactivation through hydrolytic cleavage of the phosphoryl moiety returning the active site of the enzyme to its physiologic normal state although this reaction is very slow. Another reaction can also occur, instead of hydrolysis, a dealkylation can occur leaving a monoalkoxy phosphate bound to the active site in a process termed "aging". When an enzyme undergoes aging, the inhibition is permanent and resists reactivation even in the presence of an oxime antidote (Kobayashi and Suzuki, 2010; Kassa, 2002).

This relationship between reactivation and aging varies among OP compounds and which serine esterase they are phosphorylating. For example, dimethyl OPs age more rapidly than diethyl OPs with aging half-lives of 3.7 hours and 31 hours, respectively (Milatovic and Jokanovic, 2009). The nerve agent GD, or soman, rapidly ages with 50% of the inhibited enzyme aging within 1.3 minutes as measured in red blood cell cholinesterase (Watson *et al*., 2009). This has important therapeutic implications with regard to the timing for administration of an oxime antidote after an exposure event as the structure of the OP compound is an important determinant for

oxime effectiveness and the patient's prognosis. It was demonstrated that reactivation of dimethylphosphorylated AChE with 2-PAM, as measured in RBCs, is less effective than it would be otherwise in a patient exposed to a diethyl OP compound (Hansen and Wilson, 1999). Clinical studies which do not control for the exposure time or OP compound to which a patient is exposed may be skewing results of oxime effectiveness as 2-PAM would not be expected to work for those patients exposed to a dimethyl OP compound and receiving oxime therapy after aging has already occurred (Eddleston *et al*., 2006).

The rate of aging by which phosphorylated AChE becomes resistant to nucleophilic attack by an oxime is just one of a number of factors influencing the effectiveness of oximes. Reactivation depends upon subtle differences in oxime molecular structure which influence their affinity for an OP-inhibited enzyme based on factors such as spatial orientation and steric hindrance within the active site. Additional factors affecting reactivation include inhibitory affinity of the oxime itself towards AChE, pharmacokinetics of oxime therapy, as well as inhibitory potency and concentration of the OP (Kassa, 2002; Milatovic and Jokanovic, 2009; Worek *et al*., 2016). These intricacies in the interaction between oxime and OP-phosphorylated enzyme in a real-world clinical environment with varying degrees of exposure, different OPs, timing of the initiation of treatment and available clinical resources have led to confounding studies (Eddleston *et al*., 2006).

The necessity of therapy with oximes in acute OP poisoning has been questioned in lieu of monotherapy with atropine in combination with supportive treatment such as

mechanical ventilation and intravenous fluids (Singh *et al*., 1995). A meta-analytic study analyzing over 3,000 articles reviewing the clinical treatment of OP poisoning indicated that usage of oximes had a negligible effect on the outcome of the patient. Two different oximes, obidoxime or 2-PAM, were administered in these studies and were actually associated with possible harm as those patients demonstrated an increased need for intensive care when oxime therapy was given (Peter *et al*., 2006). Pawar *et al*., compared a higher dose regimen 2-PAM treatment (1g/hour) to their standard lower dose (1g/4 hour) after a 2g loading dose. This study demonstrated that the higher 2-PAM dose group required less atropine and decreased the need and duration of ventilation in cases of moderate to severe OP insecticides poisoning (Pawar *et al*., 2006). Even partial reactivation of AChE by an oxime can significantly reduce the amount of atropine needed (Thiermann *et al*., 2011). Conversely, Eddleston *et al*. (2009) performed a study that randomized treatment of 235 patients with OP self-poisoning administering either placebo or 2-PAM using the World Health Organization's (WHO) recommended regimen (2 g loading dose over 20 minutes followed by 7 days of 0.5 g/hour constant infusion or until atropine was not needed for 12-24 hours or death occurred). The main conclusion of this trial was that there was no apparent benefit with the administration of 2-PAM versus placebo. Furthermore, there was a 69% increase in mortality associated with 2-PAM treatment. This study contrasts with Pawar *et al*. (2006) which showed high dose 2-PAM dosing beneficial to a patient's outcome. Key differences existing between these two studies include shorter term oxime administration and a higher level of intensive care available used in the Pawar *et al.* (2006) trial. It is possible that the WHO's

recommended oxime treatment guidelines include dosages that are too high or too long in duration. RBC cholinesterase activity in survivors was 25% of normal indicating that high amounts of AChE reactivation, and high doses of oxime by extension, may not be needed to improve survivability (Eddleston *et al*., 2009).

High dose oxime therapy may cause increased mortality, while if used early after OP exposure low dose oxime therapy may be beneficial (Peter *et al*., 2000). One retrospective study of 179 medical records indicated that early treatment with 2-PAM was associated with lower mortality in OP-poisoned patients which increased as time to administration of oxime therapy increased (Güllü *et al*., 2013). Droste *et al*. (2016) utilized physiologically based pharmacokinetic (PBPK) modeling to estimate treatment of inhalational sarin exposure with atropine and 2-PAM. The model indicated that at a high, incapacitating dose of sarin, oxime therapy is a beneficial, life-saving treatment while oxime therapy in a victim displaying mild symptoms is not effective. In those cases of mild to moderate exposure, supportive treatment with a benzodiazepine and intravenous fluids as determined by a physician was recommended (Droste *et al*., 2016). As mentioned before, multiple factors are responsible for apparent effectiveness of oxime treatment which make evaluation of their usage in clinical therapy problematic. These inconsistencies in the evaluation of oxime therapy can be attributed, in many cases, to limitations of the experimental design within the studies themselves; however, there are limitations associated with the clinically approved oxime in the U.S., 2-PAM (Eddleston *et al*., 2006).

Currently, there does not exist a single, broad-spectrum oxime which can be considered effective against both nerve agents and OP insecticides. For example, 2-PAM is an ineffective reactivator of AChE when inhibited by the nerve agent tabun, presumably because of the nerve agent's resistance to nucleophilic attack once bound within the active site, but is considered an effective reactivator for OP insecticide poisoning. Additionally, 2-PAM has difficulty penetrating the blood brain barrier due to its charged structure (Cabal *et al*., 2004; Kassa, 2002). It is estimated that approximately 10% of the 2-PAM dose given penetrates the blood brain barrier in a concentration-dependent manner; however, researchers are unsure if this concentration is sufficient for reactivation of OP-inhibited brain cholinesterase (Sakurada *et al*., 2003). The position of the oxime group within the pyridinium ring can be an important indicator for efficiency in crossing the blood-brain barrier. The oxime group exists in the ortho configuration or position two on the pyridinium ring within 2-PAM. Using a modified high-performance liquid chromatography method to simulate blood-brain penetration, researchers determined that the most efficient configuration of a monoquaternary oxime for passage into the brain is the para configuration with the oxime group attached to position four on the pyridinium ring which linearizes the molecule minimizing the polar surface area (Karasova *et al*., 2010).

Our laboratory has developed and patented a number of novel oximes which are designed to cross the blood-brain barrier to attenuate OP-associated inhibition within the central nervous system (Chambers *et al*., 2013). It has been demonstrated that 24-hour survivability with a lethal dose of an OP compound is increased in adult male rats when

treated with these novel oximes versus 2-PAM (Chambers *et al*., 2016). These new antidotes offer promising improvement over the current standard of oxime therapy; however, more information is needed to validate their efficacy in both sexes and in juveniles for continued development towards usage as clinical drugs.

Age and Sex-related Differences in Toxicity

Significant differences in xenobiotic metabolism have been reported between species, sex, and age which may impact the way a novel oxime is administered in cases of acute toxicity. For example, female rats have been found to be more sensitive to pesticides than their male counterparts, while juvenile rats display more varying sensitivity compared to adults. Weanling rats can be less sensitive, displaying higher LD_{50} concentrations to certain chemicals, while adults have higher toxicity towards the same compound and vice versa (Gaines and Linder, 1986). The mechanisms for these differences are not entirely explained by age/sex-related differences in xenobiotic metabolism, but can also depend on a variety of pharmacokinetic factors such as route of exposure, membrane permeability, distribution and rate of excretion (Kassa, 2002). In fact, more recent studies have demonstrated sex-hormone related differences in OP lethality which must be considered for future studies in this field (Pittel *et al*., 2018; Smith *et al*., 2015).

Often, for the sake of simplicity and to minimize variation in experimental design, many studies have utilized adult male animal models. Unfortunately, the imprecise nature in which nerve agents spread after employed as terrorist or area denial weapons lead to the indiscriminate exposure of all in the affected contamination zone including

women and children (Johnson *et al*., 2015; Watson *et al*., 2009). Additionally, initial animal studies which are disproportionately focused on males may lead to confounding data and underestimate the influence of age and sex factors on health outcomes. The National Institutes of Health (NIH) is now expecting that all research consider sex an important biological variable in vertebrate and human studies. Sex should be factored in to experimental design unless a strong scientific justification indicates otherwise (NIH, 2015).

Typically, adult female rats have been considered to be more sensitive to the lethal effects of a nerve agent such as sarin (24 hour LD_{50} = 67 μg/kg i.m.) than their male counterparts (24 hour LD_{50} = 88 μg/kg i.m.); however, this is not always the case (Pittel *et al*., 2018). Significantly higher 24 hour sarin LD_{50} (125 μg/kg s.c.) was observed in female rats while in proestrus phase of the estrus cycle versus female rats in estrus phase or those with ovaries surgically removed. Although not statistically significant, the LD_{50} of female rats in proestrus was higher than male rats (24 hour LD_{50} = 116 μg/kg s.c.) in the same experiment (Smith *et al*., 2015). In order to test new therapeutics using female animal models, it is incumbent on the researcher to know at what point in the estrus cycle the animal is in because increased levels of sex hormone such as estrogen may affect the lethality of a given dose of OP compound and thereby skew results for testing treatment effectiveness (Wright *et al*., 2016b). Furthermore, age and sex-related differences can be seen between different nerve agents and there is poor correlation between LD_{50} and cholinesterase inhibition suggesting that inactivation of

AChE cannot be the sole mechanism to explain the lethal effects of OP intoxication (Fawcett *et al*., 2009).

One of the ways hypothesized to account for these observations is that estrogen is thought to have a protective effect on astrocytes which help maintain the permeability characteristics of the blood-brain barrier (Pittel *et al*., 2018). Expression of inflammatory cytokines such as IL-6, TNF, and IL-1β in response to OP exposure within the brain increased in male astrocytes, but not in female astrocytes suggesting that female astrocytes are better protected from production of reactive oxygen species associated with inflammatory responses (Astiz *et al*., 2014). Additionally, increased concentrations of MCP-1, a monocyte chemoattractant cytokine, found in the brains of female rats indicate that this higher concentration recruits monocytes from the peripheral circulation in order to better maintain the blood-brain barrier in females after sarin exposure as opposed to males. Overall, females demonstrated better protection against brain damage induced by sarin exposure but exhibited greater overall sensitivity (lower LD_{50}) than their male counterparts when controlling for the estrus cycle (Pittel *et al*., 2018). Treatment of male rats with estrogenic compounds may induce P450 enzymes, the effect of which has been demonstrated to increase the parathion LD_{50} by 150% (Robinson *et al*., 1978). In another study, pretreatment with the estrogenic compound diethylstilbestrol enhanced the reactivating capability of the oxime HI-6 in castrated male rats after exposure to nerve agents (Lundy *et al*., 1989).

Age-related factors can have similar implications for animal studies. Carboxylesterases are important scavenger enzymes which stoichiometrically eliminate

OP compounds by acting as targets for phosphorylation. Paraoxonases are enzymes which reside on high density lipoproteins that are involved in the prevention of atherosclerosis via hydrolysis of cholesterol esters but also have demonstrated the ability to detoxify OP compounds through the same mechanism (Harel *et al*., 2004). The activity of protective enzymes in rats has been shown to dramatically increase with maturation after puberty (Atterberry *et al*., 1997). Age-related differences exist for G-series nerve agents, but not for V-series agents as measured by LD_{50}. The V-series agents do not react as well with carboxylesterases as do G-series which may account for this observation (Wright *et al*., 2016b). Additionally, the surge in sex hormones associated with puberty seem to confer a protective effect against the lethality of nerve agents. Pubescent animals have been demonstrated to have higher sarin LD_{50} than prepubescent and adult age groups (Fawcett *et al*., 2009; Wright *et al*., 2016a; Wright *et al*., 2016b). When considering age as a factor in OP lethality, however, the maturational expression of protective enzymes such as carboxylesterase or paraoxonase, may be more toxicologically relevant than rising levels of sex hormones as demonstrated in a separate study which found that younger rats were more susceptible to sarin than adult rats with minimal sex-related differences (Wright *et al*., 2017). Conversely, younger rats have been shown to be more resistant to neuronal damage from acute OP intoxication. Researchers suggest this resistance is due to the under-developed nervous system in juveniles, primarily the lower expression muscarinic receptors, which minimize the over-stimulatory effects of excess ACh thereby preventing the development of brain damaging seizures (Scholl *et al*., 2018).

There exists a need for better countermeasures against OP toxicity as these compounds represent a threat against human health as they continue to be used for legitimate agricultural purposes as well as chemical weapons. The development of improved antidotes, such as novel oximes, hinges on our ability to account for important biological variables such as age and sex. Recent developments in this area of research have pointed to clear age and sex-related differences in the responses to OP exposure. While the science is still evolving with these findings, efforts to control for important variables must be incorporated into experimental design if meaningful evaluation of new treatments is to be successful. To further enhance understanding of OP intoxication with the goal of developing more effective antidotes, new information regarding oxime reactivation of AChE inhibited by OP compounds in mammals is needed.

Research Summary

This research is designed to elucidate the differences, if any, in the efficacy of our novel oximes in females and, to a lesser extent, in juveniles which may be useful for future research in their development as new antidotes. Performance of the novel oximes will be compared against results obtained in male Sprague Dawley rats from previous studies (Chambers *et al*., 2013; Chambers *et al*., 2016). Specifically, this research investigates *in vitro* AChE oxime-mediated reactivation in brain tissue after OP inhibition in male and female rats differing in age and determines *in vivo* novel oxime reactivating efficiency and survival efficacy in the adult female Sprague Dawley rat dosed with either the sarin surrogate, 4-nitrophenyl isopropyl methylphosphonate (NIMP) or paraoxon (PXN), the active metabolite of the insecticide parathion. The *in vitro* work was

performed under the hypothesis that appreciable differences do not exist between sex and age when reactivating OP-inhibited AChE with oximes due to the conserved nature of the enzyme. Additionally, the *in vivo* treatment of adult female rats with novel oximes should have similar results as previous studies with adult males showing increased survival rates and attenuated seizure-like signs when compared to 2-PAM.

The second chapter was dedicated to the *in vitro* work in which differences in novel oxime reactivation were elucidated using pooled rat brain homogenate from 4 groups of Sprague Dawley rats: adult or postnatal day (PND) 70 males and females, and juvenile or PND 12 males and females. Species differences are seen in the inhibitory potential of OPs as well as reactivation by oximes which are believed to be due to small variations in AChE structure (Worek *et al*., 2011). Despite these variations which may exist in the non-catalytic portions of AChE, the catalytic site, responsible for enzymatic action, is almost identical across many species (Moralev *et al*., 2001). Previous studies in rats have shown that cholinergic activity rapidly increases postnatally from being relatively low at 1 week of age to near adult activity levels after 4 weeks of maturation (Coyle and Yamamura, 1976). Correspondingly, AChE activity has been demonstrated to increase with age (Tang *et al*., 1999). Despite these age-related differences in activity, the AChE enzyme is encoded by a single gene in vertebrate organisms and the active site is highly conserved in mammals which share common coding exons and introns of the same size and within the same sites on the gene (Massoulie *et al*., 1993). The target enzyme should be reactivated by oximes in the same proportions regardless of age or sex because of the consistency in functional structure.

The OPs chosen for this work include one that is representative of a nerve agent, NIMP, and PXN which is representative of a prototypical OP insecticide, both of which give an assessment of the broad spectrum capability of our novel oximes. Several surrogate compounds for nerve agents were developed for research purposes (Ohta *et al*., 2006). NIMP phosphorylates AChE with the same moiety as the nerve agent sarin, but is not volatile allowing for safer manipulation when conducting experimental protocol in facilities not certified for nerve agent testing. The concentration in which 50% AChE inhibition is achieved with NIMP is reported to be slightly higher than its nerve agent counterpart as determined in brain tissue of adult male Sprague Dawley rats (Meek *et al*., 2012). Three novel oximes designated oxime 15, 20, and 55 were selected from among many other oximes as leading candidates for further drug development all of which were first synthesized by Dr. Howard Chambers here at Mississippi State University (Meek *et al*., 2012, Chambers *et al*., 2013). Past data from this laboratory have shown that combination oxime therapy can be more effective than a single oxime alone. Combining 2-PAM with novel oximes has yielded higher 24 hour survival rates in OP lethal dosage challenge studies (Chambers *et al*., 2016). Those combination studies were performed with twice the molar equivalent of a single oxime concentration; therefore, this research compares the higher concentration of combination oxime therapy versus an equimolar concentration to help elucidate the efficacy of reactivation for differing levels of oximes in combination.

In chapter 3, the three novel oximes were used to treat adult female rats dosed with a LD_{99} concentration of either NIMP or PXN in a 24-hour survivability challenge

and compared to treatment with 2-PAM. Previous studies investigating the efficacies of the novel oximes were performed on adult male rats. Against NIMP, novel oximes 15 and 20 demonstrated odds ratios of 2.3 and 1.8 compared to 2-PAM while novel oximes 15 and 55 increased PXN survivability 5.7 and 2.3 times greater than 2-PAM, respectively (Chambers *et al*., 2016; unpublished data). Additionally, seizure-like behavior was assessed using a modified Racine scale (Lüttjohann *et al*., 2009). A decrease in the time until cessation of seizure-like behavior by monitoring signs of cholinergic toxicity, as was demonstrated in male rats, supports the hypothesis that some of these novel oximes are penetrating the blood-brain barrier (Chambers *et al*., 2016; unpublished data).

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CHAPTER II

IN VITRO AGE AND SEX-RELATED DIFFERENCES IN OXIME REACTIVATION

Introduction

Organophosphate (OP) compounds, either insecticides or nerve agents, represent a serious threat to human health when exposure occurs due to either accidental occupational exposure, suicidal self-exposure, or by intentional terroristic acts (Adinew *et al*. 2017, Bajgar *et al.*, 2015, Johnson *et al.*, 2015). The currently approved oxime used in the United States for OP intoxication, pralidoxime (2-PAM), has significant limitations with its ability to penetrate the blood-brain barrier, and broad spectrum capability against a variety of OP compounds (Cabal *et al*., 2004; Kassa, 2002; Sakurada *et al*., 2003). These reasons underscore the necessity of developing new and improved oximes as antidotes to attenuate acute OP toxicity. Our laboratory has developed novel substituted phenoxyalkyl pyridinium oximes (US Patent 9,227,937) designed to more effectively penetrate the blood-brain barrier (Chambers *et al*., 2013). New information is needed to further drug development and validate these new oximes with the ultimate goal of creating more effective antidotes.

Initial studies with our novel oximes were performed using adult male rats as an animal model (Chambers *et al*., 2013; Chambers *et al*., 2016a). If we are to further develop these oximes into drugs, experiments must be performed which take into account important biological variables such as age and sex. Differences do exist between

acetylcholinesterase (AChE) of different species. Small differences in AChE structure were detected between the fruit fly, torpedo, electric eel, mouse, and humans; however, these differences were due to non-catalytic portions of the enzyme (Moralev *et al*., 2001). These small differences were found to be relevant to an OP's inhibitory potency with 10-fold and 2-fold higher inhibitory rate constants found for human AChE inhibited by paraoxon (PXN) as compared to guinea pig and rat AChE, respectively (Worek *et al*., 2011). Additionally, expression of AChE in vertebrate animals rapidly increases as an organism matures which may confound experimental design (Coyle and Yamamura, 1976; Tang *et al*., 1999).

Despite these differences, AChE is highly conserved and expressed by a single gene in mammals (Massoulie *et al*., 1993). Reactivation of OP-inhibited AChE within the same species should be similar between age and sex groups for each oxime. The research presented in this chapter was designed to determine *in vitro* AChE oxime-mediated reactivation in brain tissue after OP inhibition in male and female rats of different in ages.

Past data from this laboratory has shown that combination oxime therapy can be more effective than a single oxime alone. Combining 2-PAM with novel oximes has yielded higher 24-hour survival rates than single oximes in OP lethal dosage challenge studies (Chambers *et al*., 2016a; Chambers *et al.,* 2016b). Those combination studies were performed with twice the molar equivalent of a single oxime concentration; therefore, an *in vitro* study comparing this higher concentration of combination oxime

therapy versus an equimolar concentration may be beneficial in elucidating the efficacy of reactivation for differing levels of oximes in combination.

Materials and Methods

Chemicals

Several surrogate compounds for nerve agents were developed for research purposes by Ohta *et al*., including the sarin surrogate, 4-nitrophenyl isopropyl methylphosphonate (NIMP) used in this study (Ohta *et al*., 2006). Novel oximes 15, 20, and 55, were first synthesized by Dr. Howard Chambers here at Mississippi State University (Meek *et al*., 2012; Chambers *et al*., 2013). These oximes were later patented (US patent 9,277,937) and are now synthesized by SRI International (Menlo Park, CA). The NIMP used in this study was also synthesized at Mississippi State University. All other reagents including 2-PAM and PXN, the active oxon of the insecticide parathion, were purchased from the commercial vendors Sigma Chemical Co (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). Figure 1 displays the chemical structures (created with MarvinSketch software, version 17.13.10) of the oximes and OP compounds used in this study.

Animals

These *in vitro* assays used whole brain tissue from naïve Sprague Dawley rats (Crl:CD(SD)BR). The rats were purchased from Envigo RMS, Inc. at postnatal day (PND) 70 while PND 12 rats were obtained from breeding colonies maintained at Mississippi State University from Envigo stock of the same strain. All animals were housed in AAALAC accredited facilities within temperature-controlled environments

with 12 hour dark-light cycle with Envigo 70-90 Sani Chips, laboratory grade bedding. Rats had free access to Envigo Rodent Diet, 18% protein laboratory rat chow and tap water. Procedures performed to harvest tissue for research purposes received prior approval by the Mississippi State University Animal Care and Use Committee.

For these *in vitro* assays, the estrus cycle in adult (PND 70) female rats was not controlled because the physiological barriers presumably bolstered by peaks in estrogen, such as the blood-brain barrier, would be destroyed in the process of making homogenate and the tissue is pooled from 5 animals in random stages of their cycle (Pittel *et al*., 2018; Smith *et al*., 2015). Representing prepubescent, juvenile animals, the PND 12 age was selected because at this stage the brain is still rapidly developing; however, cholinergic activity is high enough to be easily assayed for comparison with the adults (Coyle and Yamamura, 1976).

Tissue Collection and Preparation

Sprague Dawley rats were humanely euthanized utilizing CO_2 for anesthesia followed by decapitation. Harvested brain tissue was snap frozen in liquid nitrogen and stored at -80°C until further preparation could be performed. Pooled homogenates of whole rat brain from 5 animals were created for experimental replication utilizing a motorized homogenizer and normalized via dilution in 50 mM Tris HCl buffer (pH 7.4 at 25°C) for approximately equivalent cholinesterase activity between the two age groups as determined via a procedure described below. Three pools were created for both age groups and for both sexes (12 pools consisting of 60 animals total). For PND 70 pooled brain homogenate, the stock solution was 40 mg/mL wet weight equivalent with a

cholinesterase assay final concentration (FC) of 1 mg/ml, while stock and final concentrations of PND 12 animals were 65 mg/mL and 1.6 mg/ml wet weight equivalent, respectively

Measurement of Cholinesterase Activity

Central to this research is the quantification of cholinesterase activity. This cholinesterase assay is performed in conjunction with reactivation assays, which are described later in this chapter, and performed to assay harvested tissues from the *in vivo* studies outlined in the next chapter. To accomplish this measurement, a modified form of the Ellman discontinuous spectrophotometric cholinesterase assay (Ellman *et al.*, 1961) was utilized.

Stock concentration of rat brain homogenate (40 mg/mL wet weight equivalent for PND 70, 65 mg/mL wet weight equivalent for PND 12 in 50 mM Tris HCl buffer, pH 7.4 at 25°C) was aliquoted into 4 separate glass test tubes for each sample to be tested diluted in 50 mM Tris HCl buffer for a 2 mL total assay volume (FC, 1 mg/mL and 1.6 mg/mL for PND 70 and PND 12, respectively). One tube was designated as a tissue blank by the addition of 20 µL of eserine sulfate (FC, 0.01 mM) which inhibits all cholinesterase activity in the tissue. This blank serves as measurement of background interference caused by any non-AChE hydrolysis of the colorimetric substrate which is subtracted from the final result. The other three tubes contain the sample in which tissue cholinesterase is measured. These three tubes allow the assay to be performed in triplicate for experimental replication. All tubes were vortexed and incubated at physiologic relevant temperature, 37°C, in a shaking water bath to ensure the assay

remains well mixed for 15 minutes. Next, 20 µL of acetylthiocholine (ATCh) was added (FC, 1 mM) to all tubes which serves as a substrate for the tissue AChE within the rat brain homogenate. This mixture was vortexed and incubated again for another 15 min at 37°C in a shaking water bath. Finally, 250 µL of a 4:1 mixture of 0.24 M 5,5'-dithio bis(2-nitrobenzoic acid) (DTNB) and 5% sodium dodecyl sulfate (SDS) is added to each tube. SDS is a detergent which disrupts proteins thereby terminating the reaction, while DTNB reacts with thiocholine produced by the hydrolysis of ATCh substrate producing a yellow color measurable by a Thermo Scientific Biomate 3 spectrophotometer at 412 nm. This reaction produces absorbance values (A) directly proportional to the amount of substrate hydrolyzed by AChE thereby allowing quantitation of cholinesterase activity within the tissue.

The concentration of rat brain homogenate for PND 70 rats is consistent with previous studies (Chambers *et al*., 2013) while the concentration for PND 12 rats was determined experimentally by performing range finding assays with various dilutions of rat brain homogenate until the cholinesterase activity was approximately similar to adults. An additional age range was tested (PND 1); however, the cholinesterase activity was determined to be too low for inclusion in this research under this experimental design paradigm.

Reactivation of Acetylcholinesterase

In order to determine and compare the efficacy of the novel oximes, a reactivation assay was performed utilizing AChE contained within rat brain homogenate consistent with previous studies performed in this laboratory (Chambers *et al*., 2013). AChE was

inhibited by one of two OP compounds, PXN or NIMP, with cholinesterase activity at approximately 80% of maximum. This ensures that the rat brain homogenate is not saturated with OP, which would falsely depress the apparent reactivation efficacy of the oximes, while maintaining a large enough experimental range to adequately compare reactivation capability between them. Each age/sex group was reactivated by each oxime singularly or by combination of two oximes which are outlined in Table 1.

The reactivation assay procedure begins by placing 1 mL of stock concentration rat brain homogenate into a 1.5 mL plastic centrifuge tube. This tissue homogenate is incubated with 10 µL of ethanol (1% EtOH v/v) vehicle or OP (PXN or NIMP in EtOH). At this point in the assay, the inhibition step, the concentration of PXN and NIMP was 100 nM and 56 nM, respectively, which produced about 80% cholinesterase inhibition in the rat brain homogenate. The centrifuge tube receiving EtOH vehicle serves as the tissue control which will retain maximal cholinesterase activity. Another tube will serve as an inhibition control which will display the maximum inhibition produced by the OP while multiple reactivation tubes are used for each oxime/oxime combination to be tested. All tubes are vortexed and incubated in a shaking water bath at 37°C for 15 min. Next, in the reactivation step, 10 µL of a 1:1 mixture of dimethyl sulfoxide (DMSO):EtOH vehicle (1% v/v) is added to the tissue control tube and inhibition control tube while 10 µL of the single oximes and 5 µL of each oxime for the oxime combinations are added to each reactivation tube. All tubes are vortexed and incubated in a shaking water bath at 37°C for 30 min. The tissue control tube ensures there is no significant enzyme inhibition from the vehicles themselves while providing a measurement of maximal cholinesterase

activity to which the reactivation tubes were compared. Finally, 250 µL from each centrifuge tube is diluted in ice cold 50 mM Tris HCl buffer, pH 7.4 at 25°C (10 mL total volume) to produce the final concentrations of rat brain homogenate, 1 mg/mL and 1.6 mg/mL for PND 70 and PND 12, respectively. For each sample, 2 mL is aliquoted into 4 separate glass test tubes and cholinesterase activity is assayed as described above.

Reactivation is calculated as a percentage of control determined by comparing absorbance in the reactivation tubes against the tissue and inhibition control tubes. Total cholinesterase inhibition percentage (I_{total}) is calculated from absorbance values (A) of the tissue control and inhibition control, less the background (blank):

$$I_{total}\ \% = \frac{(Tissue\ Control - Blank) - (Inhibition\ Control - Blank)}{(Tissue\ Control - Blank)} * 100 \qquad (1.1)$$

Next, the percentage of cholinesterase inhibition in each sample (I_{sample}) is calculated from absorbance values (A) of the tissue control and reactivation samples, less the background interference (blank):

$$I_{sample}\ \% = \frac{(Tissue\ Control - Blank) - (Reactivation\ Sample - Blank)}{(Tissue\ Control\ Avg - Blank)} * 100 \qquad (1.2)$$

Finally, the total reactivation percentage for each sample is calculated:

$$Reactivation\ \% = \frac{I_{total} - I_{sample}}{I_{total}} * 100 \qquad (1.3)$$

Oxime Control Studies

To ensure that the oximes or vehicles used in these experiments would not significantly lower cholinesterase activity themselves, control studies were performed to measure cholinesterase activity in rat brain homogenate with oximes in the absence of any OP. The protocol for this experiment is very similar to the reactivation assay

outlined above. Two separate pools of adult male rat tissue and female rat tissue were utilized (4 pools total). Briefly, in 1.5 mL centrifuge tubes, 1 mL of rat brain homogenate was added to the tissue control tube and each oxime control tube. There is no inhibition control tube in this experiment. Next, 10 µL EtOH (1% v/v) was added to all centrifuge tubes, vortexed, and incubated in a shaking water bath at 37°C for 15 minutes. From this point forward, the procedure is identical to the above reactivation assay. Oximes were tested at the same concentrations as outlined in Table 1. If an OP compound was present, inhibited AChE would be reactivated by the oximes. Instead, all of the oxime control samples were compared to the tissue control to ensure approximately equivalent cholinesterase activity.

Oxime Effective Concentration

Reactivation and cholinesterase assays were performed using the procedures above with various concentrations of oxime to determine the effective concentration at which they achieve 25% reactivation (EC_{25}). This experiment utilized brain homogenate (40 mg/ml, 3 pools total) from naïve, adult male rats in which 80% of the cholinesterase activity was inhibited by either PXN or NIMP (FC in 1 mL total volume, 100 nM and 56 nM, respectively). Experimental protocol was identical to the reactivation assay outlined above. Reactivation efficacy for 2-PAM, novel oximes 15, 20, and 55 were tested at six different concentrations in each pool of rat brain homogenate. Range-finding, reactivation assays were performed to find the concentration at which maximal reactivation activity was possible for each oxime. Step-wise, lower concentrations were

then tested to yield a dose-response curve from which the EC_{25} could be calculated using statistical software.

Statistical Methods

Each pool of rat brain homogenate served as an experimental unit for statistical analysis in this research. Comparison of cholinesterase activity between the age/sex groups was performed by a two-way ANOVA test to assess the difference in mean absorbance utilizing the PROC GLM function in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC) on a personal computer. The distribution of data was assessed via the Shapiro-Wilk test utilizing PROC UNIVARIATE and determined to be approximately normal. An alpha level of 0.05 was used to determine statistical significance. Significant findings were explored by pairwise comparison using the LSMEANS function and TUKEY adjustment.

For the oxime control studies, the distribution of data was assessed utilizing PROC UNIVARIATE in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC) on a personal computer. From assessment of the Shapiro-Wilk test and histograms, these data were determined to be non-parametric due to the number of replicates (n = 4) for each oxime tested. The PROC UNIVARIATE procedure was also used to carry out a Wilcoxon signed rank test to determine if there was a significant difference in mean absorbance between the oxime controls and tissue control. An alpha level of 0.05 was used to determine statistical significance.

The determination of oxime EC_{25} was performed by graphing a scatterplot of reactivation percentage as the dependent variable versus the independent variable oxime

concentration in Sigma Plot/SigmaStat 13 software on a personal computer. A hyperbola was fit to the scatterplot in Sigma Plot regression wizard using the following equation:

$$y = y_0 + \frac{ax}{b+x} \quad (1.4)$$

The EC_{25} was calculated by assigning reactivation efficacy, the dependent variable, as 25% and solving for the independent variable, concentration. The accuracy of this method was checked via linear regression by logit transformation of the data and plotting the log of the concentration versus the logit value of the reactivation percentage. This check was performed in Microsoft excel on a personal computer.

Finally, for the *in vitro* reactivation studies, 3 pools of each age/sex group were assessed for a total 12 pools. Distribution of the data was assessed utilizing PROC UNIVARIATE in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC) and determined to be non-parametric from assessment of the Shapiro-Wilk test and histograms. The non-parametric data from the *in vitro* reactivation studies was transformed via PROC RANK and a two-way ANOVA performed with PROC GLM. Significant findings were explored by pairwise comparison using the LSMEANS function and TUKEY adjustment. Comparison of the two oxime combination dose groups (50 μM vs. 100 μM) was assessed by a two-way ANOVA using a group by block factor method in PROC GLM. An alpha level of 0.05 was used to determine statistical significance.

Results

To determine cholinesterase activity in rat brain homogenate at various age/sex groups, a cholinesterase assay was performed utilizing whole rat brain from 3 different naïve age groups (PND 1, PND 12, and PND 70) at the same concentration. Activity for

the pooled rat brain homogenate at a concentration of 40 mg/ml (FC, 1 mg/mL) increased with age as shown in Figure 2. Statistically significant differences ($p < 0.05$) were seen between the PND 1 group versus all other groups and the PND 12 female group versus the PND 70 male group. When rat brain homogenate for the PND 12 group was diluted to 65 mg/ml (FC, 1.6 mg/mL), approximately equivalent cholinesterase activity to PND 70 was achieved and this concentration was utilized in the rest of the *in vitro* assays.

Before beginning any reactivation assays, the oximes and vehicles needed to be tested to ensure that they did not significantly alter cholinesterase activity by themselves in the absence of OP. Results from oxime control studies confirmed that cholinesterase activity in the rat brain homogenate was not significantly ($p > 0.05$) altered from control for any of the oximes or vehicles used in these experiments.

Dose-response curves were created from oxime-mediated reactivation of rat brain homogenate inhibited by OP as shown in Figure 3 and 4. Reactivation percentages produced by increasing concentrations of oxime were plotted and best-fit to a hyperbola model. The models displayed a good fit to these data ($R^2 > 0.97$) from which the EC_{25} for each oxime was calculated. The greatest *in vitro* reactivation efficacy was displayed by 2-PAM against PXN and NIMP with EC_{25} values of 10.03 μM and 18.59 μM, respectively. The novel oximes 15 and 55 were effective reactivators of PXN with EC_{25} values of 27.49 and 23.62 μM, respectively. All the oximes were not as effective against NIMP and Ox 15 failed to achieve 25% maximal reactivation at the highest concentration tested (FC, 1000 μM). High concentrations (FC, >250 μM) of several oximes exhibited an inhibitory effect on reactivation and at concentrations higher than 0.1 M, most would

not stay in solution utilizing DMSO:EtOH solvent. The calculated EC_{25} values for each oxime are displayed in Table 2.

Reactivation studies were performed for each oxime against both OPs (PXN and NIMP) utilizing rat brain homogenate pools for each of the age/sex groups (PND 12 female, PND 12 male, PND 70 female, PND 70 male) as previously described. Oximes were tested singularly, and in combination with each other at an equimolar concentration (FC, 100 μM) to the single oxime dose or at double this concentration (FC, 200 μM). Data for these reactivation studies are contained in Tables 3 and 4 and represented graphically in Figures 5 – 10 for easier interpretation.

Oximes used alone versus PXN-inhibited rat brain homogenate (Table 3) indicate consistent reactivation percentages in all age/sex categories with 2-PAM garnering the highest at 83% to 88% while the novel oximes yielded 46% to 52%, 26% to 29%, and 35% to 40% for oximes 15, 20, and 55, respectively. Statistically significant differences ($p < 0.05$) were seen between some age/sex groups for oxime 15 and 55; however, there does not appear to be a distinguishable pattern to the statistically different results highlighted in Figure 5, a trend which also continues when observing the oxime combinations used against PXN.

Statistically significant differences ($p < 0.05$) exist for the combinations of 2-PAM and Oxime 20 as well as Oxime 15 and 20 against PXN in both the 100 μM and 200 μM concentrations as displayed in Figures 6 and 7; however, these differences are very small. For example, for the combination of 2-PAM and Oxime 20 at 100 μM, the statistically significant result is between the PND 70 male and PND 12 male groups with

reactivation percentages of 71% versus 63%, respectively. The statistically significant result for the other oxime combination at 100 μM, oxime 15 and 20, is even smaller at 44% versus 39% for the PND 70 male and PND 12 female groups, respectively. The significant differences seen in single oxime therapy against PXN do not seem to carry over in any way to the reactivation data for the oximes used in combination. The relatively small amount of variation among the tested pools seems to be the main driver of these statistically significant results with standard deviation reaching 1% or lower in many cases.

When comparing oxime combinations against PXN at the two different therapy concentrations, equimolar (Figure 6) versus double the single oxime dose (Figure 7), these data indicate statistically significant ($p < 0.05$) increases in reactivation at the higher concentration for all the combinations except for 2-PAM and oxime 15. None of the oxime combinations at the equimolar dose met or exceeded a reactivation as high as that achieved by a single oxime used alone. For example, for PND 70 males, 2-PAM used alone (FC, 100 μM) achieved 88% reactivation while the highest reactivation for the equimolar oxime combinations in that same age/sex group is 2-PAM + Ox55 at 77%. The same is not true for the higher concentration at double the single dose (FC, 200 μM). These data show that oxime 15 and 20 seem to have an inhibitory effect on reactivation when combined with themselves or 2-PAM. Oxime 55, on the other hand, does not display this same inhibitory trend on reactivation efficacy when combined with the other oximes and achieves about the same cholinesterase activity when combined with oxime 15 or 2-PAM as compared to those oximes alone.

When incubated with NIMP-inhibited rat brain homogenate, reactivation efficacy for all the oximes (Table 4) was lower than that seen against PXN. For these *in vitro* experiments, 2-PAM garnered the highest reactivation percentages across all age/sex groups ranging from 65% to 72%, while both oxime 15 and 55 yielded comparatively much lower reactivation activities. Comparison of the single oxime doses (FC, 100 μM) yielded statistical significance ($p < 0.05$) with a noticeable pattern as displayed in Figure 8. For 2-PAM, oxime 15 and 20, reactivation in the adult (PND 70) brain homogenate was statistically different from that in the juvenile (PND 12) tissue. While 2-PAM seemed slightly more effective in PND 70 tissue, novel oximes 15 and 20 both displayed significant increases in reactivation in PND 12 tissue versus PND 70.

When examining the oxime combinations against NIMP-inhibited rat brain homogenate, this same pattern between age/sex groups remains consistent as demonstrated in Figures 9 and 10. In almost all instances where oxime combinations were used in equimolar concentration (FC, 100 μM) to the single oxime dose, there was statistically significant differences ($p < 0.05$) between reactivation in PND 70 versus PND 12 tissue. Sex-related statistically significant differences were detected for two oxime combinations at this concentration; however, no consistent pattern in these data suggest that these are not biologically significant variations. Oxime combinations at 200 μM used against NIMP again show a pattern of differences consistent with the other oxime therapy concentrations. All combinations of novel oximes display increased reactivation of PND 12 tissue versus PND 70, while combinations of 2-PAM at 200 μM did not show any significant differences between age/sex groups. The latter may be due

to an overall increased reactivation efficacy at higher concentrations of 2-PAM which may be masking any potential differences.

Overall, regardless of age and sex, combinatorial oxime therapy at 100 μM does not seem to confer any reactivation benefit when used against NIMP-inhibited tissue *in vitro*. None of the oxime combinations at this concentration produced reactivation which significantly increased as compared to the reactivation produced by the oximes when used singularly; however, there are relevant results from the higher concentration (200 μM) oxime combinations. Oxime 15 in combination with 2-PAM at 200 μM significantly reduced reactivation than that achieved by 2-PAM alone. Interestingly, oxime 20 when combined with 2-PAM at 200 μM produced reactivation that was not significantly different from 2-PAM alone, and only oxime 55 in combination with 2-PAM at 200 μM significantly increased ($p > 0.05$) reactivation of NIMP-inhibited tissue greater than that of 2-PAM alone.

Discussion

Much of these data presented in this chapter rely on accurate comparison of cholinesterase activities between the age/sex groups of adult (PND 70) male and female rat brain tissue and juvenile (PND 12) male and female rat brain tissue. We know from previous reports that cholinesterase activity increases with age as rats mature (Coyle and Yamamura, 1976). Data garnered from these experiments verify significant differences in cholinesterase activity from PND 1, PND 12, and PND 70 rat brain tissue. While reactivation assays report results as a percent of control, it was important to increase the concentration of brain homogenate in PND 12 samples to be approximately equivalent to

the cholinesterase activity of PND 70 rat brain tissue. This allows added oxime to react with approximately the same amount of target enzyme *in vitro*, prevents shrinking the analytical range of the assay, and mitigates a potential source of experimental bias which could confound results.

In addition to ensuring an accurate comparison of cholinesterase activity, it was also important to compare the effective concentration of the oximes themselves. Data from previous experiments in this laboratory indicated that 2-PAM was a superior *in vitro* reactivator of cholinesterase activity as compared to the novel oximes (Chambers *et al*., 2013; Chambers *et al*., 2016b). The EC_{25} values calculated in this study confirm that 2-PAM is more effective than novel oxime 15, 20, or 55 reactivating 25% of brain tissue cholinesterase activity inhibited by PXN or NIMP at 10.03 μM and 18.59 μM, respectively, whereas the EC_{25} values for all three novel oximes are higher. Despite the effectiveness of 2-PAM *in vitro*, studies performed *in vivo* have demonstrated that our novel oximes have provided enhanced survivability over 2-PAM in most cases against a lethal dose of OP in rats. Both oximes 15 and 55 improved survivability against PXN over 2-PAM, while oxime 15 and 20 improved survivability over 2-PAM against NIMP (Chambers *et al*., 2016a; unpublished data). The EC_{25} values calculated in this study are consistent with those previous findings showing that among the novel oximes, 15 and 55 had the best performance against PXN while oxime 20 was more effective against NIMP as outlined in Table 2.

Previous studies in this laboratory investigating oxime reactivation of OP-inhibited cholinesterase activity were conducted using adult male rats as the animal

model (Chambers *et al*., 2013; Chambers *et al*. 2016b). In the present study, both male and female rats of various ages were assessed. There was no statistical difference demonstrated between males and females in either age category for any oxime-OP combination which is consistent with the highly conserved nature of AChE described in mammals (Massoulie *et al*., 1993).

Reactivation of PXN-inhibited AChE in rat brain with all the oximes indicated consistent efficacy in each age/sex group. Although statistically significant differences were observed, no clear pattern emerged with respect to age or sex. Furthermore, these differences in reactivation within the groups do not seem to be physiologically relevant and are likely due to the low amount of variability seen among the tested tissue pools. These statistically significant differences seen in oxime reactivation of PXN-inhibited rat brain AChE would likely diminish with a larger sample size.

Overall, the oximes were less effective reactivating NIMP-inhibited AChE, and 2-PAM performed better than the novel oximes, again consistent with EC_{25} values calculated. Interestingly, while 2-PAM showed slightly better efficacy in adults than juveniles, the novel oximes 15 and 20 performed better in the latter groups. Statistical significance ($p < 0.05$) was achieved with reactivation of NIMP-inhibited AChE and these data indicate a pattern suggestive of an age-related difference present between adults and juveniles but not between the sexes. A possible explanation for this age-related difference may lie with proposed "non-classical", morphogenic functions of AChE beyond simple hydrolysis of acetylcholine. An association was found between AChE and a glutamate receptor subtype, α-amino-3-hydroxy-5-methylisoxazole-4-

propionic acid (AMPA), within the brain of developing rats to promote synaptogenesis which diminishes with maturity. This is an example of an AChE-protein binding interaction found primarily in juvenile rats which may sterically affect the reactivation of AChE dependent upon which OP phosphorylates the enzyme and which oxime is used for reactivation. Inhibitors targeting peripheral and catalytic binding sites on AChE ameliorated the modulating effect of the enzyme on AMPA receptors (Soreq and Seidman, 2001; Olivera *et al*., 2003). If the catalytic and peripheral binding sites are involved with AMPA receptors promoting synaptogenesis in juvenile rats, this could induce subtle deviations in or around the molecular structure of AChE in maturing rat brain which may increase effectiveness of these novel oximes in juvenile tissue.

Comparison of the oxime combinations against either OP revealed that there was no apparent benefit to reactivation at the lower dose (100 μM), which was equimolar to the single oxime dose. In these combination studies, each oxime was added separately to the reaction tube at 50 μM, both of which when added together yielded 100 μM total oxime FC which was equimolar to the single dose as previously mentioned. It is therefore, unclear if there was competitive inhibition between the oximes or if they were acting as true chemical antagonists against each other; however, what is clear is that at this concentration, there were no synergistic effects on reactivation.

With respect to the higher concentration oxime combinations, both oximes were added separately to the reactivation assay at 100 μM, both of which when added together yielded 200 μM FC. It is not surprising then that reactivation percentages at this concentration significantly increased as compared to the lower concentration oxime

combinations. One would also expect reactivation percentages to exceed those achieved by the oximes used singly because there was twice the molar concentration of an equivalent single concentration present; however, this was not observed for most of the oximes. For this reason, the higher concentration oxime combinations may give better insight as to which oximes work together and which do not. For example, addition of oxime 15 to 2-PAM or oxime 20 significantly lowered reactivation when compared to 2-PAM or oxime 20 used alone. Against either OP, oxime 55 did not appear to decrease reactivation when combined with any oxime as compared to those oximes alone. Even with the low reactivation of 8% to 11% garnered by oxime 55 alone against NIMP, when combined with 2-PAM at the 200 μM concentration, oxime 55 seemed to have an additive effect significantly increasing reactivation as compared to 2-PAM alone. These data indicate that oxime 55 may be a good candidate for *in vivo* oxime combination studies while oxime 15 may be inhibitory towards other oximes if used in combination.

Conclusion

The results obtained thus far indicate that the novel oximes will be equally efficacious in males and females. There was no significant difference in reactivation by any oxime in males versus females at either age tested. Performance of these oximes depend upon the OP which is inhibiting AChE which was consistent with previous research and confirmed in both the EC_{25} calculations as well as reactivation studies. These data also indicate an age-related difference in reactivation of NIMP-inhibited enzyme which could be confirmed with future *in vivo* studies if it were to be found that these novel oximes improve survivability in juveniles. Additionally, these data seem to

point to oxime 55 as a good candidate for combination therapy. The combination of 2-PAM, as a potent peripheral reactivator, and oxime 55, as a reactivator which could cross the blood-brain barrier to attenuate central nervous system inhibition has the potential to boost survivability against an OP exposure event.

Table 1 Assayed Oximes and Oxime Combinations

Oxime (s)	Final Concentration (μM)
Pralidoxime (2-PAM)	100
Novel Oxime 15 (Ox 15)	100
Novel Oxime 20 (Ox 20)	100
Novel Oxime 55 (Ox 55)	100
2-PAM + Ox 15	100
2-PAM + Ox 20	100
2-PAM + Ox 55	100
Ox 15 + Ox 20	100
Ox 15 + Ox 55	100
Ox 20 + Ox 55	100
2-PAM + Ox 15	200
2-PAM + Ox 20	200
2-PAM + Ox 55	200
Ox 15 + Ox 20	200
Ox 15 + Ox 55	200
Ox 20 + Ox 55	200

Single oximes and oxime combinations dissolved in 1:1 ethanol/ dimethyl sulfoxide vehicle. Oximes used in combination added separately at 50 μM or 100 μM for the low or high concentration, respectively to yield the final concentrations as calculated in 1 mL total volume rat brain homogenate (40 mg/mL and 65 mg/mL for postnatal day (PND) 70 and PND 12, respectively) during the reaction step of the reactivation assay.

Table 2 Oxime Effective Concentration for 25% Reactivation of OP-inhibited Rat Brain

	PXN		NIMP	
Oxime	**EC_{25} (µM)**	**R^2**	**EC_{25} (µM)**	**R^2**
2-PAM	10.03	0.99	18.59	1.00
Ox 15	27.49	0.98	…	…
Ox 20	91.57	0.98	176.93	0.99
Ox 55	23.62	0.99	269.60	0.99

Multiple oxime concentrations were used to reactivate 1 mL adult male rat brain homogenate (FC, 40 mg/mL) inhibited by either paraoxon (PXN) (FC, 100 nM) or 4-nitrophenyl isopropyl methylphosphonate (NIMP) (FC, 56 nM). A dose-response relationship curve was plotted and a hyperbola model was best-fit to these data yielding the R^2 values. Calculated EC_{25} values were determined from this model. Ox 15 could not achieve 25% maximal reactivation against NIMP with concentrations up to 1 mM; therefore, an EC_{25} value could not be calculated.

Table 3 Reactivation Percentages of PXN-inhibited Rat Brain

Oxime	FC (μM)	PND 70 Male	SD	PND 70 Female	SD	PND 12 Male	SD	PND 12 Female	SD
2-PAM	100	87.80	0.8	84.63	5.1	82.53	1.7	85.08	1.9
Ox 15	100	51.86 [a]	3.7	45.51 [b,c]	0.9	47.87 [a,b,c]	0.7	49.58 [a]	1.1
Ox 20	100	28.80	1.6	26.16	1.0	29.22[a]	2.1	27.74	1.8
Ox 55	100	39.86 [a]	0.9	35.45 [b]	1.5	37.34 [a]	1.0	35.98 [b]	3.0
2-PAM + Ox 15	100	71.45	2.0	66.67	3.8	70.35	0.5	67.99	3.8
2-PAM + Ox 20	100	70.90 [a]	2.9	66.08 [a,b]	3.7	62.49 [b]	2.2	65.79 [a,b]	2.5
2-PAM + Ox 55	100	77.20	2.6	71.91	5.4	69.28	1.8	72.65	5.7
Ox 15 + Ox 20	100	44.07 [a]	0.8	41.35 [a]	1.4	40.34 [b]	1.8	39.36 [b]	1.9
Ox 15 + Ox 55	100	52.00	2.0	47.48	3.3	46.26	1.2	44.73	3.4
Ox 20 + Ox 55	100	36.35	2.6	32.18	0.3	33.41	2.6	32.28	3.0
2-PAM + Ox 15	200	74.88	0.8	72.12	2.7	70.85	6.7	69.37	3.3
2-PAM + Ox 20	200*	77.51 [a]	2.7	74.38 [b]	0.6	68.46 [c]	0.6	72.38 [d]	1.7
2-PAM + Ox 55	200*	84.83	2.6	80.36	3.9	79.64	2.4	80.28	2.6
Ox 15 + Ox 20	200*	46.51 [a]	0.5	43.29 [a,b]	0.4	41.08 [b]	2.4	40.48 [a,b]	5.8
Ox 15 + Ox 55	200*	54.16	1.7	51.70	4.0	49.17	3.4	47.15	3.8
Ox 20 + Ox 55	200*	41.17	0.6	40.94	1.3	37.97	3.1	38.11	3.2

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with paraoxon (PXN) (100 nM). Reactivation with 2-PAM, novel oximes 15, 20, 55 (100 μM) or in combination with each other at equimolar (100 μM) or twice the equimolar concentration (200 μM) of the single oxime concentration. Reactivation results expressed as a percentage (%) of control (standard deviation = SD). Differences in means between PND/sex groups compared via two-way ANOVA. Mean reactivation percentages not indicated by the same letter are significantly different ($p < 0.05$). Differences in means between oxime combination groups compared via two-way ANOVA (* = significantly different from 100 μM oxime combination).

Table 4 Reactivation Percentages of NIMP-inhibited Rat Brain

Oxime	FC (μM)	PND 70 Male	SD	PND 70 Female	SD	PND 12 Male	SD	PND 12 Female	SD
2-PAM	100	71.66 [a]	1.9	74.95 [a]	2.3	65.18 [b]	2.2	67.22 [b]	2.4
Ox 15	100	2.54 [a]	3.2	5.80 [a]	1.7	14.81 [b]	1.4	15.71 [b]	1.2
Ox 20	100	34.99 [a]	0.5	34.46 [a]	5.3	44.80 [b]	2.5	44.50 [b]	2.7
Ox 55	100	9.18	1.5	8.10	2.8	10.24	1.1	10.77	0.7
2PAM + Ox 15	100	4.26 [a]	1.4	11.60 [b]	3.7	19.49 [c]	2.1	21.49 [d]	1.6
2PAM + Ox 20	100	37.14 [a]	4.1	39.86 [a]	5.5	46.79 [b]	1.5	49.74 [b]	2.7
2PAM + Ox 55	100	50.81 [a]	1.9	57.29 [b]	3.2	48.51 [c]	1.7	52.74 [d]	2.3
Ox 15 + Ox 20	100	18.35 [a]	0.5	20.72 [a]	3.5	32.66 [b]	2.5	33.54 [b]	0.5
Ox 15 + Ox 55	100	0.46 [a]	2.9	1.95 [a]	2.2	12.35 [b]	0.8	11.88 [b]	1.4
Ox 20 + Ox 55	100	22.06 [a]	0.8	20.98 [a]	5.3	33.92 [b]	1.9	35.99 [b]	2.4
2PAM + Ox 15	200*	42.19	2.1	44.73	6.2	48.49	4.4	54.00	9.3
2PAM + Ox 20	200*	70.77	1.5	70.87	1.2	72.09	2.9	73.55	1.2
2PAM + Ox 55	200*	75.76	1.5	81.35	0.4	74.76	2.2	75.02	2.6
Ox 15 + Ox 20	200*	11.90 [a]	1.5	15.79 [a]	2.9	27.26 [b]	2.1	28.58 [b]	1.2
Ox 15 + Ox 55	200*	9.09 [a]	1.6	13.24 [a]	3.3	20.42 [b]	4.0	23.82 [b]	1.4
Ox 20 + Ox 55	200*	15.13 [a]	3.1	14.59 [a]	4.4	25.37 [b]	0.9	25.01 [b]	1.6

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with 4-nitrophenyl isopropyl methylphosphonate (NIMP) (56 nM). Reactivation with 2-PAM, novel oximes 15, 20, 55 (100 μM) or in combination with each other at equimolar (100 μM) or twice the equimolar concentration (200 μM) of the single oxime dose. Reactivation results expressed as a percentage (%) of control (standard deviation = SD). Differences in means between PND/sex groups compared via two-way ANOVA. Mean reactivation percentages not indicated by the same letter are significantly different ($p < 0.05$). Differences in means between oxime combination groups compared via two-way ANOVA (* = significantly different from 100 μM oxime combination).

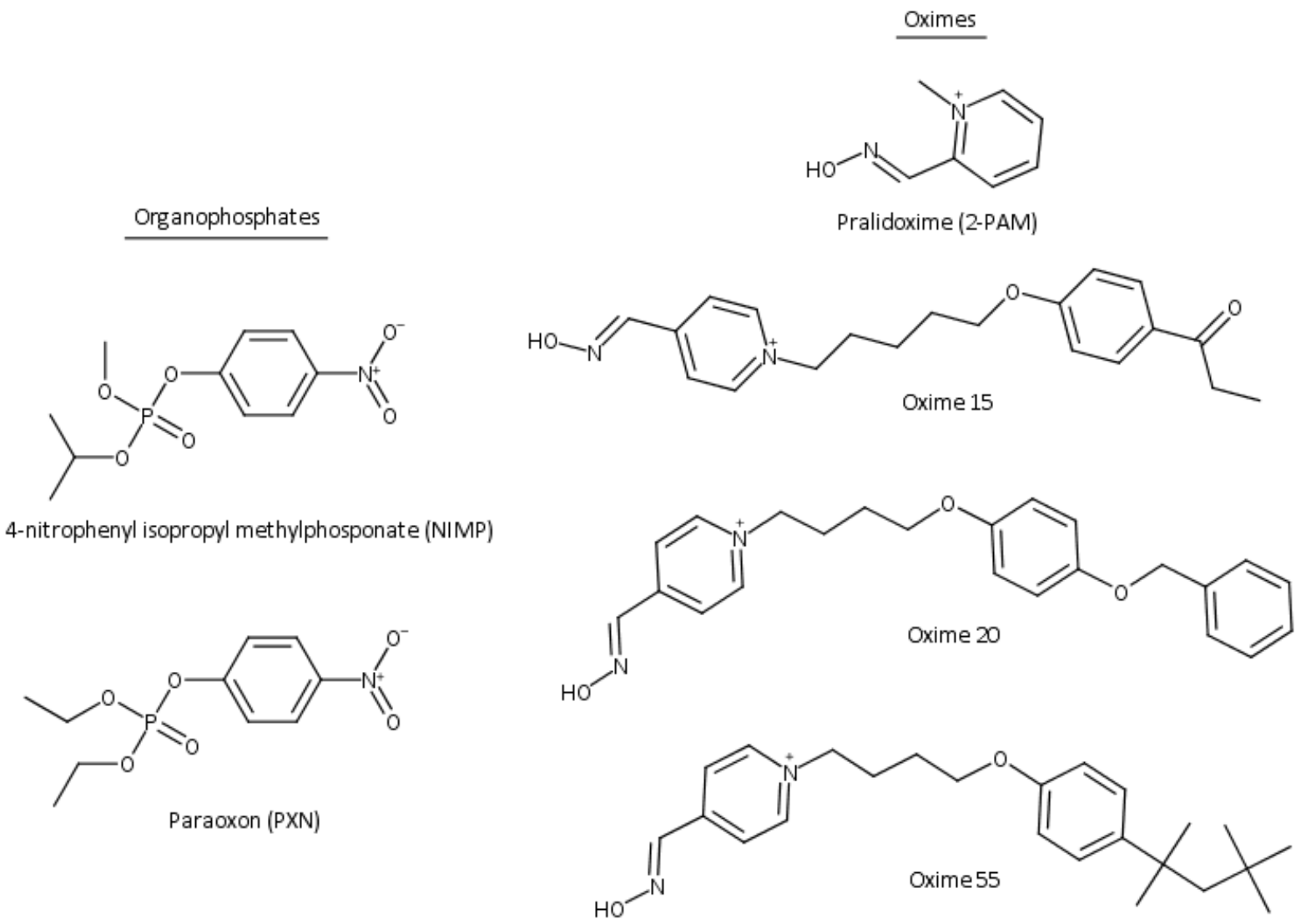

Figure 1 Structure of Organophosphates and Novel Oximes (US patent 9,277,937)

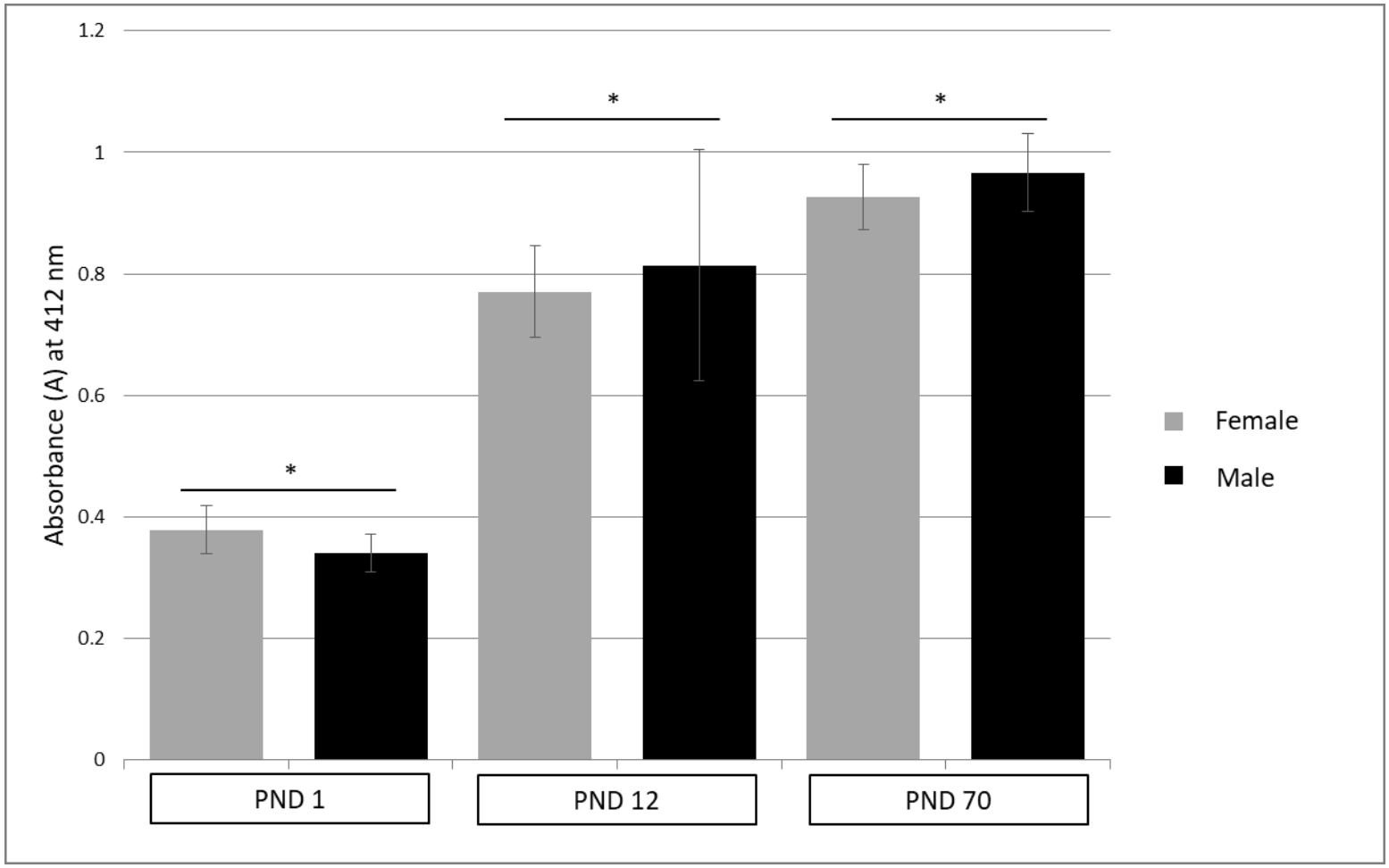

Figure 2 Rat Brain Cholinesterase Activity by PND/Sex Group

Rat brain homogenate (1 mg/mL) with vehicle control (1% EtOH v/v). Cholinesterase activity determined with 1 µM ATCh substrate by modified Ellman assay. A two-way ANOVA assessed differences in mean absorbance between the multiple postnatal day (PND)/sex groups. * = statistically different ($p < 0.05$) mean absorbance between PND groups

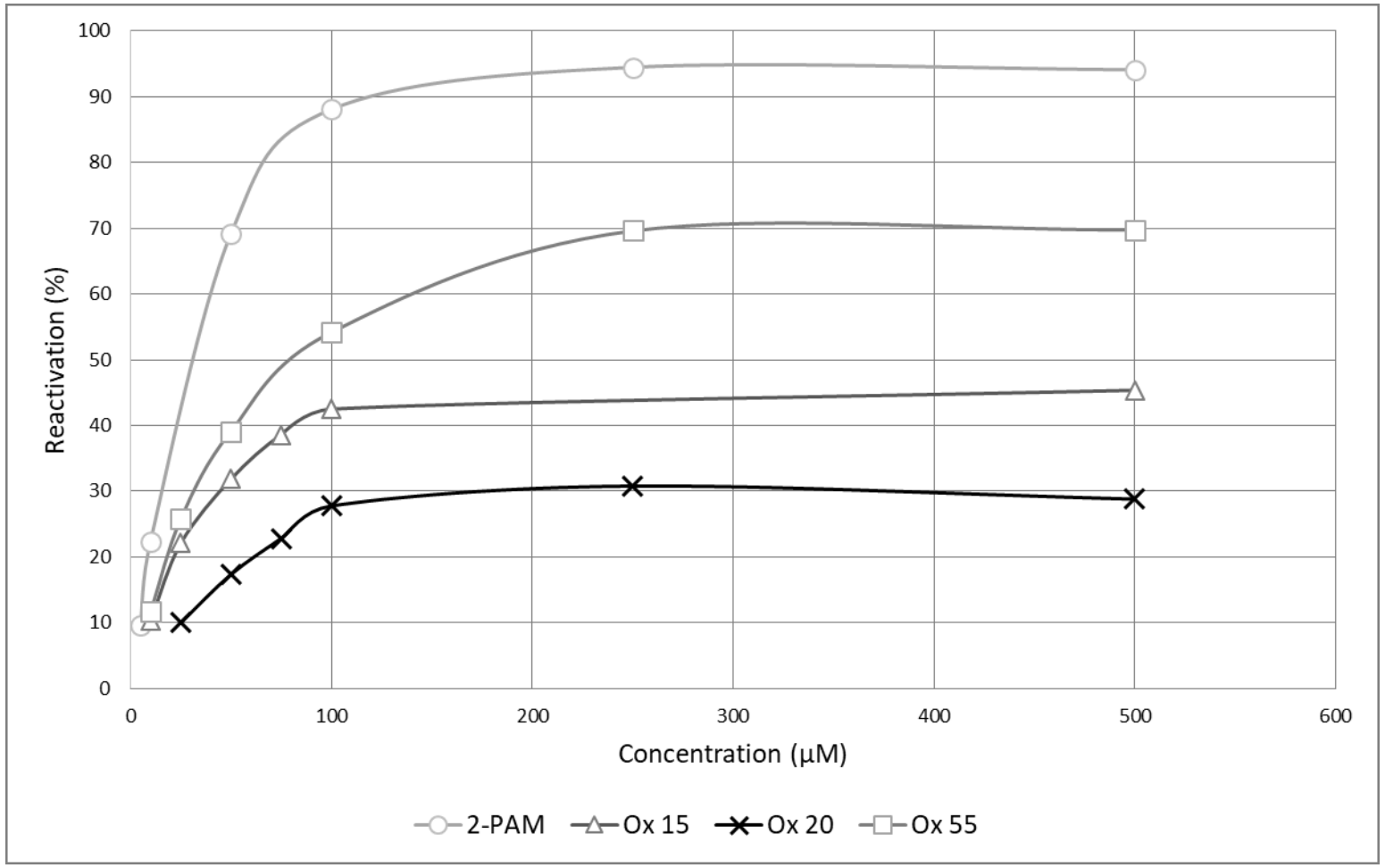

Figure 3 Oxime Dose-response Curve of Reactivation in PXN-inhibited Rat Brain Homogenate

Adult male rat brain homogenate (40 mg/mL) inhibited by paraoxon (PXN) (100 nM) and reactivated with increasing concentrations of 2-PAM, novel oximes 15, 20, or 55.

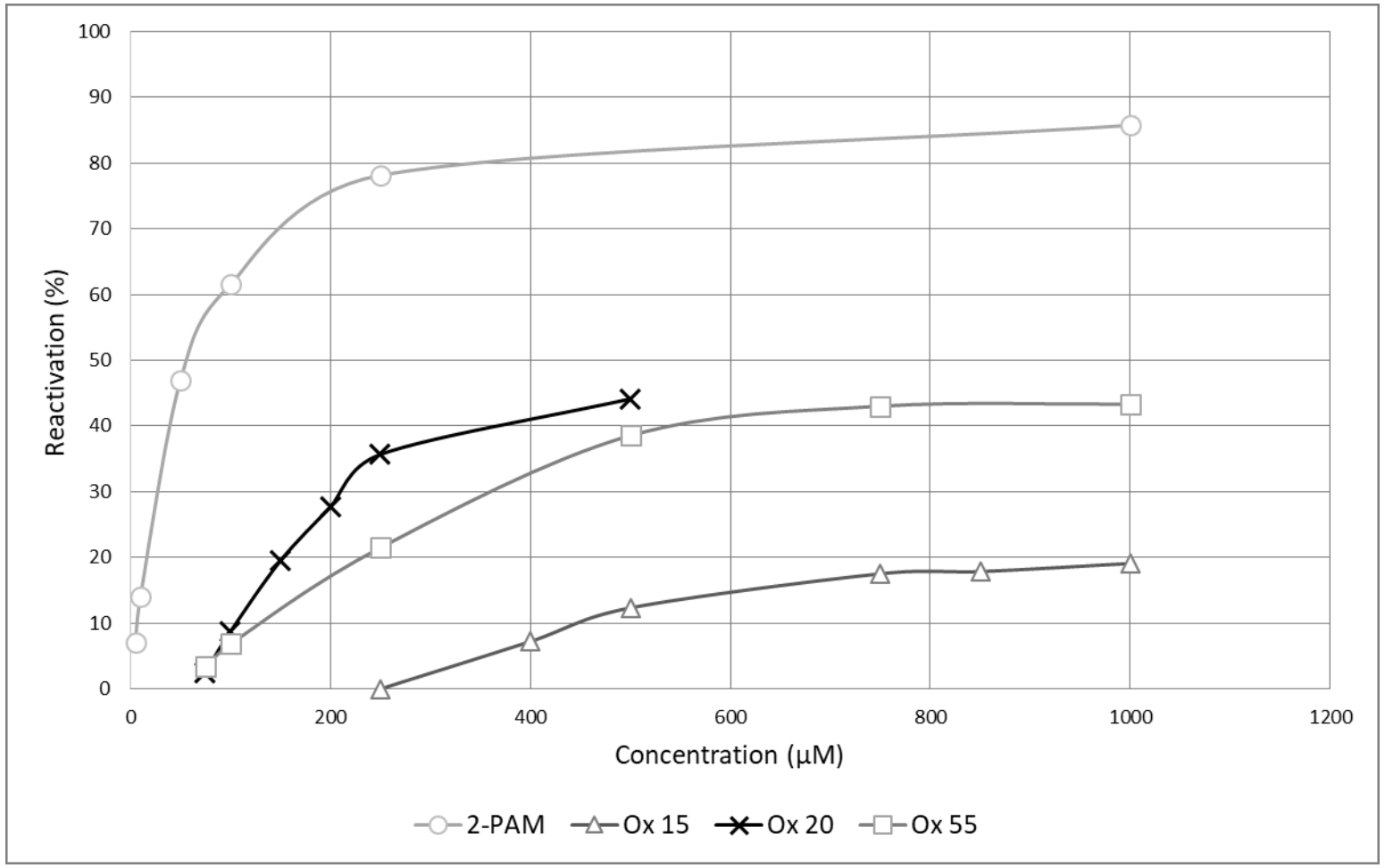

Figure 4 Oxime Dose-response Curve of Reactivation in NIMP-inhibited Rat Brain Homogenate

Adult male rat brain homogenate (40 mg/mL) inhibited by 4-nitrophenyl isopropyl methylphosphonate (NIMP) (56 nM) and reactivated with increasing concentrations of 2-PAM, novel oximes 15, 20, or 55. Oxime 20 produced maximum reactivation at 500 µM, demonstrated reduced reactivation at 750 µM, and would not remain in solution at 1000 µM.

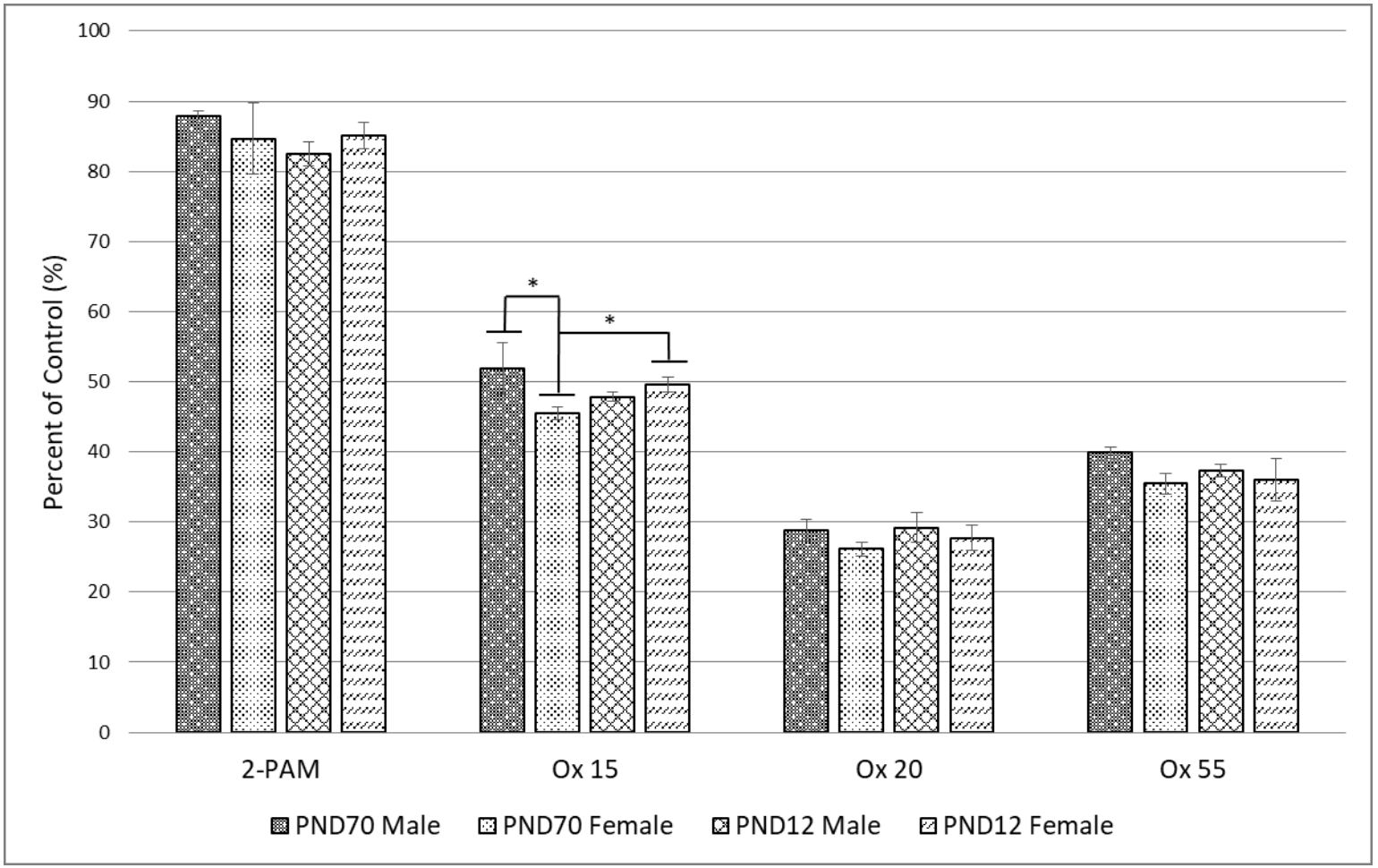

Figure 5 Reactivation of PXN-inhibited Rat Brain

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with paraoxon (PXN) (100 nM). Reactivation with 2-PAM, novel oximes 15, 20, or 55 (100 µM). Reactivation expressed as a percentage (%) of control. Differences in means between PND/sex groups for each oxime compared via two-way ANOVA. * = statistical significance ($p < 0.05$)

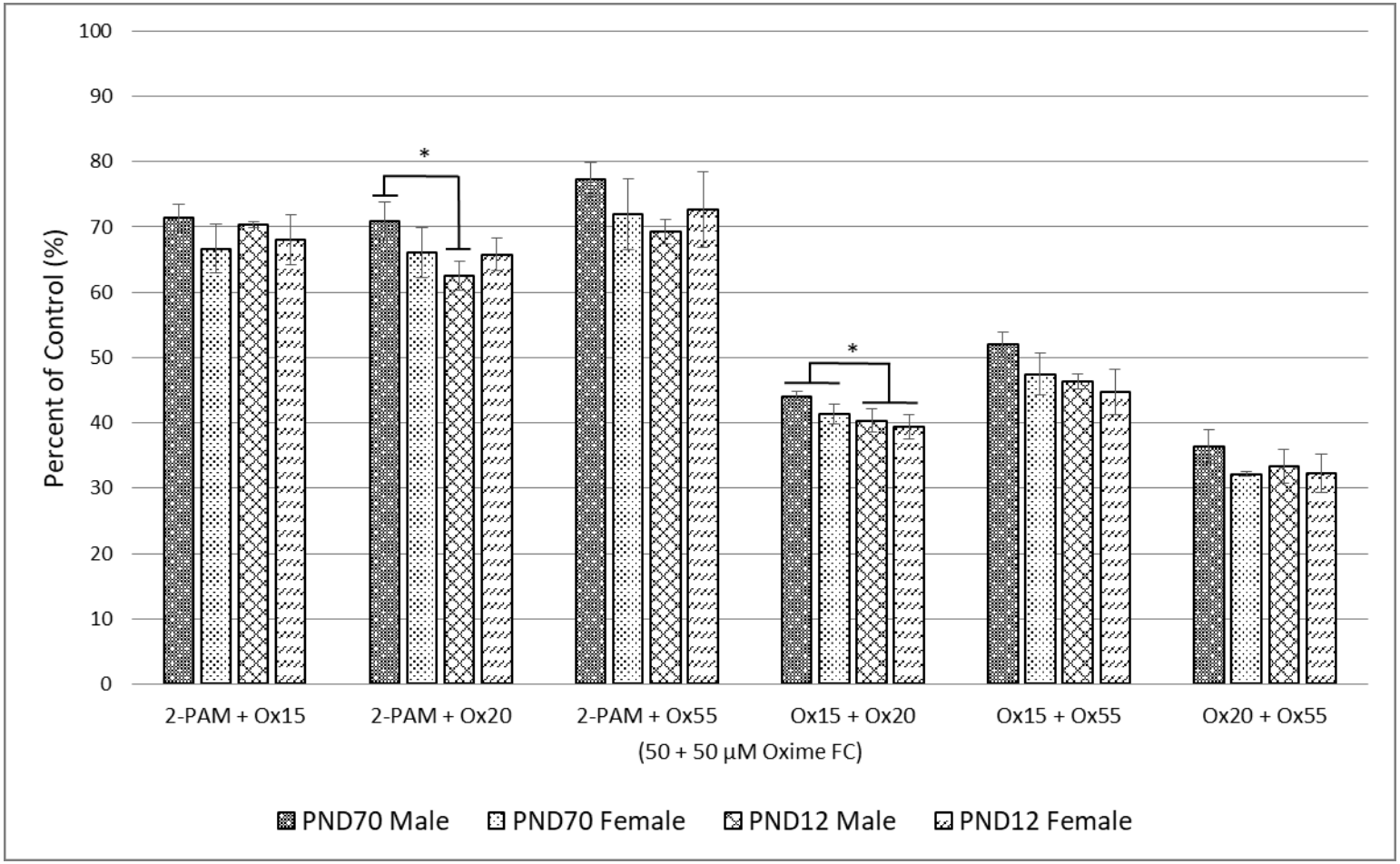

Figure 6 Reactivation of PXN-inhibited Rat Brain with Oxime Combinations

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with paraoxon (PXN) (100 nM). Reactivation with 2-PAM, novel oximes 15, 20, or 55 (50 µM) in combination with each other at an equimolar concentration (100 µM) to the single oxime dose. Reactivation results expressed as a percentage (%) of control. Differences in means between PND/sex groups for each oxime combination compared via two-way ANOVA. * = statistical significance ($p < 0.05$)

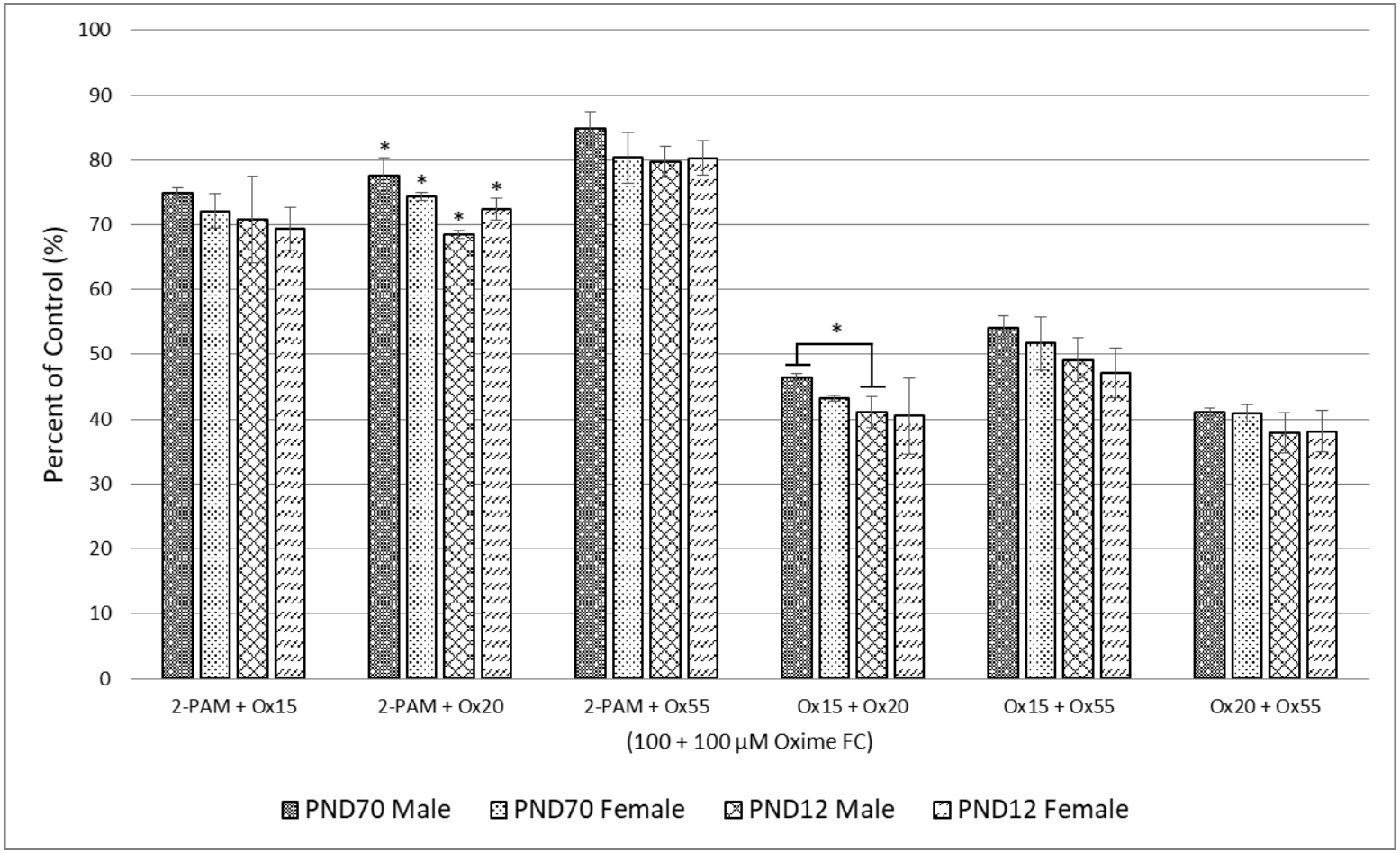

Figure 7 Reactivation of PXN-inhibited Rat Brain with Oxime Combinations at High Concentrations

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with paraoxon (PXN) (100 nM). Reactivation with 2-PAM, novel oximes 15, 20, or 55 (100 µM) in combination with each other at a concentration double (200 µM) the single oxime dose. Reactivation results expressed as a percentage (%) of control. Differences in means between PND/sex groups for each oxime combination compared via two-way ANOVA. * = statistical significance ($p < 0.05$)

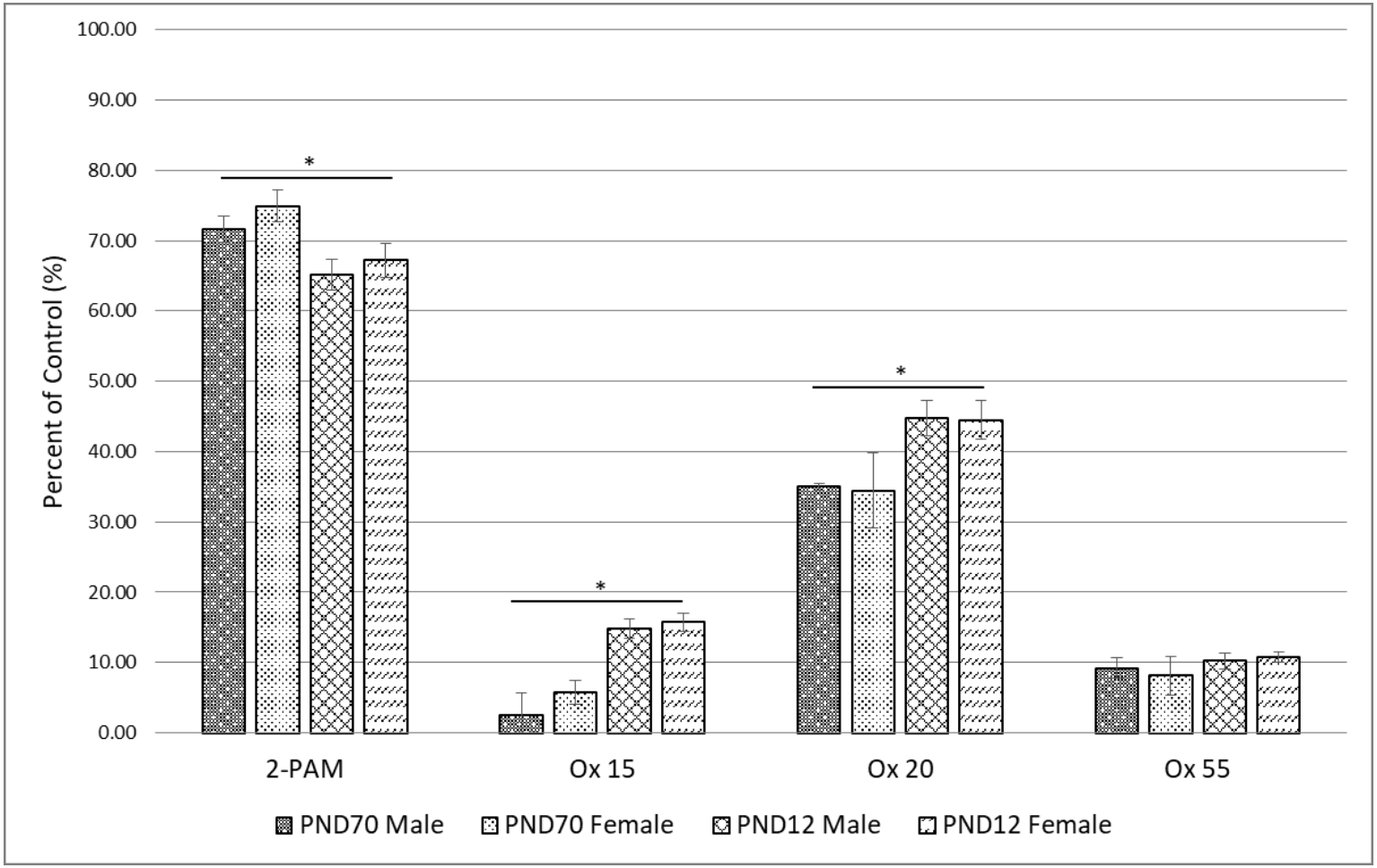

Figure 8 Reactivation of NIMP-inhibited Rat Brain

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with 4-nitrophenyl isopropyl methylphosphonate (NIMP) (56 nM). Reactivation with 2-PAM, novel oximes 15, 20, or 55 (100 μM). Reactivation expressed as a percentage (%) of control. Differences in means between PND/sex groups compared via two-way ANOVA. * = statistical significance ($p < 0.05$) between PND groups

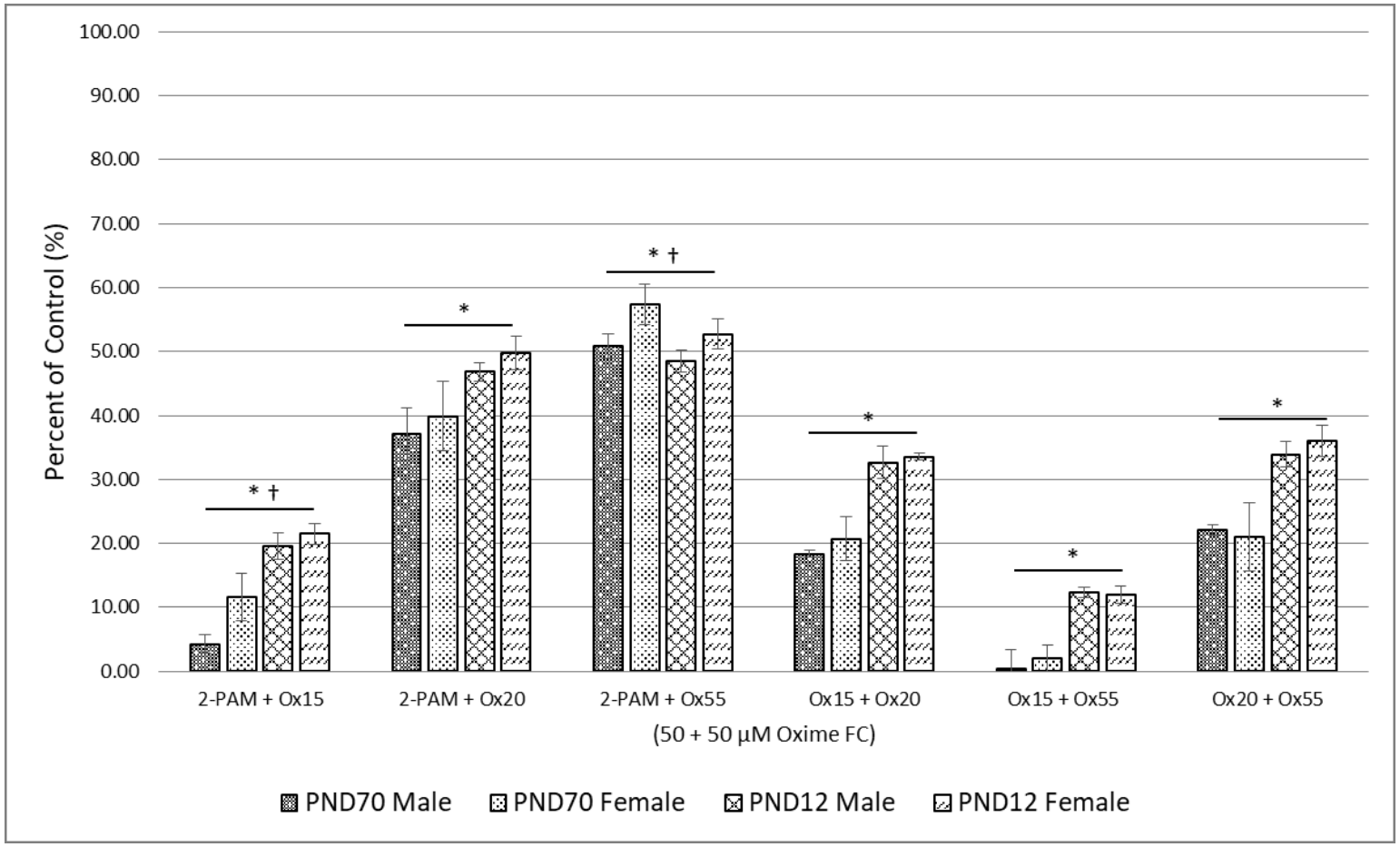

Figure 9 Reactivation of NIMP-inhibited Rat Brain with Oxime Combinations

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with 4-nitrophenyl isopropyl methylphosphonate (NIMP) (56 nM). Reactivation with 2-PAM, novel oximes 15, 20, or 55 (50 µM) in combination with each other at an equimolar concentration (100 µM) to the single oxime dose. Reactivation results expressed as a percentage (%) of control. Differences in means between PND/sex groups for each oxime combination compared via two-way ANOVA. * = statistical significance ($p < 0.05$) between PND groups; † = statistical significance ($p < 0.05$) between sex groups

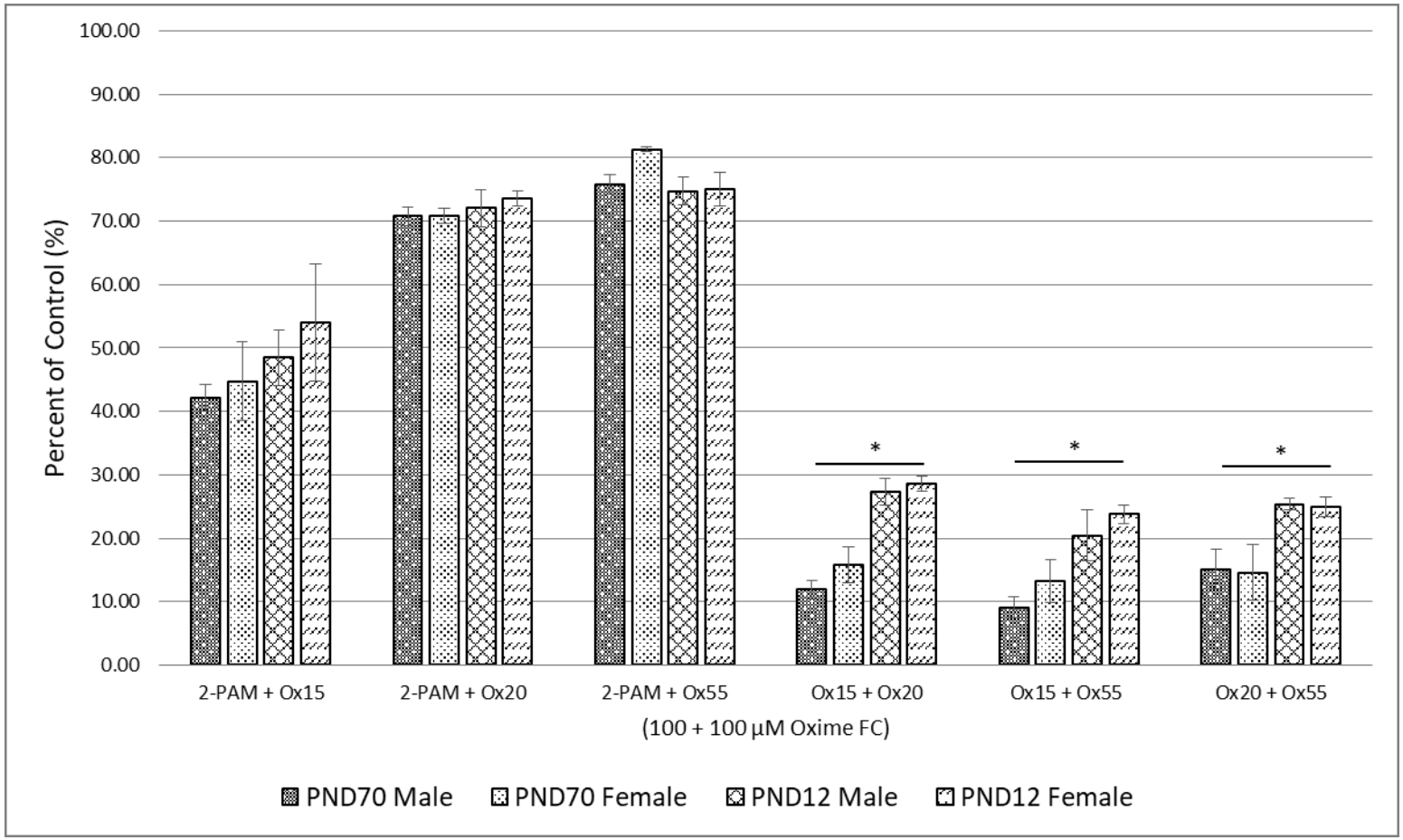

Figure 10 Reactivation of NIMP-inhibited Rat Brain with Oxime Combinations at High Concentrations

Postnatal day (PND) 70 (40 mg/ml) and PND 12 (65 mg/ml) rat brain homogenate incubated with 4-nitrophenyl isopropyl methylphosphonate (NIMP) (56 nM). Reactivation with 2-PAM, novel oximes 15, 20, or 55 (100 µM) in combination with each other at a concentration double (200 µM) the single oxime dose. Reactivation results expressed as a percentage (%) of control. Differences in means between PND/sex groups for each oxime combination compared via two-way ANOVA.
* = statistical significance ($p < 0.05$) between PND groups

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CHAPTER III

ADULT FEMALE RAT SUSCEPTIBILITY TO A LETHAL DOSE OF ORGANOPHOSPHATE WHEN TREATED WITH NOVEL PYRIDINIUM OXIMES

Introduction

The focus of this chapter is the performance of the novel pyridinium oximes as compared to pralidoxime (2-PAM) in adult female Sprague Dawley rats challenged with a lethal dose of organophosphate (OP). As mentioned in the previous chapter, sex-related differences in susceptibility to OP insecticides have been known for quite some time with female rats typically regarded as more susceptible as determined by LD_{50} studies (Gaines and Linder, 1986). Adult female rats have been shown to be more sensitive to the parathion, the parent compound of paraoxon (PXN), than adult males. Presumably, this is due to increased levels of testosterone in males which favorably oxidize parathion more towards non-toxic metabolites as opposed to bioactivation to PXN. Male rats treated with parathion were more resistant to acetylcholinesterase (AChE) inhibition as measured in brain tissue (196 nmol/min·mg protein) versus their female counterparts (80 nmol/min·mg protein). However, gonadectomized female rats exposed to parathion and treated with testosterone demonstrated higher resistance to AChE inhibition (193 nmol/min·mg protein) than gonadectomized male rats treated with estrogen (91 nmol/min·mg protein). There were no significant differences noticed when treating the

animals directly with PXN indicating that the sex-related differences in susceptibility to an OP is related to the metabolism of the parent compounds (Agarwal *et al.*, 1982).

More recently, studies have been conducted to better characterize sex-related differences in rats exposed to nerve agents which hypothesize that estrogenic sex hormones are a determining factor in susceptibility (Pittel *et al*., 2018; Smith *et al*., 2015; Wright *et al*., 2015). Although the exact mechanisms by which estrogens confer their protective effects against OP compounds remains unclear, it was demonstrated that female rats displayed significantly higher 24-hour sarin LD_{50} while in the proestrus phase of their estrus cycle as compared to male rats. Because estradiol peaks during this phase, it is thought that the higher concentration of estrogens better protected this group against the nerve agent (Smith *et al*., 2015). For this reason, experimental design of research involving OP exposure in females must take into account and control for the estrus cycle.

The estrus cycle of the female Sprague Dawley rat is considerably shorter than the menstrual cycle in a human averaging about 4 – 5 days in length (Goldman *et al.*, 2007). There are four major stages of the estrus cycle: proestrus, estrus, metestrus, and diestrus. Distinguishing between these stages can be easily performed via light microscopy (Cora *et al*., 2015). By controlling for the stage of the estrus cycle, the experiments performed in this research add another level of insurance that the true efficacy of oxime performance will be measured. The known cyclical increases in sex hormone can be avoided which could otherwise lead to an overestimation of protection conferred by an oxime in the absence of such a control measure.

Materials and Methods

Chemicals

The organophosphates used for the *in vivo* studies in this research, 4-nitrophenyl isopropyl methylphosphonate (NIMP) and paraoxon (PXN), were synthesized by Dr. Howard Chambers which structures were confirmed by nuclear magnetic resonance at the Department of Chemistry at Mississippi State University. The novel oximes, which were first synthesized by Dr. Howard Chambers, have been scaled up for production and have been recently synthesized by SRI International (Menlo Park, CA). Both atropine and 2-PAM were purchased from Sigma Chemical Co (St. Louis, MO). All other reagents used in this research were purchased from the commercial vendors Sigma Chemical Co (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Animals

Adult female Sprague Dawley rats (Crl:CD(SD)BR) at postnatal day (PND) 70 were purchased from Envigo RMS, Inc. and housed in AAALAC accredited facilities at Mississippi State University. All animals were held in temperature-controlled environments with a 12-hour dark-light cycle, used Envigo 70-90 Sani Chips, laboratory grade bedding and had free access to Envigo Rodent Diet, 18% protein laboratory rat chow and tap water. Procedures performed for this research received prior approval by the Mississippi State University Animal Care and Use Committee.

Estrus Cycle Check

The estrus cycle of each female rat was checked prior to advancing to the dosing protocol. This check ensured that each animal was in the diestrus phase of their estrus

cycle. This phase was chosen for two reasons. First, higher tolerance to OP lethality was only seen during the proestrus phase which was presumed to be due to the surge of estrogen associated with this stage of the estrus cycle (Smith *et al*., 2015). Second, the diestrus phase has been estimated to be the longest in duration of all the stages lasting about 48 – 72 hours which gives the largest window of opportunity to proceed to the dosing protocol (Cora *et al*., 2015). An increased concentration of estrogen sex hormone has the potential to cause overestimation of oxime efficacy. By performing the estrus cycle check and confirming that each animal is in the diestrus stage, this allows for the control of one potentially confounding variable.

In order to check the estrous cycle, a syringe was loaded with 100 µL of 0.9% saline solution. The syringe was then gently placed into the vaginal orifice and the saline was flushed into the vagina 2-3 times. Next, this lavage fluid was placed on a glass slide and allowed to air dry with the help of a slide warmer. After completely dry, the slide was stained in a three-step process utilizing Mercedes Medical QUIK-DIP™ hematological stain. The slide was first dipped in a Coplin jar containing the first reagent, 80% methyl alcohol, 5 times for 1-2 seconds per dip. The excess reagent was drained off the slide with absorbent paper. Next, the slide was dipped in a Coplin jar containing the second reagent, xanthene dye (1 g/L), 5 times for 1-2 seconds per dip. The excess reagent was drained off the slide with absorbent paper. Finally, the slide was dipped in a Coplin jar containing the third reagent, thiazine dye (1.1 g/L), 5 times for 1-2 seconds per dip. The excess reagent was drained off the slide with absorbent paper and allowed to completely dry. The slides were then read by standard light microscopy using

10x magnification to assess cell density and 40x magnification to identify cells using a Fisher Scientific Micromaster® light microscope.

Dosing Protocol

The experimental paradigm used for the adult female rats closely followed the same design used for adult males from previous studies (Chambers *et al*., 2016a). Initial testing was performed to ensure the correct dose of PXN or NIMP to achieve LD_{99} results after 24 hours with only the administration of 0.65 mg/kg atropine free base in saline. That dose was determined to be 0.8 mg/kg and 0.6 mg/kg for PXN and NIMP, respectively, which was the same as that used previously for the adult male rats. Each rat was dosed with OP subcutaneously (s.c.), 2 minutes apart, except for one animal, which was dosed with only vehicle (Multisol, a biocompatible solvent consisting of 48.5% water, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol). Atropine and oximes were administered intramuscularly (i.m.) 25-30 minutes after dosing with OP or after onset of seizure-like signs (whichever comes first). One animal received only atropine (0.65 mg/kg, i.m.) as an OP control, while the remaining animals additionally received 1 of 4 oximes in Multisol (0.146 mmol/kg, i.m.): 2-PAM, oxime 15, oxime 20, or oxime 55. Animals were closely monitored and assessed for signs of cholinergic toxicity which followed a modified Racine scale for scoring seizures (Luttjohann *et al*., 2009). These signs were scored as follows: (0) normal behavior; (1) oro-alimentary movements (chewing); (2) anterior limb clonus and rearing; (3) loss of balance (falling) and shaking; (4) tonic convulsions; (5) death. The observer was blinded as to which oxime each animal received. Animals were observed closely for the first 8 hours

following treatment to assess and score signs of toxicity, and scored one last time at the 24-hour mark to terminate the *in vivo* portion of the experiment. Animals which did not exhibit seizure-like behavior after challenged with a lethal dose of OP were removed from the study.

Tissue Collection and Preparation

Rats which survived the 24-hour survivability challenge were humanely euthanized utilizing CO_2 for anesthesia followed by decapitation. Animals which died during the 8-hour surveillance period and those that survived 24 hours had tissues harvested to include the brain, skeletal muscle, liver, and diaphragm which were snap frozen in liquid nitrogen and stored at -80°C until they could be assayed. Whole blood was collected from these animals and allowed to clot in a 1.5 mL plastic centrifuge tube while kept on ice. The whole blood was then spun down using a centrifuge at 10,000 RCF at 4°C for 8 minutes. The serum was removed and kept frozen at -80°C until assayed.

Whole rat brain were homogenized separately utilizing a motorized homogenizer in 50 mM Tris HCl buffer (pH 7.4 at 25°C) to a stock solution of 40 mg/mL wet weight equivalent. Skeletal muscle was cut into small pieces and then homogenized utilizing a motorized homogenizer with blades in 50 mM Tris HCl buffer (pH 7.4 at 25°C) to a stock solution of 100 mg/ml wet weight equivalent. This homogenate was then filtered to remove any large particulate prior to assaying.

Measurement of Cholinesterase Activity

As described in the previous chapter, cholinesterase activity in harvested tissues was assayed utilizing a modified form of the Ellman discontinuous spectrophotometric cholinesterase assay (Ellman *et al.*, 1961). Rat brain or skeletal muscle homogenate was added at volumes of 50 µL or 100 µL, respectively to 50 mM Tris HCl buffer (pH 7.4 at 25°C) for a total volume (TV) of 2 mL and final concentration (FC) of 1 or 5 mg/mL wet weight equivalent, respectively. For each experimental unit, tissue was added to 5 tubes for experimental replication with 2 tubes serving as tissue blanks and 3 tubes in which the cholinesterase activity was measured. Both blanks received 20 µL of eserine sulfate (FC, 0.01 mM) which inhibits all cholinesterase activity in the tissue and served as a measurement of background interference caused by any non-AChE hydrolysis of the colorimetric substrate. All tubes were vortexed and incubated at physiologically relevant temperature, 37°C, in a shaking water bath to ensure the assay remains well mixed for 15 minutes. Next, 20 µL of acetylthiocholine (ATCh) was added (FC, 1 mM) to all tubes which serves as a substrate for the tissue AChE. This mixture was vortexed and incubated again for another 15 min at 37°C in a shaking water bath. Finally the reaction was terminated and color developed by the addition of 250 µL of a 4:1 mixture of 0.24 M 5,5'-dithio bis(2-nitrobenzoic acid) (DTNB) and 5% sodium dodecyl sulfate (SDS) to each tube. Each sample was read at 412 nm utilizing a Thermo Scientific Biomate 3 spectrophotometer which produced absorbance values (A) directly proportional to the amount of substrate hydrolyzed by AChE.

The enzymatic activity of AChE was targeted for assay in all harvested tissues, however, butyrylcholinesterase (BChE) activity was also a target for assay in serum

samples as well. Serum assays used 5 µL or 10 µL of undiluted serum for quantifying AChE or BChE activity, respectively, which were added to 50 mM Tris HCl buffer (pH 7.4 at 25°C) for a TV of 1 mL. Similar to the protocol outlined above for other tissues, five tubes were used for each experimental unit while all other reagents were added in amounts scaled to a 1 mL TV assay. The two blanks received 10 µL of eserine sulfate (FC, 0.01 mM) and all tubes were vortexed and incubated for 15 min at 37°C in a shaking water bath. Next, 10 µL of ATCh or butyrylthiocholine (BTCh) were added to each tube (FC, 1 mM) with BTCh acting as the preferred substrate for BChE to assess enzymatic activity (Masson and Lockridge, 2010). This mixture was vortexed and incubated again for another 15 min at 37°C in a shaking water bath. Finally, the reaction was terminated and color developed by the addition of 125 µL of 0.24 M DTNB/5% SDS to each tube. Each sample was read at 412 nm.

The method of Lowry *et al*. (1951) was used to quantify protein content of each tissue sample using bovine serum albumin as the standard. Specific activities for all tissues were calculated as nmoles min^{-1} mg P^{-1} using the equation outlined below:

$$Specific\ Activity\ = A \times \varepsilon \times \frac{TV\ (mL)}{T_c\ (mg)} \times \frac{T_p\ (mg)}{Lowry\ protein\ (\mu g)} \times \frac{1}{t\ (\text{min})} \times \frac{1000\ \mu g}{1\ mg} \quad (3.1)$$

The absorbance (A) of the tissue homogenate is multiplied by the molar extinction coefficient (ε) for SDS/DTNB which was determined to be 115.16 nmoles/mL·A by Dr. Howard Chambers. This product is multiplied by the TV divided by the total amount of tissue (T_c) in the cholinesterase assay. Next, the product is multiplied by the total amount of tissue (T_p) used in the Lowry protein assay divided by the determined protein content. Finally, the product is multiplied by the reciprocal of time used for incubation in the

cholinesterase assay (t = 15 min) and by a conversion factor to arrive at the desired units for specific activity.

Statistical Methods

The effect of oxime treatment on survival and odds ratios were calculated using 2-PAM as the referent with PROC LOGISTIC in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC.). Statistical significance was determined using an alpha level of 0.1 rather than 0.05 in order to reduce the number of animals needed for the dosing protocol while still allowing for statistical power. Differences in mean time of cessation of seizure-like behavior were determined using PROC LIFETEST in SAS for Windows 9.4. Data were censored if the animal died before the end of the 8-hour observational period or if seizure-like behavior continued past this window. The log-rank test statistic was used to assess significance and Dunnett's adjustment used for multiple comparisons of the novel oximes against 2-PAM. Differences in mean cholinesterase activity from harvested tissues between oxime treatments were determined by ANOVA using PROC GLM in SAS for Windows 9.4 with TUKEY adjustment, and an alpha level of 0.05 was used to determine statistical significance.

Results

Determination of the rat estrus cycle was performed prior to initiation of the dosing protocol. Each phase of the estrus cycle was identified by examining the vaginal cytology of each rat as outlined in Cora *et* al. (2015). The proestrus phase was characterized by small nucleated epithelial cells of low to moderate density found individually or in clumps (Figure 11). Estrus was defined by the presence of

predominantly anucleated keratinized epithelial cells (Figure 12). Metestrus was rarely seen as this phase is very short lasting about 6 – 8 hours. A combination of anucleated keratinized epithelial cells and neutrophils are seen in this phase with the number of neutrophils often outnumbering the epithelial cells 10:1. Finally, the last stage of interest, diestrus, was characterized by a substantial decrease in the number of epithelial cells seen with varying numbers of neutrophils present. This phase has low to moderate cell density and although the number of neutrophils can vary, they are always present in diestrus (Figure 13).

After the female rats were determined to be in the diestrus phase of the estrus cycle, the experiment proceeded to the dosing protocol ensuring that enough animals were available to represent each oxime in every experimental group of rats. All animals were administered with a LD_{99} dosage of either PXN or NIMP except those serving as controls receiving only Multisol and atropine. Of those animals dosed with OP receiving atropine only as therapy, none survived when challenged with NIMP while only one survived against PXN. Most died shortly after being challenged with OP during the 8-hour observational period with the average time to death being about 1 hour for both PXN and NIMP.

Animals treated with novel oximes showed improvement over 2-PAM against both OP compounds in 24-hr survivability. The percentage of improvement for the novel oximes as compared to 2-PAM is shown in Figure 14. Novel oximes 15 and 55 demonstrated the best performance against PXN increasing the odds of survival by 6.5 and 3.1 times that of survivability with 2-PAM, respectively. Novel oxime 20 performed

the best against NIMP significantly increasing ($p < 0.1$) survivability with an odds ratio of 3.2 as compared to 2-PAM. Only oxime 55 did not increase 24-hour survivability as compared to 2-PAM when the animals were challenged with a lethal dose of NIMP. Survival data for all treated groups are summarized in Table 5.

Time to seizure cessation were evaluated for all four oximes against both PXN and NIMP for rats surviving the 8-hour observational period (Table 6). Novel oxime 55 performed the best against PXN significantly reducing ($p < 0.05$) seizure-like behavior by 2 hours on average as compared to 2-PAM. A Kaplan-Meier plot demonstrates the performance of all the oximes against PXN in reducing seizure-like behavior in Figure 15. Novel oxime 20 performed the best against NIMP significantly reducing ($p < 0.05$) seizure-like behavior by 1.8 hours on average as compared to 2-PAM. A Kaplan-Meier plot demonstrates the performance of all the oximes against NIMP in reducing seizure-like behavior in Figure 16. Overall, the novel oximes produced an average reduction in the duration of seizure-like behavior of about 1-1.5 hours by comparison with 2-PAM.

The cholinesterase activity of brain, skeletal muscle, and serum was assessed for those adult female rats surviving 24 hours (Tables 7-9). In most of the assayed tissues, there was no difference in mean activity among the oxime treatment groups. There was one statistical difference ($p < 0.05$), mean serum activity of AChE and BChE was higher in the oxime 15 treated group than the other oxime groups when rats were challenged with NIMP. Correspondingly, mean inhibition percentages were lower while recovery of enzymatic activity was higher for NIMP-dosed animals treated with oxime 15 as compared to the other oximes. These differences in the serum of NIMP-dosed animals

were not seen in the PXN-treated group as all the oximes seem to better protect against cholinesterase inhibition by PXN.

Discussion

In order to further the development of these novel oximes as a potential improvement to current organophosphate treatment, new information was needed to ensure that they have similar effects between males and females. This study was designed to mimic previous studies done in this laboratory which tested the efficacy of the novel oximes in adult male rats (Chambers *et al*., 2016a). Data from additional studies on adult male rats which have not been published as of the writing of this dissertation were also consulted for comparison with the data garnered in this research involving adult female rats.

Compared to adult male rats, their female counterparts in this study displayed very similar 24-hour survivability improvements when treated with the novel oximes as compared to 2-PAM. These similarities were evident even with the fact that the same lethal doses of PXN (0.8 mg/kg, s.c.) and NIMP (0.6 mg/kg, s.c.) were used on both sexes. The percentage of adult male rats dosed with PXN surviving 24 hours and treated with novel oximes 15, 20, and 55 was 85%, 30%, and 70%, respectively (unpublished data). In this study, the percentage of adult female rats dosed with PXN surviving 24 hours and treated with novel oximes 15, 20, and 55 was 94%, 78%, and 89%, respectively. Looking at oxime efficacy against a lethal dosage of the sarin surrogate, NIMP, the percentage of adult male rats surviving 24 hours and treated with novel oximes 15, 20, and 55 was 60%, 55%, and 30%, respectively (Chambers *et al*., 2016a;

unpublished data). In this study, the percentage of adult female rats dosed with NIMP surviving 24 hours and treated with novel oximes 15, 20, and 55 was 56%, 67%, and 28%, respectively.

From the comparison above, it has been demonstrated that these novel oximes all improve survivability as compared to 2-PAM except for oxime 55 against lethal doses of NIMP. All oximes, including 2-PAM, seemed to perform better in adult female rats dosed with PXN in this study compared to the male rats. Since these animals were dosed with PXN, and not the parent compound parathion, a mechanism based on OP metabolism seems an unlikely explanation for this difference. Indeed, as mentioned previously, Agarwal *et al*. (1982) demonstrated there was no difference seen in PXN toxicity between male and female rats.

During the initial 8-hour observation period of the dosing protocol, the female rats were closely monitored to record the time of cessation of seizure-like behavior. Novel oximes decreased the time to cessation of seizure-like behavior as compared to 2-PAM with oxime 55 and 20 significantly reducing ($p < 0.05$) seizure time against PXN and NIMP, respectively. This reduction in seizure-like behavior aligned with the increased survival percentages seen with the novel oximes, although this seizure-like behavior cessation was not as pronounced as that seen earlier in male rats. The novel oximes in male rats reduced seizure-like behavior on average 2-3 hours sooner in comparison with 2-PAM which offered little to no protection (unpublished data). For female rats, again 2-PAM performed better against PXN in this study such that the novel oximes produced an

average reduction in the duration of seizure-like behavior of about 1-1.5 hours by comparison.

While it is difficult to determine if these differences seen between the sexes in two separate studies are significant, what is clear is that oxime therapy is more successful against PXN than NIMP. This is most likely due to the fact that experimentally determined inhibition kinetics show that NIMP is indeed more potent than PXN (Coban *et al*., 2016). Additionally, aging probably occurs much faster with NIMP-inhibited AChE than it does with PXN. Aging halftimes for sarin have been experimentally determined to be about 3 hours while aging of diethyl-phosphorylated AChE, as a result of PXN inhibition, occurs in about 32 hours (Worek *et al*., 2004). It appears from these data that adult female rats dosed with PXN seem to respond slightly better to therapy than the adult males demonstrating slightly improved survivability and cessation of seizure-like behavior with all oximes, including 2-PAM. This observation may be indicative of the ability of estrogenic sex hormones to bolster physiological defenses against OPs, such as the blood-brain barrier, as proposed in other reports which indicate that females are better protected against experimentally-induced brain damage than males (Pittel *et al*., 2018).

Analysis of harvested tissues from 24-hour survivors challenged with a lethal OP dose show that there was largely no difference in mean inhibition or reactivation of cholinesterase activity between the oxime treatment groups with the one exception being the performance of oxime 15 in NIMP-inhibited serum. In the absence of any additional trends in cholinesterase activity from the other tissues, it is difficult to ascertain if this

statistically significant result bears any physiological significance. The lack of any other appreciable differences are most likely due to the experimental design of this research which uses high concentrations of OP and relatively quick administration of oxime (usually within 25-30 min of dosing with OP). These high concentrations of circulating OP would be available to re-inhibit cholinesterase enzyme reactivated by oxime.

Other studies have been performed which were designed to prevent this phenomenon of re-inhibition by using a smaller, sub-lethal dose of OP and waiting about 1 hour before oxime administration when maximal enzyme inhibition presumably should have occurred. This allows for the circulating concentration of OP to diminish before oxime administration mitigating the risk of re-inhibition (Chambers *et al*., 2013). Using this paradigm, oxime 20 has demonstrated about 25% reactivation of NIMP-inhibited rat brain AChE with little to no reactivation by 2-PAM which would be more consistent with the 24-hour survivability data seen in this research (Chambers *et al*., 2016b).

Conclusion

The data presented here demonstrate improved survivability with our novel oximes as compared to 2-PAM against both OP compounds. All the novel oximes tested against PXN increased 24-hour survivability as compared to 2-PAM at the same molar equivalent. Novel oxime 20 performed the best against the sarin surrogate, NIMP, significantly ($p < 0.1$) improving 24-hour survivability 3.2 times greater than 2-PAM. While the 24-hour survival percentages in these data with female rats were not statistically significant at $p < 0.05$, the results are trending towards similar patterns of improvements over 2-PAM as those seen in male rats from previous studies (Chambers *et*

al., 2016a; unpublished data). Reduced seizure-like behavior was seen in animals treated with novel oximes as compared to 2-PAM. Novel oximes 55 and 20 significantly reduced ($p < 0.05$) the time of cessation of seizure-like behavior against PXN and NIMP, respectively. These data provide additional support to the concept that our novel oximes can enter the brain.

Table 5 24-Hour Survival of Treated Rats with Lethal Dosages of Organophosphate

	PXN			NIMP		
Oxime	**Surv./Trted.**	**%Surv.**	**Odds Ratio**	**Surv./Trted.**	**%Surv.**	**Odds Ratio**
None	1/18	6		0/18	0	
2-PAM	13/18	72		7/18	39	
Oxime 15	17/18	94	6.5	10/18	56	2.0
Oxime 20	14/18	78	1.4	12/18	67	3.2*
Oxime 55	16/18	89	3.1	5/18	28	0.6

Percent survival after 24 hours of adult (PND 70) female rats treated with paraoxon (PXN) (0.8 mg/kg s.c.) or 4-nitrophenyl isopropyl methylphosphonate (NIMP) (0.6 mg/kg s.c.) followed at 30 min by atropine (0.65 mg/kg i.m.) and oxime (0.146 mmol/kg i.m.) or Multisol vehicle control. Odds ratios were calculated for survival against 2-PAM. * = statistical significance ($p < 0.1$) vs 2-PAM

Table 6 Cessation of Seizure-like Behavior in Rats Surviving 8 Hours

	PXN			NIMP		
Oxime	**n**	**Time**	**p Value**	**n**	**Time**	**p Value**
2-PAM	15	7.3 ± 1.2		9	8.0 ± 0	
Oxime 15	17	5.9 ± 2.0	0.097	13	7.6 ± 0.9	0.858
Oxime 20	15	6.3 ± 1.6	0.115	14	6.2 ± 2.2	0.001
Oxime 55	17	5.2 ± 1.7	0.001	8	6.9 ± 1.7	0.343

Results expressed as average time (hours) until the cessation of seizure-like behavior in female rats treated with paraoxon (PXN) (0.8 mg/kg s.c.) or 4-nitrophenyl isopropyl methylphosphonate (NIMP) (0.6 mg/kg s.c.) followed at 30 min by atropine (0.65 mg/kg i.m.) and oxime (0.146 mmol/kg i.m.). Statistical significance ($p < 0.05$) determined vs 2-PAM.

Table 7 Tissue Specific Activity from Adult Female Rats Surviving 24 Hours

	PXN					NIMP				
			Serum					Serum		
Oxime	n	Brain	AChE	BChE	SKM	n	Brain	AChE	BChE	SKM
Control	10	97.9 ± 9.4	34.6 ± 7.1	12.8 ± 2.5	10.3 ± 2.3	10	95.0 ± 12.8	30.9 ± 5.6	11.6 ± 1.9	12.7 ± 1.9
2-PAM	13	37.0 ± 7.8	26.4 ± 5.4	9.9 ± 2.5	7.6 ± 2.3	7	17.7 ± 1.5	13.6 ± 4.4	5.3 ± 2.1	4.1 ± 0.7
Oxime 15	17	36.3 ± 3.8	27.0 ± 5.7	10.0 ± 2.4	7.2 ± 1.7	10	14.8 ± 3.5	20.3 ± 5.5*	8.5 ± 2.7*	5.3 ± 1.6
Oxime 20	14	37.2 ± 2.9	26.9 ± 6.4	10.2 ± 2.9	6.3 ± 1.3	12	19.0 ± 12.3	15.6 ± 4.0	6.0 ± 1.9	5.2 ± 1.4
Oxime 55	16	37.8 ± 5.8	28.0 ± 9.1	11.0 ± 4.8	11.0 ± 4.8	5	14.3 ± 1.3	16.7 ± 2.1	7.0 ± 1.1	4.3 ± 1.7

Specific activities expressed as nmoles min^{-1} mg P^{-1}, means ± standard deviation in rats surviving 24 hours after treatment with paraoxon (PXN) (0.8 mg/kg s.c.) or 4-nitrophenyl isopropyl methylphosphonate (NIMP) (0.6 mg/kg s.c.) followed at 30 min by atropine (0.65 mg/kg i.m.) and oxime (0.146 mmol/kg i.m.). The control animals were not treated with OP, only with Multisol vehicle and atropine (0.65 mg/kg i.m.), and are referenced here for comparison against OP treated animals.

* = statistical significance ($p < 0.05$) vs 2-PAM

AChE = acetylcholinesterase; BChE = butyrylcholinesterase; SKM = skeletal muscle

Table 8 Tissue Inhibition from Adult Female Rats Surviving 24 Hours

	PXN					NIMP				
			Serum					Serum		
Oxime	n	Brain	AChE	BChE	SKM	n	Brain	AChE	BChE	SKM
2-PAM	13	62 ± 8.0	24 ± 15.2	25 ± 16.4	27 ± 20.4	7	81 ± 1.8	60 ± 11.4	59 ± 15.1	68 ± 5.7
Oxime 15	17	63 ± 3.9	22 ± 16.0	24 ± 18.8	31 ± 15.1	10	84 ± 3.6	36 ± 17.9*	29 ± 23.3*	60 ± 12.5
Oxime 20	14	62 ± 3.0	23 ± 16.1	23 ± 18.8	38 ± 12.5	12	80 ± 12.9	50 ± 13.0	49 ± 16.8	59 ± 11.3
Oxime 55	16	61 ± 5.9	23 ± 17.9	23 ± 20.8	36 ± 18.7	5	85 ± 1.4	46 ± 8.9	40 ± 8.9	66 ± 13.2

Inhibition expressed as a percentage (%), means ± standard deviation in rats surviving 24 hours after treatment with paraoxon (PXN) (0.8 mg/kg s.c.) or 4-nitrophenyl isopropyl methylphosphonate (NIMP) (0.6 mg/kg s.c.) followed at 30 min by atropine (0.65 mg/kg i.m.) and oxime (0.146 mmol/kg i.m.).

* = statistical significance ($p < 0.05$) vs 2-PAM

AChE = acetylcholinesterase; BChE = butyrylcholinesterase; SKM = skeletal muscle

Table 9 Tissue Recovery from Adult Female Rats Surviving 24 Hours

	PXN					NIMP				
			Serum					Serum		
Oxime	n	Brain	AChE	BChE	SKM	n	Brain	AChE	BChE	SKM
2-PAM	13	36 ± 8.3	69 ± 19.9	64 ± 23.9	68 ± 23.7	7	15 ± 1.6	24 ± 19.5	21 ± 22.5	7 ± 6.6
Oxime 15	17	35 ± 4.0	71 ± 20.9	65 ± 22.9	64 ± 17.6	10	12 ± 3.9	53 ± 24.3*	58 ± 33.8*	19 ± 16.8
Oxime 20	14	36 ± 3.1	69 ± 21.1	67 ± 27.4	56 ± 14.5	12	17 ± 13.7	32 ± 17.7	27 ± 24.2	18 ± 15.7
Oxime 55	16	36 ± 6.1	70 ± 23.4	67 ± 30.4	58 ± 21.7	5	12 ± 1.4	37 ± 9.4	40 ± 13.8	11 ± 15.6

Recovery expressed as a percentage (%), means ± standard deviation in rats surviving 24 hours after treatment with paraoxon (PXN) (0.8 mg/kg s.c.) or 4-nitrophenyl isopropyl methylphosphonate (NIMP) (0.6 mg/kg s.c.) followed at 30 min by atropine (0.65 mg/kg i.m.) and oxime (0.146 mmol/kg i.m.).

* = statistical significance ($p < 0.05$) vs 2-PAM

AChE = acetylcholinesterase; BChE = butyrylcholinesterase; SKM = skeletal muscle

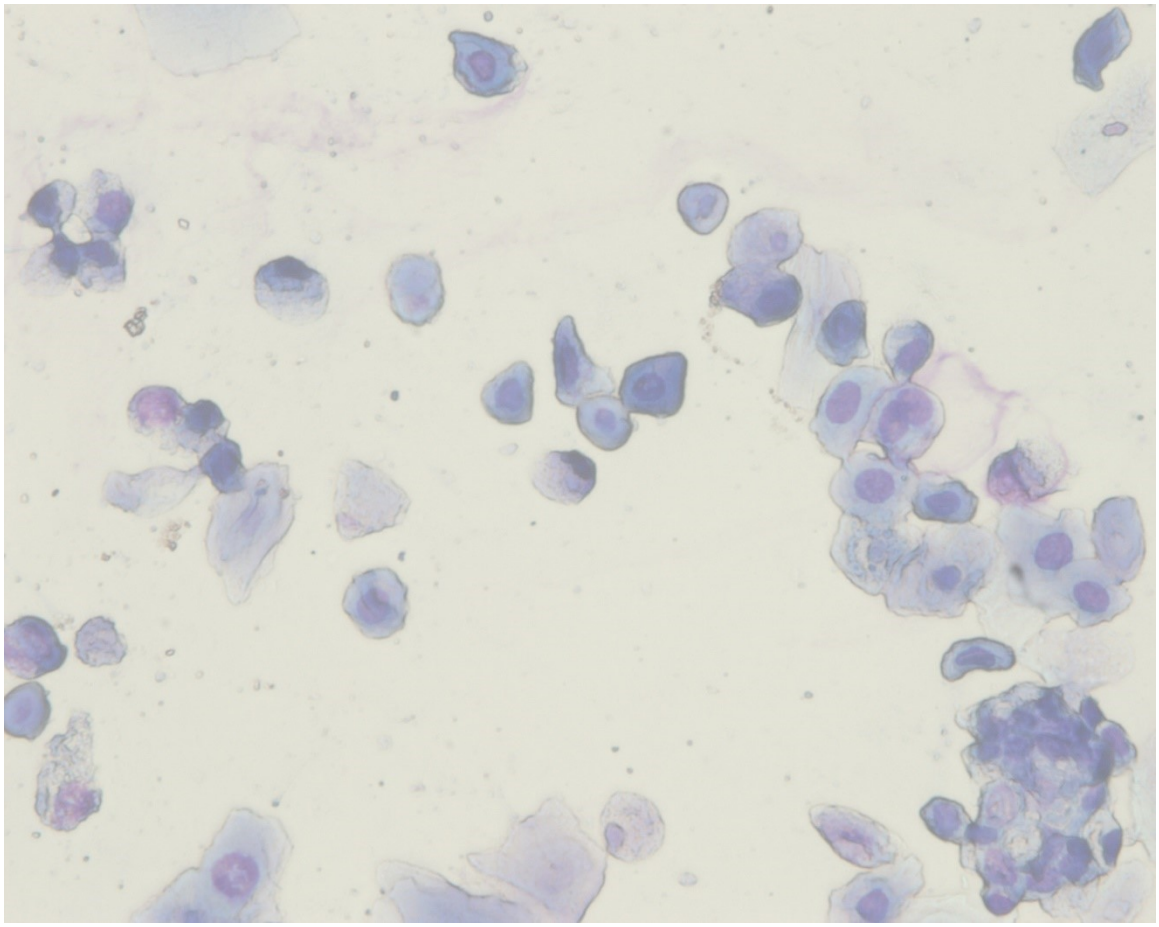

Figure 11 Vaginal Cytology of Proestrus Phase at 40x Magnification in an Adult Female Sprague Dawley Rat.

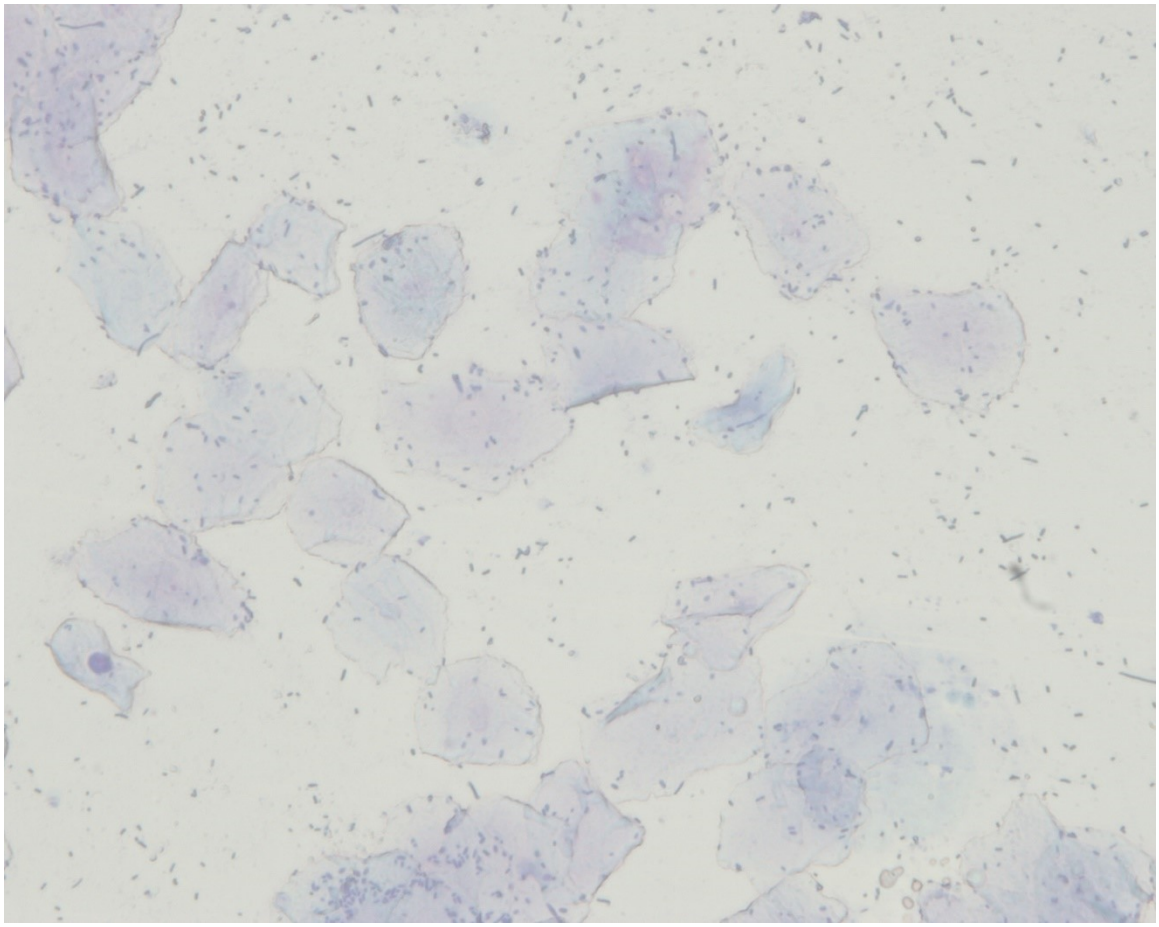

Figure 12 Vaginal Cytology of Estrus Phase at 40x Magnification in an Adult Female Sprague Dawley Rat.

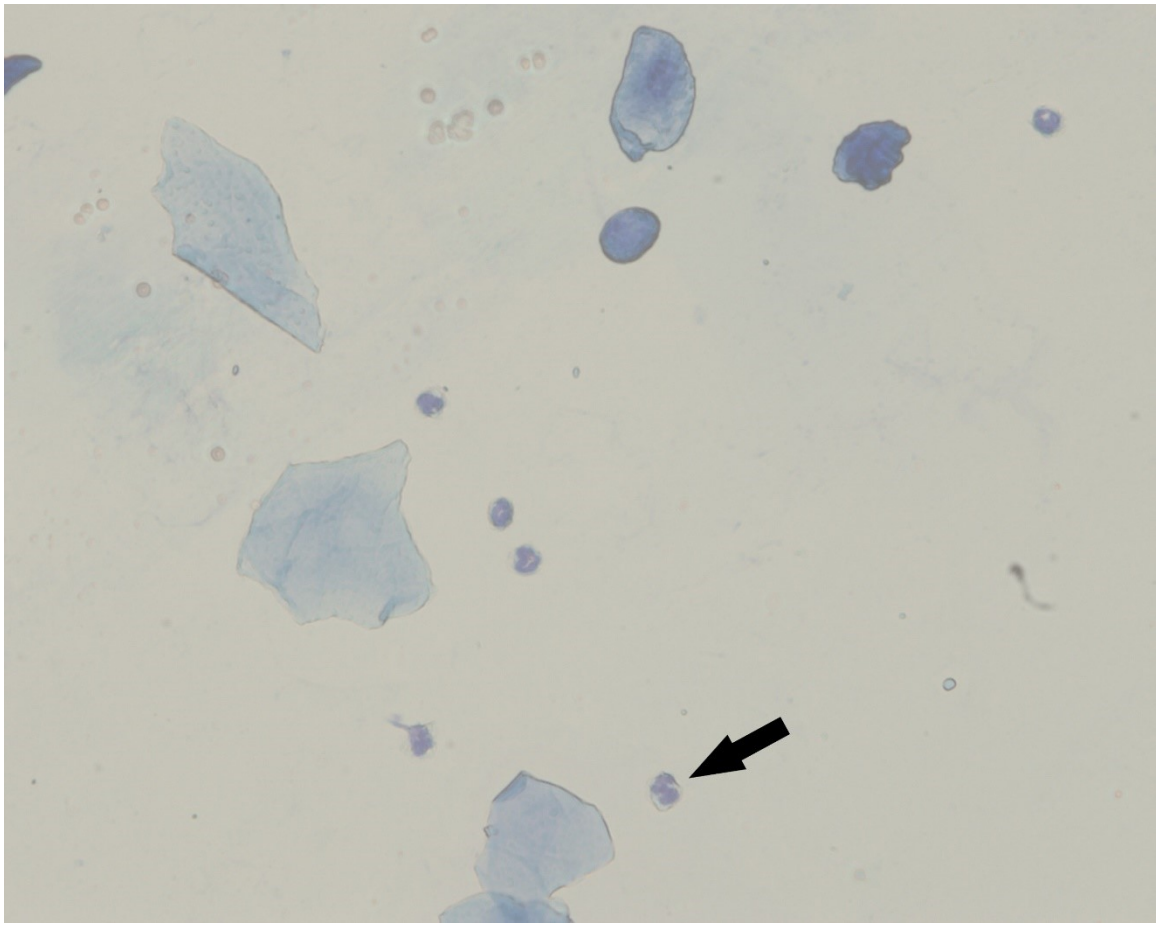

Figure 13 Vaginal Cytology of Diestrus Phase at 40x Magnification in an Adult Female Sprague Dawley Rat. A neutrophil is indicated with an arrow.

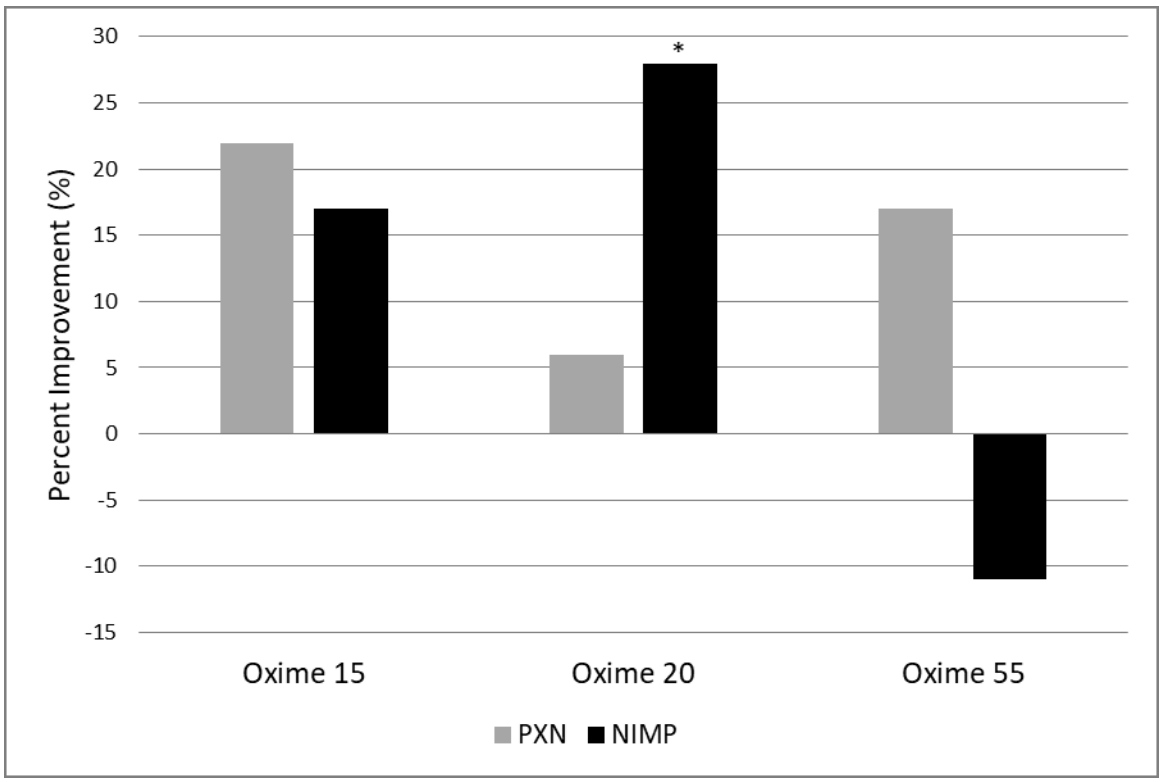

Figure 14 24-Hour Survival Improvement in Treated Rats Compared to 2-PAM.

Percent improvement in survival (24-hour) of treated rats with lethal dosages of paraoxon (PXN) (0.8 mg/kg, s.c.) and 4-nitrophenyl isopropyl methylphosphonate (NIMP) (0.6 mg/kg, s.c.), atropine (0.65 mg/kg, i.m.), and novel oximes (0.146 mmol/kg, i.m.) compared to 2-PAM.
* = statistical significance ($p < 0.1$) vs 2-PAM

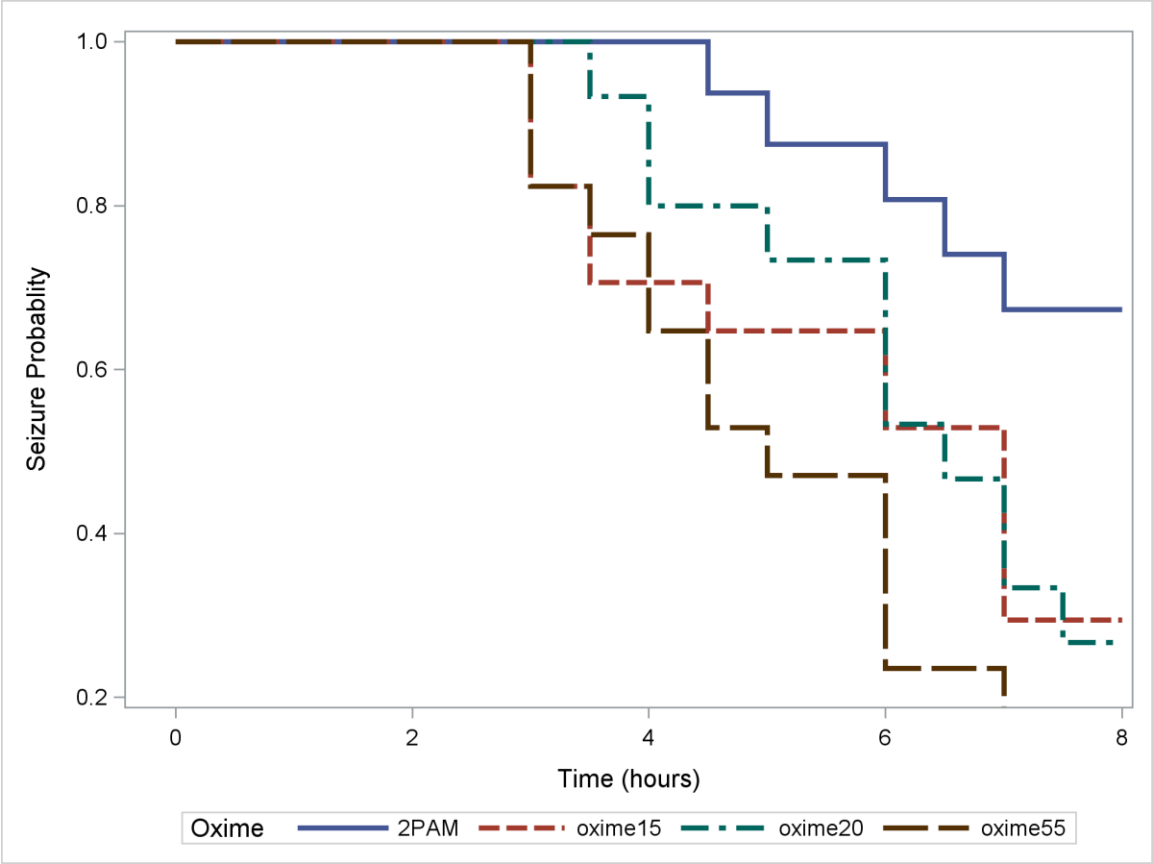

Figure 15 Novel Oxime Cessation of Seizure-like Behavior Against PXN.

Kaplan-Meier analysis of time to cessation of seizure-like behavior during the first 8 hours following paraoxon (PXN) administration (0.8 mg/kg, s.c.) in rats treated with atropine (0.65 mg/kg, i.m.) and oximes (0.146 mmol/kg, i.m.). Statistical analysis results of novel oximes compared to 2-PAM alone: Oxime 15, $p = 0.097$; Oxime 20, $p = 0.115$; Oxime 55, $p = 0.001$.

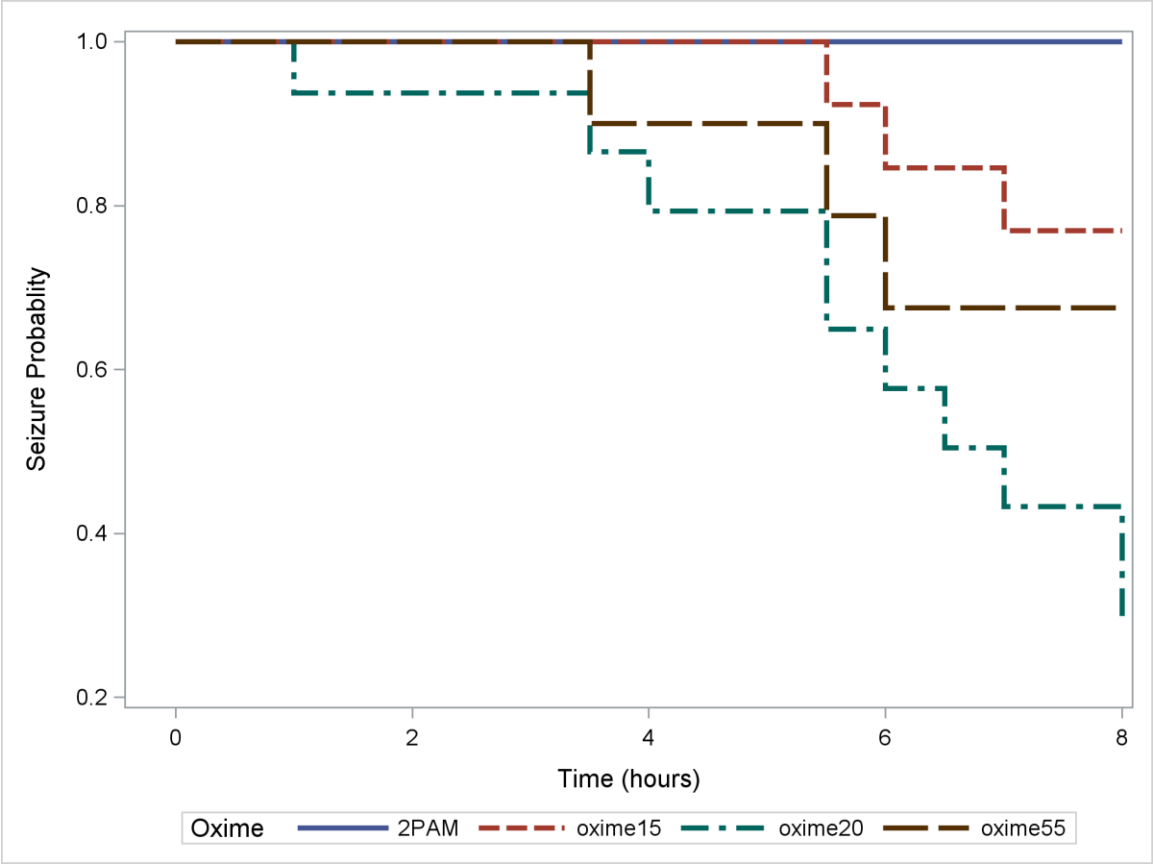

Figure 16 Novel Oxime Cessation of Seizure-like Behavior Against NIMP.

Kaplan-Meier analysis of time to cessation of seizure-like behavior during the first 8 hours following 4-nitrophenyl isopropyl methylphosphonate (NIMP) administration (0.6 mg/kg, s.c.) in rats treated with atropine (0.65 mg/kg, i.m.) and oximes (0.146 mmol/kg, i.m.). Statistical analysis results of novel oximes compared to 2-PAM alone: Oxime 15, $p = 0.858$; Oxime 20, $p = 0.001$; Oxime 55, $p = 0.343$.

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CHAPTER IV

CONCLUSION

The usage of organophosphate (OP) compounds continues to be a threat to human health, particularly when employed as chemical weapons. Recently, on February 3, 2017, two women with ties to North Korea used their bare hands to apply nerve agent to the face of Kim Jong-nam, the older brother of North Korean Dictator, Kim Jong-un. The attack ended with the death of their target as well as one of the women becoming severely ill. It is thought that they used a derivative of the nerve agent VX, VX2, which consists of two compounds that are harmless by themselves, but become toxic when mixed together (Paddock *et al*., 2017). On March 4, 2018, an obscure OP nerve agent, Novichok, was used in an assassination attempt against a former Russian spy. This nerve agent was presumably developed by the Russians and is estimated to be 5 times more potent than VX or sarin (Sang, 2018). Despite the continued usage of OP nerve agents, and their apparent novel formulations, drug therapy countermeasures such as treatment using oximes has not changed since the early 1960s. Current OP therapy in the United States still relies on the first oxime utilized in a clinical setting, pralidoxime (2-PAM), as the reactivator of choice which was developed in 1955 (Childs *et al*., 1955; Worek *et al*., 2016).

The primary goal of this research, and other previously mentioned research from this laboratory, is providing evidence that our novel oximes are better reactivators which demonstrate improved survivability due to an improved ability to better penetrate the

blood-brain barrier as compared to 2-PAM. Much of the initial studies conducted in this laboratory, as well as some unpublished data, were performed using adult male rats as the animal model (Chambers *et al*., 2013; Chambers *et al*., 2016a; Chambers *et al*., 2016b). Therefore, the focus of these experiments was to demonstrate that our novel oximes perform with similar efficacy in adult female rats and, to a lesser extent, juvenile rats of both sexes.

In the first part of this study, a number of *in vitro* experiments were performed to assess cholinesterase activity of rat brain tissue, and to compare oxime efficacy and oxime-mediated reactivation of OP-inhibited AChE in different age and sex groups. Data from these experiments indicate that all of the novel oximes reactivate OP-inhibited acetylcholinesterase (AChE) with equal efficacy in male and female rats of both age groups tested, post-natal day (PND) 70 and 12. There was one statistically significant difference ($p < 0.05$) detected when comparing the age groups. Oximes 15 and 20 demonstrated about a 10% improvement in reactivation of PND 12 brain AChE inhibited by 4-nitrophenyl isopropyl methylphosphonate (NIMP) as compared to the PND 70 age group. While the mechanism for this improvement in juvenile tissue is unknown, future *in vivo* experiments could confirm this age-dependent increase in reactivation efficacy if juvenile rats dosed with NIMP displayed improved survivability in comparison to their adult counterparts.

Oxime efficacy was compared via calculation of the effective concentration at which 25% reactivation (EC_{25}) was achieved. For both OPs, paraoxon (PXN) and NIMP, 2-PAM required the lowest concentration to achieve an EC_{25}. This observation was confirmed in the other *in vitro* studies where 2-PAM consistently performed the best

against both OPs with the highest reactivation percentages of all the oximes studied. This performance, however, does not translate into improved survivability during *in vivo* studies where the novel oximes outperform 2-PAM at the same molar equivalent dosage in this research with adult female rats as well as past reports with adult male rats (Chambers *et al*., 2013; Chambers *et al*., 2016a; Chambers *et al*., 2016b). This is the main difference between 2-PAM and the novel oximes which lends evidence towards the theory that these new oximes are better suited to cross the blood-brain barrier to reactivate inhibited brain AChE. Even though 2-PAM consistently outperforms the novel oximes during *in vitro* reactivation studies, small improvements in blood-brain permeability can have large impacts on therapy against OP toxicity as even partial reactivation has been shown to significantly reduce the amount of atropine needed to treat a patient (Thiermann *et al*., 2011).

The second part of this study focused on demonstrating *in vivo* efficacy of our novel oximes as compared to 2-PAM in adult female rats. The novel oximes demonstrated improved survivability approximately equivalent to that seen in adult male rats. Survivability data from the adult female rats in this experiment show that oxime 15 and 55 perform the best against PXN, while oxime 20 had the best odds ratio against NIMP. The EC_{25} values determined in the first part of this study predicted that Oximes 15 and 55 would be more effective against PXN than Oxime 20 while the latter would be the most effective of the novel oximes against NIMP. These *in vitro* predictions matched with the improvements seen in the *in vivo* survivability experiments.

More evidence from this research towards the idea that these novel oximes better penetrate the blood-brain barrier as compared to 2-PAM is the time to cessation of

seizure-like behavior demonstrated in the *in* vivo study among the treatment groups. All of the novel oximes reduced the time to cessation of seizure-like behavior as compared to 2-PAM against both OPs. Significant reductions ($p < 0.05$) in seizure-like behavior were seen for the oxime 20 and 55 treatment groups when the rats were challenged with NIMP and PXN, respectively.

This research utilized two OP compounds, PXN which is the active metabolite of the insecticide parathion, and the other, NIMP, a surrogate for the nerve agent, sarin. This was done to demonstrate broad spectrum effectiveness, and improvement of our novel oximes over 2-PAM. These data indicate improved survivability and reduced duration of seizure-like behavior against both compounds. While more studies will be needed to further development and to garner evidence of improved efficacy against traditional oxime therapy, this research demonstrates that these novel oximes show great promise as the first improvements to antidotes against acute OP toxicity in 60 years.

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APPENDIX A

DISCLAIMER

The views expressed in this dissertation are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.