Changes in Circulating Levels of the Traumatic Brain Injury Biomarkers S100B and UCH-L1 in Soman Exposed Sprague Dawley Rats

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This work was performed by Battelle Memorial Institute (Columbus, OH)

This work was supported by the NIH Chemical Countermeasures Research Program and funded by the NIH Office of the Director through an interagency agreement (OD#: Y1- OD-0387-01) between NIAID and Department of Defense (DoD) and prepared under the auspices of the NIH, NIAID, and the DoD Defense Technical Information Center (DTIC) under the CBRNIAC program, Contract No. SP0700-00-D-3180, Delivery Order Number 0794, CBRNIAC Task 689/CB-13-0689.

Report submitted to DTIC on July 27, 2020

HIGHLIGHTS

- Goal: Identify circulating proteins as potential indicators of OP exposure and -induced toxicity
- Evaluated markers of CNS injury (BDNF, GFAP, NSE, NMDAR, S100B, and UCH-L1)
- Significant changes observed in the serum levels of S100B and UCH-L1

ABSTRACT

Organophosphorus (OP) chemical warfare nerve agents (CWNAs) and pesticides exert toxicity primarily through the inhibition of acetylcholinesterase. If left uncontrolled, hypersynchronous activity within the central nervous system can result in neuronal loss, neuroinflammation, and network reorganization. While these pathological outcomes are common to many neurodegenerative conditions, it remains unresolved whether OP-induced toxicity also results in detectable changes in circulating protein levels similar to other CNS injuries. The goal of this pilot study was to identify circulating proteins that may serve as indicators of OP-induced toxicity. Adult male Sprague-Dawley rats were challenged with the CWNA soman and monitored over 72h post-exposure. Blood was collected at 6h, 24h, and 72h post-exposure and analyzed for candidate biomarker levels. The circulating protein candidates selected were based on other central injury models and included BDNF, GFAP, NSE, NMDAR, S100B, and UCH-L1. Significant changes were observed in the serum levels of S100B and UCH-L1. Collectively, this study demonstrates that in addition to quantifying cholinesterase activity, circulating proteins could be utilized to corroborate OP exposure. Additional studies are warranted to identify whether changes in the levels of these circulating proteins may also correlate with the extent of neuronal injury following OP exposure to guide therapeutic treatment.

KEYWORDS

Biomarkers, organophosphates, chemical warfare nerve agents, CNS

ABBREVIATIONS

OP: Organophosphorous

CNS: Central Nervous System

CWNA: Chemical Warfare Nerve Agent

SD: Sprague-Dawley rat

BDNF: Brain Derived Neurotrophic Factor

GFAP: Glial Fibrillary Acidic Protein

NSE: Neuron Specific Enolase

NMDAR: N-Methyl-D-Aspartate Receptor

S100B: S-100 Calcium-Binding Protein B

UCH-L1: Ubiquitin C-Terminal Hydrolase-L1

IMS: Intermediate Syndrome

OPIDN: OP-Induced Delayed Neuropathy

AMN: Atropine Methyl Nitrate

2-PAM Cl: oxime, 2-pralidoxime chloride

HI-6: oxime, 1-[[[4-(Aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]-pyridinium chloride,4-Carbamoyl-1-[[[2-[(hydroxyimino)methyl]pyridinium-1-yl]methoxy]methyl]pyridinium dichloride

LD_{50}: Administered dose which results in 50% lethality

1.0 INTRODUCTION

Organophosphorus-containing chemicals (OPs), which include many pesticides and chemical warfare nerve agents (CWNAs), are some of the most potent neurotoxicants known. Deliberate and/or accidental exposure to OP compounds is a significant threat to human health worldwide. For example, OPs have been intentionally employed in large scale acts of terror and war as well as suicide attempts [1-4]. Unintentional exposures resulting from industrial, agricultural, and occupational accidents have also been reported [5-7]. Depending on the OP, route of exposure, and dose, acute poisoning can rapidly lead to life-threatening neurological complications due to cholinergic crisis. Even if these severe toxic effects are successfully mitigated with the standard of care treatments (e.g., an antimuscarinic, an oxime acetylcholinesterase reactivator, and an anticonvulsant drug if convulsions are observed [8]), central neuropathology with associated long-term neurological deficits may still occur [9]. Similarly, while not as commonly reported as the acute signs and symptoms of toxicity, delayed chronic neurotoxic morbidities in the peripheral and central nervous system and/or mortality may also ensue days to weeks after initial exposure and treatment [10].

These delayed onset syndromes caused by OP intoxication include intermediate syndrome (IMS) and OP-induced delayed neuropathy (OPIDN). Unfortunately, the etiology of each of these syndromes is currently poorly understood. IMS typically develops within 24-96 hours after OP exposure and is characterized by widespread muscle weakness that can compromise respiratory muscle function and, if untreated, may ultimately lead to respiratory failure [11-13]. OPIDN generally involves muscle pain, numbness, and weakness in the lower limbs (and upper limbs in severe cases) that appears 1-3 weeks post-exposure [14]. Most individuals exposed to OP pesticides do not develop significant IMS or OPIDN, and there is currently no widely accepted means for predicting which patients will develop these morbidities nor a specific treatment approach [11-14]. Furthermore, the cause of the development of (or absence of) IMS and OPIDN following an OP nerve agent exposure remains unclear and requires additional study [15, 16].

In alignment with the thrust of the National Institutes of Health Chemical Countermeasures Research Program (CCRP) as detailed by Yeung and colleagues[17], the goal of this preliminary, exploratory/proof-of-feasibility study was to determine whether an acute systemic challenge with a convulsion-inducing dose of the OP CWNA soman generates a detectable change in the levels

of several select circulating proteins. Candidate circulating proteins were rationally identified from previous reports that demonstrated their potential utility as either diagnostic and/or prognostic markers of health outcomes in various central injury models, such as traumatic brain injury (TBI) [18-20], stroke [21-23], *status epilepticus*, seizures [24, 25], and OP exposure [26-28]. These circulating proteins included: Brain Derived Neurotrophic Factor (BDNF), Glial Fibrillary Acidic Protein (GFAP), Neuron Specific Enolase (NSE), N-Methyl-D-Aspartate Receptor (NMDAR), S-100 Calcium-Binding Protein B (S100B), and Ubiquitin C-Terminal Hydrolase-L1 (UCH-L1).

2.0 MATERIALS AND METHODS

2.1 Test Materials

A list of the study materials, the individual components, and the prepared concentrations are detailed in Table 1. The concentration of the soman challenge solution was confirmed prior to use on the study. No additional dose verification or purity confirmation of the treatment materials were conducted.

Table 1 Test Materials

Material	Concentration	Manufacturer
Soman	101 µg/mL	U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD).
HI-6 DMS*	125 mg/mL	Synthesized by Southwest Research Institute, San Antonio, TX.
AMN (atropine methyl nitrate)	4 mg/mL	BOC Sciences Shirley, NY
2-PAM (pralidoxime chloride)	102.8 mg/mL	King Pharmaceuticals, St Louis, MO
Soman Vehicle (saline)	0.9%	Hospira, Austin, TX
HI-6 DMS vehicle (saline)	0.9%	Hospira, Austin, TX
AMN vehicle (saline)	0.9%	Hospira, Austin, TX
2-PAM vehicle (saline)	0.9%	Hospira, Austin, TX

*(4-carbamoyl-1-[({2-[(E)-(hydroxyimino) methyl)] pyridinium-1-yl}methoxyl) methyl] pyridinium dimesylate

2.2 Animals

Male Sprague-Dawley rats approximately 8-10 weeks in age and weighing 256-323 grams, when placed on study, were obtained from Charles River Laboratories (Raleigh, NC) and

quarantined in accordance with Battelle procedures. Animals were weighed during quarantine and randomized by weight to treatment groups. Prior to challenge, animals were observed a minimum of twice daily for clinical signs, morbidity, and general husbandry. Challenge and treatment doses were based on weights taken the day before challenge. The study design is summarized in Table 2.

2.3 Experimental Design

For this study, the challenge level and therapeutic regimen were based on a soman-induced rat seizure model established at the U.S. Army Medical Research Institute for Chemical Defense (USAMRICD) [29]. This 1.6 x LD_{50} soman seizure model was used to maximize the chance of determining detectable changes in circulating protein biomarkers elicited by a nerve agent. The model was well suited for achieving the goals of this study for a number of reasons: 1) the relatively high dose of soman used was expected to readily cross the blood brain barrier (BBB) and induce *status epilepticus* leading to neuronal damage, 2) the model had been previously shown to cause seizures in 100% of the animals tested [30], and 3) the therapeutics (atropine methyl nitrate and the oximes, 2-pralidoxime chloride and HI-6) have poor BBB permeability and were expected to increase survival without exerting direct and potentially confounding effects in the CNS. Additionally, given that acute CNS damage could be expected to play a role in the development of IMS and OPIDN, benzodiazepines (which are typically indicated to ameliorate seizures) were not included in the treatment regimen in order to avoid the introduction of a complicating factor. However, given a higher than expected mortality (67%) in initial experiments at 1.6 x LD_{50}, the soman challenge level was decreased to 1.2 x LD_{50} (135 µg/kg) for subsequent experiments. At 1.2 x LD_{50} soman, all animals exhibited moderate to severe signs of OP intoxication (tremors and convulsions, respectively) with a decreased mortality rate (35%). Electroencephalogram (EEG) monitoring was not included in this proof of concept study, so it could not be determined with certainty whether the animals experienced seizures (especially non-convulsive seizures). However, previous studies have demonstrated seizures, cognitive deficits, and significant neuropathology following a 1.2 x LD_{50} soman exposure [31, 32], suggesting that the model employed in this study likely resulted in similar central damage.

Table 2 Experimental Design

Group Number	Pre-Treatment	Challenge Level	Countermeasure (CM) Treatments	Number of Animals per Group	Number of Animals Utilized for ELISA
1 (Naïve)	None	None	None	n=20 [7 (6 hrs), 7 (24 hrs), 6 (72 hrs)]	n=20 [7 (6 hrs), 7 (24 hrs), 6 (72 hrs)]
2 (Vehicle Control)	HI-6 vehicle	Soman Vehicle	Vehicle	n=36 (12 per time point)	n=36 (12 per time point)
3 (Countermeasure Control)	HI-6 (125 mg/kg)	Soman Vehicle	AMN, 2-PAM	n=36 (12 per time point)	n=36 (12 per time point)
4 (Soman Challenged)	HI-6 (125 mg/kg)	135 µg/kg Soman	AMN, 2-PAM	n=40 [12 (6 hrs), 12 (24 hrs), 16 (72 hrs)]	n=26 [8 (6 hrs), 9 (24 hrs), 9 (72 hrs)]

Group 1 consisted of 20 naïve animals that were used to establish baseline values for the proteins to be analyzed at sacrifice time points of 6, 24, and 72 hours. These animals received no treatment, challenge agent, or vehicle. Group 2 animals were pretreated with an intraperitoneal (IP) injection of the HI-6 oxime vehicle (saline) 30 minutes prior to a subcutaneous (SC) administration of the soman vehicle (saline). Approximately 1 minute after administration with the soman vehicle, the animals received intramuscular (IM) injections of atropine methyl nitrate (AMN) and 2-PAM Cl vehicles (saline was used for both vehicles). Group 2 animals were administered vehicle (saline) to control for the stress induced by handling and injections.

Group 3 animals were used to account for the effects of the countermeasures on the proteins of interest. Animals were pretreated with HI-6 (125 mg/kg, IP) 30 minutes prior to administration of the soman vehicle. Approximately 1 minute after the vehicle administration, the animals received countermeasures AMN (2.0 mg/kg, IM) and 2-PAM Cl (25 mg/kg, IM).

Forty Group 4 animals were pretreated with HI-6 (125 mg/kg, IP) 30 minutes prior to SC challenge with 135 µg/kg (1.2 x LD_{50}) of soman. Approximately 1 minute after challenge, the animals received AMN (2.0 mg/kg, IM) and 2-PAM Cl (25 mg/kg, IM).

2.4 Blood Sample Collection

Surviving animals from each group were serially sacrificed and blood was collected at each of the 3 sample collection time points (6 hrs, 24 hrs, and 72 hrs post-challenge). The animals were anesthetized with a ketamine/xylazine mixture and approximately 5-10 mL of blood was collected from each animal. The whole blood was drawn into serum separation tubes (SST) and retained at 2-8ºC until processing. Samples were placed into a centrifuge and spun for 10 minutes at 1,300xg to partition the serum. The serum was removed and stored at ≤ -70ºC until analysis.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA) Testing

ELISA kits for each of the previously selected proteins were obtained (Table 3) and optimized for the samples used. Manufacturer's instructions served as guiding documents for each of the ELISA kits used throughout the study. Variations from the manufacturer provided protocols were minimal and typically involved deviating from the recommended starting dilution of a sample to obtain results within the reference standard range provided.

Table 3 ELISA Kits

Target Protein	Manufacturer	Product Description	Catalog #
Brain Derived Neurotrophic Factor (BDNF)	Promega	BDNF Emax Immunoassay System	G7610, G7611
Glial Fibrillary Acidic Protein (GFAP)	Lifespan	Rat GFAP ELISA Kit	LS-F11532
S-100 Calcium-Binding Protein B (S100B)	Millipore	Human S100B ELISA Kit	EZHS100B
Ubiquitin C-Terminal Hydrolase-L1 (UCH-L1)	Abbexa	Rat Ubiquitin Carboxyl Terminal Hydrolase L1 ELISA Kit	ABX256085
N-Methyl-D-Aspartate Receptor (NMDAR)	MyBioSource	Rat N-methyl-D-aspartate receptor 1 ELISA Kit	MBS724735
Neuron Specific Enolase (NSE)	Lifeome / Cusabio	Rat Neuron Specific Enolase ELISA Kit	CSB-E07963r

2.7 Statistical Analysis

ELISA results, figures, and statistics were prepared using GraphPad Prism® Version 5. The arithmetic mean ± the standard error of the mean (SEM) is reported in each of the ELISA results figures. Statistical analysis was performed as an analysis of variance (ANOVA) with a Dunnett's Multiple Comparison Post Test. Group 2 (vehicle) was defined as the control group for the Dunnett's Post Test.

3.0 RESULTS

3.1 Clinical Observations

All animals in the naïve, vehicle control, and countermeasure treatments only control groups displayed normal clinical signs at all time points, with the exception of four animals in the countermeasure treatments only group. These animals exhibited respiratory distress (increased respirations, gasping, labored breathing) out to 60 minutes post-treatment. It is unclear why these isolated animals showed these abnormal clinical signs, but it is worth mentioning that atropine, 2-PAM, and HI-6 are all known to induce toxic signs in laboratory animals [33-35].

For the soman-challenged animals, 78% (31 of 40) showed signs of OP intoxication by 10 minutes, 98% (39 of 40) by 30 minutes, and 100% (40 of 40) by 60 minutes. Every animal demonstrated tremors (moderate sign) and/or convulsions (severe sign), with mortality observed in 35% (14 of 40) of the soman-challenged animals. The clinical signs observed among the soman-challenged animals included: agonal breathing, ataxia, convulsions, chewing, death, decreased respirations, exophthalmos, fasciculations, gasping, increased respirations, lethargy, labored breathing, lacrimation, milky lacrimation, mydriasis, nasal discharge, prostration, salivation, tremors, and weakness. The severe manifestation of toxic signs (i.e., convulsions) following the 1.2 x LD_{50} challenge observed were consistent with a lethal nerve agent challenge and similar to those reported by Shih and colleagues when a 1.6 x LD_{50} challenge model was used for seizure induction [29], thereby suggesting that seizures and subsequent damage to the hippocampus, amygdala, and piriform cortex were likely occurring.

3.2 ELISA Results

3.2.1 Brain-Derived Neurotrophic Factor (BDNF)

BDNF is a secreted protein that is involved with the development, maintenance, survival, and regeneration of neurons. In humans, elevated BDNF levels appear to reduce secondary brain injury following TBI [18, 36]. The Promega BDNF Emax® Immunoassay System was used to evaluate BDNF levels in the serum of each of the four treatment groups at 6 hrs, 24 hrs, and 72 hrs post-treatment. As depicted in Figure 1, with the exception of Group 4 at the 24 hrs timepoint, there was no statistically significant difference between naive, treatment control, or soman-

challenged samples compared to the vehicle control samples. However, a moderate (not statistically significant at 6 hrs or 72 hrs post-challenge) elevation of BDNF levels in the soman-challenged animals was observed at all time points.

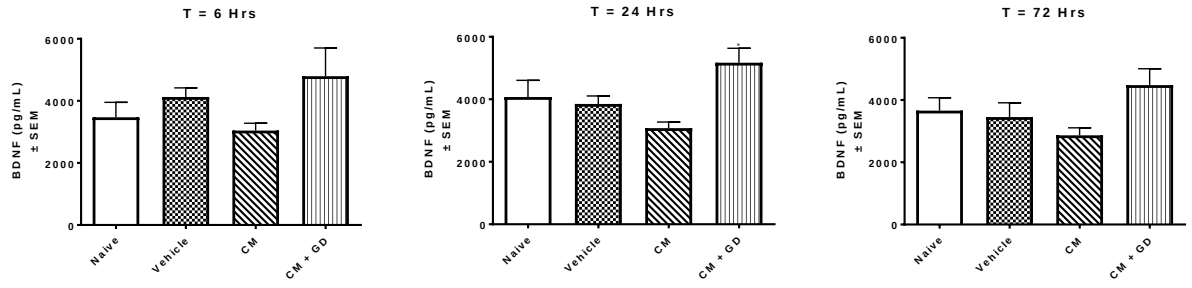

Fig. 1. BDNF Levels in Rat Serum Following Treatment and Soman Challenge. Serum samples were analyzed once through serial dilution of each sample on an assay plate. A minimum of two data points were within the reference standard range for all samples. Data was interpolated from the reference standard curve and the mean value ± SEM of each group is reported. Each group corresponds to the groups identified in Table 2 [Group 1: Naïve control, Group 2: Vehicle control, Group 3: Countermeasure (CM) control, and Group 4: Countermeasure-treated, soman-Challenged (CM+soman)]. At each timepoint a 1-way ANOVA with a Dunnett's Post Test (Control = Group 2, Vehicle Control) was performed to determine statistical significance. No statistical significance was observed. For 6 hr analysis; degrees of freedom (DF) = 35, F = 2.575. 24 hr; DF = 33, F = 6.52. 72 hr; DF = 32, F = 2.765.

3.2.2 S100B

S100B is a calcium-binding protein primarily produced by astrocytes in the CNS and has been implicated in the development and maintenance of the nervous system. It has been suggested that increased expression of S100B may exacerbate neuronal injury, and it is widely considered a relevant biomarker for CNS damage or dysfunction [37]. The Millipore Human S100B ELISA Kit was used to evaluate S100B levels in the serum for each of the four treatment groups.

A statistically significant decrease ($p < 0.05$) in S100B of approximately 30% in the serum was observed in the soman-challenged animals (Group 4) at each of the three sample collection time points when compared to the vehicle control (Figure 2).

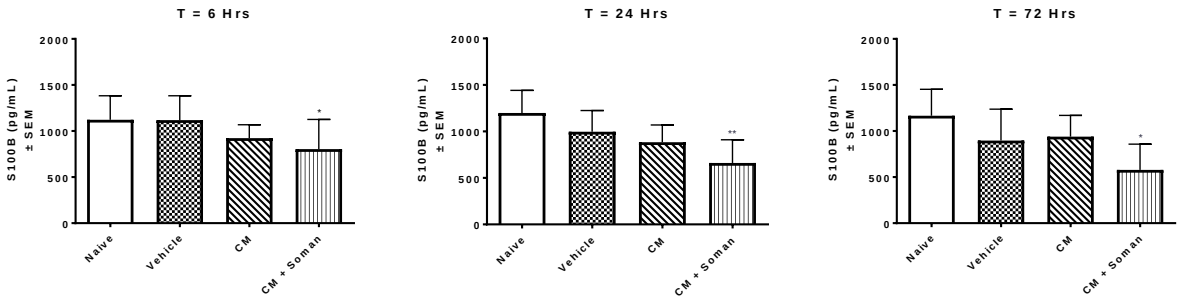

Fig. 2. S100B Levels in Rat Serum Following Treatment and Soman Challenge. Serum was analyzed in duplicate over at least three independent experiments. Data was interpolated from the reference standard curve and the mean value ± SEM of each group is reported. Each group corresponds to the groups identified in Table 2 [Group 1: Naïve control, Group 2: Vehicle control, Group 3: Countermeasure (CM) control, and Group 4: Countermeasure-treated, soman-Challenged (CM+soman)]. At each timepoint a 1-way ANOVA with a Dunnett's Post Test (Control = Group 2, Vehicle Control) was performed to determine statistical significance. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). For 6 hr analysis; degrees of freedom (DF) = 38, F = 3.557. 24 hr; DF = 39, F = 8.224. 72 hr; DF = 38, F = 5.459.

3.2.3 Ubiquitin Carboxyl Terminal Hydrolase L1 (UCH-L1)

UCH-L1 is one of the most abundant proteins in the mammalian brain, constituting 1-5% of the total soluble protein concentration [38]. UCH-L1 is a bi-functional protein that plays a substantial role in the removal of excessive, oxidized, or misfolded proteins via the proteasome pathway [39, 40]. UCH-L1 functions in this pathway as both a deubiquitinating enzyme and a ubiquityl ligase. Additionally, UCH-L1 binds to monoubiquitin in neurons, thereby stabilizing neuronal monoubiquitin levels. [39, 41-43]. Chen and colleagues also showed that UCH-L1 is required to maintain the structure and function of the neuromuscular junction in mice [44]. Multiple studies have indicated that altered levels of UCLH-L1 play a role in the pathogenesis of neurologic disorders. For example, mutated or depressed levels of UCH-L1 have been implicated in various neurodegenerative disease states, including Alzheimer's and Parkinson's disease [45]. Conversely, elevated levels of UCLH-L1 in the CSF and serum appear to be a promising candidate biomarker for TBI [39, 40]

To investigate whether UCH-L1 could serve as an indicator of OP exposure, protein levels were evaluated in the serum using the Abbexa Rat Ubiquitin Carboxyl Terminal Hydrolase L1 ELISA Kit. A highly significant ($p < 0.001$), approximately 2-fold decrease in UCH-L1 values in the soman-challenged rat serum (Group 4) compared to the vehicle control (Group 2) values was

found at the 72-hour time point (Figure 3). A statistically significant increase ($p < 0.05$) in UCH-L1 levels in the countermeasure treated animals was also observed at the 6-hour time point. (Figure 3).

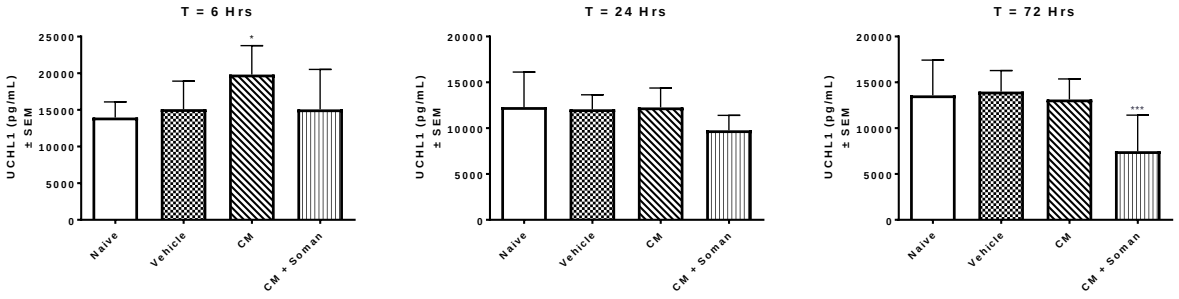

Fig. 3. UCH-L1 Levels in Rat Serum Following Treatment and Soman Challenge Serum samples were analyzed twice by serial dilution of each sample on an assay plate. Data was interpolated from the reference standard curve and the mean value ± SEM of each group is reported. Each group corresponds to the groups identified in Table 2 [Group 1: Naïve control, Group 2: Vehicle control, Group 3: Countermeasure (CM) control, and Group 4: Countermeasure-treated, soman-Challenged (CM+soman)]. At each timepoint a 1-way ANOVA with a Dunnett's Post Test (Control = Group 2, Vehicle Control) was performed to determine statistical significance. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). For 6 hr analysis; degrees of freedom (DF) = 37, F = 4.253. 24 hr; DF = 37, F = 2.93. 72 hr; DF = 38, F = 9.705.

3.2.4 Glial Fibrillary Acidic Protein (GFAP), N-Methyl-D-Aspartate Receptor (NMDAR), and Neuron specific enolase (NSE)

Based on their diverse reported roles in the neurodegenerative process, GFAP, NMDAR1, and NSE were candidate proteins selected for evaluation by ELISA in soman-challenged animals. GFAP, a ubiquitously expressed component of the structural network of astrocytic glial cells, has been positively correlated as a biomarker of neurodegeneration in TBI [46]. NMDAR1, a subunit of N-methyl-D-aspartate receptors, plays a key role in both neuroplasticity as well as glutamate-mediated excitotoxicity [47]. NSE, a dominant enolase found in neuronal and neuroendocrine tissues, is not typically secreted from healthy cells. However, following axonal damage, NSE appears to be upregulated and thus may serve as a prognostic marker following neuronal injuries [48]. Despite their respective roles in neuronal pathology, ELISA analysis of serum samples failed to indicate any statistical differences in serum levels of GFAP, NMDAR, or NSE between treatment groups at any post-treatment time points.

4.0 DISCUSSION

Prognostic biomarkers represent a promising strategy that could potentially be employed to guide medical intervention strategy. For example, efforts have already identified multiple protein and non-protein biomarkers with potential clinical and prognostic relevance in cases of CNS damage [49-52]. These putative biomarkers have been identified from complex biological matrices including serum, cerebrospinal fluid, and urine. As such, the goal of this study was to evaluate whether exposure to the CNS poison soman induces similar detectable changes in the levels of candidate protein biomarkers in circulation. A potential broader, long-term goal of this line of research is to determine whether the identified biomarkers have potential prognostic values in predicting the long-term and/or delayed neurological consequences following OP exposure.

In this study, six circulating proteins previously identified as potential diagnostic and/or prognostic biomarkers of human CNS injuries were evaluated using commercially available ELISA kits for their potential utility as biomarkers following an OP exposure. Three of the proteins (GFAP, NSE1, and NMDAR1) showed no statistical differences in serum levels at the various time points studied. Conversely, the circulating levels of BDNF, S100B, and UCH-L1 did demonstrate a statistically significant change following a 1.20 x LD_{50} soman challenge.

BDNF is a secreted protein shown to be involved with the development, maintenance, survival, and regeneration of neurons. In humans, elevated BDNF levels appear to reduce secondary brain injury following TBI putatively by providing neuroprotection through restoration of neural connectivity [18, 36]. Given the putative role of BDNF in recovery from neuronal injury, it may be surprising that the circulating level of BDNF did not display a statistically significant change for longer than 24 hours post-challenge. However, it is interesting to note that the mean BDNF level was lowest in the countermeasure control group and highest in the soman-challenged group at each time point. One possibility is that the soman challenge caused an increase in BDNF levels that was offset by a small decrease triggered by the treatment regimen. If true, the data from this study could support the observations from previous groups regarding BDNF and TBI and suggest that elevated BDNF levels may exert a neuroprotective effect following OP intoxication [18, 36]. Unfortunately, this hypothesis could not be evaluated within the current study design, where countermeasure treatments were essential to mitigate the otherwise highly lethal dose of soman necessary to elicit a convulsive response. Nonetheless, these data suggest that an elevation

of BDNF levels in circulation may be observed in soman-exposed animals in the absence of countermeasure treatment (albeit at a less lethal challenge dose). As such, further studies with BDNF at lower OP doses should be considered before eliminating it as a biomarker candidate.

Numerous studies have suggested that S100B serum levels have value as a prognostic indicator of brain damage and subsequent survival and recovery. Specifically, multiple studies have shown the positive correlation of elevated S100B levels with the severity of the trauma and poor clinical outcomes [53-57]. A comparison of S100B levels in human serum and urine from healthy individuals and those suffering from moderate to severe head trauma found elevated S100B levels in those individuals suffering from physical trauma [58]. Similar results were observed by Vos and colleagues who compared survival and outcome to S100B and GFAP serum levels at the time of admission to the hospital for TBI [56]. Therefore, it is surprising that in this study, a statistically significant decrease ($p < 0.05$) of approximately 30% in S100B protein levels in the serum was observed in the 1.2 x LD_{50} soman-challenged group at each of the three sample collection time points when compared to the vehicle control group (Figure 3). It must be acknowledged that S100B is known to be expressed in adipose tissue and testes as well as the CNS [59], and the source of the S100B in circulation cannot be determined using the study design reported herein. While this may complicate efforts to elucidate the biology underlying the change in circulating S100B levels, it does not necessarily limit the utility of S100B as a biomarker, since identifying the specific point of origin of a circulating protein is not necessarily an important consideration for a clinical biomarker.

One may speculate why a decrease of S100B was observed in this study when previous studies clearly indicate elevation of S100B as a biomarker for brain injury. Potential explanations for the conflicting results include species differences (rat versus human), a difference in the type of brain injury (physical trauma versus chemical insult), the timing of sample collection, or perhaps a compound-specific effect. There is evidence in the literature that contradicts the first three explanations: Multiple studies indicate that S100B serum levels increase in rats exposed to the OPs chloropyrifos oxon [60] and diisopropyl fluorophosphate (DFP) [61], although it is worth mentioning that the sublethal OP doses employed in these studies would not be expected to induce seizures and did not require medical countermeasure treatment for survival. Additionally, S100B levels are elevated and correlate to survival in humans exposed to various OPs [28]. It could be argued that the timing of sample collection plays a significant role and that S100B levels increase

over time as most studies observe this elevation weeks to months post-injury. However, it has also been shown that an increase in S100B levels in the immediate hours following traumatic brain injury correlate with patient death [54, 56, 62, 63]. The observed decrease in S100B following a 1.2 x LD_{50} challenge of soman may be specific to the OP/CWNA, the dose of challenge agent, the treatments employed, and/or the duration of convulsive activity.

UCH-L1 is one of the most abundant and significant proteins in the mammalian brain. UCH-L1 functions to target excessive, oxidized, or misfolded proteins for removal via the ATP-dependent proteasome pathway – a critical function for the cell. Various groups in the last several years have shown that early detection of elevated levels of UCH-LI in both the cerebrospinal fluid (CSF) and serum are indicative of ischemic injury and TBI [39, 40]. Furthermore, UCH-L1 has been a topic of interest recently as work performed by Banyan Laboratories has led to a recent FDA announcement authorization of the first blood test for the evaluation of concussion in adults, whereby UCH-L1 and GFAP were identified as biomarkers of particular interest [64]. In the current study, a surprising, highly significant ($p < 0.001$), approximately 2-fold decrease in UCH-L1 in the rat serum was found at the 72-hour sample collection time point. This is contradictory to previous reports in which following a controlled cortical impact or a middle cerebral artery occlusion, an initial 2-6-hour elevation of UCH-L1 is observed. Even at the 72-hour time point, Liu and colleagues continue to show elevation of UCH-L1 in the serum [39]. However, in the case of their study, a physical injury is involved, whereas in the study described herein, a chemically induced brain injury is generated in the presence of medical countermeasures. It is possible that that difference in study design is accounting for the differences observed here.

While our data did not identify a soman-related elevation of UCH-L1 levels in circulation, it has been shown that unavailable UCH-L1, caused by downregulation, mutation, aggregation in inclusion bodies, or oxidative modification plays a role in neurodegenerative diseases – namely Alzheimer's and Parkinson's disease [44, 45, 65]. These data suggest that UCH-L1 downregulation may be the result of oxidative stress induced by the prolonged hypersynchronous firing of neuronal ensembles in the brain following soman exposure. These results provide evidence that UCH-L1 may prove to be a positive indicator of OP exposure and/or a potential early biomarker of OP-induced neuropathology. Just as for S100B, it should be mentioned that UCH-L1 is expressed outside the CNS (e.g., kidney and pancreas), which does not necessarily disqualify it as a potential biomarker for OP-induced neuronal damage [66, 67].

While this study focused on using serum derived from whole blood, which would likely be more readily available during a mass casualty situation using minimally invasive means to obtain, additional matrices should be considered in future studies. For example, the concentration of various biomarkers found in cerebrospinal fluid (CSF) has been shown to be one of the most sensitive indicators of pathology within the central nervous system (CNS) in both children and adults [68-70]. Elevated levels of various CSF proteins have been seen in infections, intracranial hemorrhages, multiple sclerosis, a variety of inflammatory conditions, and neurodegenerative diseases including Alzheimer's disease and Creutzfeldt Jakob Disease [68, 71-73], and are therefore likely to be increased followed soman-induced seizures. As such, it is reasonable that CSF may be a better surrogate than blood (serum) for determining the response of brain tissue. Evaluation of the CSF protein levels may, therefore, allow for a more sensitive detection matrix for each of these proteins to potentially provide early diagnosis and/or prognosis of an acute OP intoxication event. This is certainly an approach that could be utilized in-hospital after victims have been evacuated from the site of exposure. Another limitation of the current study is that behavior and neuropathology were not evaluated, so no analysis of the relationship between protein levels and behavioral or pathological effects of the nerve agent could be performed. These endpoints were not explored as they were beyond the contractual scope of the funded effort since this was intended to be a pilot proof of principle study to determine whether changes in circulating levels of putative protein biomarkers could be detected following a significant nerve agent challenge. The positive results reported here warrant further investigations to include assessments of behavior, neuropathology, and long-term effects.

5.0 CONCLUSION

The overall goal of this study was to determine whether circulating proteins previously shown to have prognostic values as biomarkers in common CNS injuries may potentially have the same utility as indicators of neuropathology following acute OP exposure. Ultimately, well characterized and validated prognostic biomarkers could be used to inform medical caregivers on which patients should be prioritized for extended care or more thorough follow-up with the appropriate treatment plan to improve clinical outcomes. The need for such an approach is especially critical in mass casualty scenarios, where triage decisions would be critical. The data

presented herein demonstrate that the circulating levels of several of the proteins investigated, at least two of which currently provide value as prognostic biomarkers of TBI, are similarly affected following OP-induced brain injury. However, to fully understand the potential clinical value of these proteins (S100B, UCH-L1, and possibly BDNF) as potential prognostic biomarkers following a civilian OP exposure where countermeasure treatment is likely to be delayed, additional studies using OP and/or nerve agent doses adequate to produce toxic manifestations while allowing for sufficient survival without countermeasure intervention would be necessary. Furthermore, where differences in the circulating levels of these proteins are established, it would then be critical to also correlate those changes to the severity of the injuries to the central nervous system. Lastly, while the results of the current study are contradictory in some regards to previous reports, the importance of evaluating multiple compounds and conditions before drawing conclusions regarding the clinical utility of a biomarker must be emphasized.

Acknowledgements

The authors wish to acknowledge the hard work of Jeremy Majocha, Ellen Viereckl, Tyson Winters, Nancy Niemuth, and John Mitchell. The authors also wish to thank members of the Countermeasures Against Chemical Threats (CounterACT) Program Steering Committee (CPSC) for their collective expertise and input in the design of this study.

Disclosure Statement

The authors declare that there are no conflicts of interest.

Funding

This work was supported by the NIH Chemical Countermeasures Research Program and funded by the NIH Office of the Director through an interagency agreement (OD#: Y1- OD-0387-01) between NIAID and Department of Defense (DoD) and prepared under the auspices of the NIH, NIAID, and the DoD Defense Technical Information Center (DTIC) under the CBRNIAC program, Contract No. SP0700-00-D-3180, Delivery Order Number 0794, CBRNIAC Task 689/CB-13-0689. The authors have no known conflicts of interest. The views expressed in this

article are those of the authors and do not reflect the official policy of the NIH, HHS, DoD, or the U.S. Government. No official support or endorsement of this article by the NIAID or NIH is intended or should be inferred. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Battelle. All procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The sponsor developed the concept of the study, contributed to its design, and the interpretation of the data as well as the preparation of the manuscript and the decision to submit it for publication. The sponsor also made similar contributions to other studies occurring at Battelle during the same time frame.

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