Summary

Purpose: The aim of our study was to determine and compare the activity of acetylcholinesterase (AChE) in different parts of dog brain after the exposure to nerve agent sarin with or without HI-6 oxime treatment.

Material and methods: Before intoxication, beagle dogs were intravenously anaesthetized and premedicated with atropine sulphate (0.01 mg/kg). Three experimental groups were established – control, sarin (0.03 mg/kg, intramuscularly, 5 min after anaesthesia onset), and sarin + HI-6 dichloride (11.4 mg/kg, intramuscularly, 30 min after sarin poisoning). Brain (amygdaloid body, head of caudate nucleus, somatosensory cortex, Amon’s horn of hippocampus, hypothalamus, brain stem ventral respiratory group, and medial nuclei of thalamus) samples were taken 4 h after sarin administration. AChE activity was detected by histochemistry using the Karnovsky-Roots method and computer image analysis.

Results: Sarin poisoning decreased AChE activity in all selected brain areas. HI-6 did not affect this outcome.

Conclusion: HI-6 does not reactivate brain AChE in dogs when administered 30 min after sarin poisoning.

Key words: sarin; HI-6; quantitative histochemistry; dog; acetylcholinesterase

INTRODUCTION

Nerve agents are highly toxic organophosphates, which represent potential threats to both military as well as civilian population. The basic mechanism of nerve agents’ toxicity lies in irreversible binding
to and inactivation of acetylcholinesterase (AChE, EC 3.1.1.7). This is associated with accumulation of acetylcholine at the synapses and overstimulation of central as well as peripheral nervous system manifesting clinically as the acute cholinergic crisis [1].

The treatment of nerve agent poisoning consists of administration of parasympathomimetics (e.g. atropine), anticonvulsants (e.g. diazepam), live support therapy such as artificial ventilation, infusion therapy and causal treatment using reactivators (e.g. HI-6) [2]. Bispyridinium oxime HI-6 seems to be a reactivator capable of reactivating almost the whole spectrum of nerve agents [3]. However, the efficacy of oximes to penetrate blood-brain barrier and reactivate AChE-nerve agent complexes in vivo has been discussed in literature [4].

To utilize a large mammal model, AChE activity was measured in dog brain 4 h after the exposure to nerve agent sarin with or without HI-6 oxime treatment administered 30 min after poisoning using conventional histochemistry and computer image analysis, which yields morphological data with suitable accuracy [5 - 7]. Due to non-uniform distribution of AChE activity and varying sensitivity of different brain structures to nerve agent’s poisoning [5, 8], seven different brain regions were selected for analysis including amygdaloid body, caudate nucleus, somatosensory cortex, Ammon’s horn of hippocampus, hypothalamus, ventral respiratory nuclei in medulla oblongata, and thalamus.

M ATER IAL A ND METHODS

Animals

Before the experiment, beagle dogs aged 1 – 5 years and weighing 11 – 18 kg (Velaz, Unetice, Czech Republic) were housed in the animal facility of our institution, in compliance with guidelines set for the care and use of laboratory animals according to the Czech Republic laws. Experimental animals were handled under supervision of the Ethics Committee (Faculty of Military Health Sciences, Hradec Kralove, Czech Republic).

Chemicals

Sarin (GB; O-isopropyl methylphosphonofluoridate) was obtained from the Military Technical Institute of Protection (Brno, Czech Republic) and its purity (97 – 98%) was assayed by acidimetric titration. Oxime HI-6 dichloride was synthesized at the Department of Toxicology of the Faculty of Military Health Sciences (Hradec Kralove, Czech Republic) and its purity was analyzed using the HPLC (Spectra-Physics Analytical, Fremont, CA, USA). The solutions of agents for experimentation were prepared immediately before use. All other chemicals of analytical purity were obtained commercially and used without further purification. All chemicals were administered at a volume of 1 ml/kg of body weight.

Experimental setup

The dogs were randomly divided into 3 experimental groups. Before the intoxication, the animals were intravenously anaesthetized by butorphanol (0.2 mg/kg; Richter Pharma, Wels, Austria), xylazine (2 mg/kg; Vetoquino, Gorzow, Poland) and ketamine (10 mg/kg; Vetoquinol) and premedicated with atropine sulphate (0.01 mg/kg; Fatro, Ozzano Emilia, Italy) to prevent respiratory arrest. To prolong the anaesthesia, xylazine (1 mg/kg) and ketamine (5 mg/kg) were used every 40 min. If seizures occurred, diazepam (Zentiva, Prague, Czech Republic) was intravenously applied. Five minutes after the onset of anesthesia, the tested compounds were intramuscularly administered: (1) physiological solution (control group – 3 males and 3 females), (2) sarin (0.03 mg/kg – 2 males and 2 females), and (3) sarin (0.03 mg/kg – 2 males and 2 females) followed by HI-6 dichloride (11.4 mg/kg; the dose was derived from Antiva antidotum containing 800 mg of HI-6 and an average bodyweight of 70 kg in the European population) 30 min after sarin poisoning [9].

Histochemical Examinations

The dogs were euthanized 4 h after physiological solution or sarin exposure. Samples of brain were taken, rapidly frozen and cut into 20µm thick sections using a Leica CM 1900 cryostat (Leica, Wetzlar, Germany) at -20°C. Based on the neuroanatomical mapping according to the dog brain atlas [10], the following areas or nuclei of the dog brain (amygdaloid body, head of caudate nucleus, somatosensory cortex, hippocampus, hypothalamus, ventral respiratory nuclei in medulla oblongata, and thalamus) were chosen (Figure 1). Karnovsky-Roots method with Lojda’s modification was used to assess AChE activity [11]. Iso-OMPA (tetraisopropylpyrophosphamide; Sigma, St. Louis, MO, USA) was added into
the Baker’s fixative solution (100 ml 40% formaldehyde, 20 g calcium chloride [both from Sigma], and 900 ml distilled water) in a 10 µM concentration to inactivated butyrylcholinesterase (BChE, EC 3.1.1.8.). Fixation time was set at 5 minutes. Incubation with thiocholine (Sigma) lasted 3 hours at 36.6°C.

**Image Analysis**

For image analysis, 8 randomly selected microscopic fields per selected area were taken at 600× magnification using a BX-51 microscope and the ImagePro 5.1 computer image analysis system (Media Cybernetics, Bethesda, MD, USA). The AChE activity was evaluated in the inverted 8 bit (0 – 255) gray scale, where 0 is white and 255 is black colour, and expressed by mean optical density (mOD).

**Statistical analysis**

The Mann-Whitney test using SigmaStat 3.1 software (Systat Software Inc., Erkhart, Germany) were used for the statistical analysis. Results are presented as mean ± 2 × standard error of mean (SEM). The Mann-Whitney U test was used at the significance level alpha = 0.01.

**Table 1.** Mean optical density per microscopic field in different parts of the dog brain.

<table>
<thead>
<tr>
<th>group</th>
<th>control</th>
<th>sarin</th>
<th>sarin + HI-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>amygdaloid body</td>
<td>180 ± 5</td>
<td>151 ± 10 *</td>
<td>152 ± 12 *</td>
</tr>
<tr>
<td>caudate nucleus</td>
<td>183 ± 5</td>
<td>162 ± 8 *</td>
<td>165 ± 7 *</td>
</tr>
<tr>
<td>somatosensory cortex</td>
<td>66 ± 3</td>
<td>35 ± 2 *</td>
<td>37 ± 5 *</td>
</tr>
<tr>
<td>hippocampus</td>
<td>101 ± 6</td>
<td>65 ± 6 *</td>
<td>74 ± 8 *</td>
</tr>
<tr>
<td>hypothalamus</td>
<td>122 ± 8</td>
<td>83 ± 5 *</td>
<td>78 ± 8 *</td>
</tr>
<tr>
<td>medulla oblongata - ventral respiratory group</td>
<td>122 ± 6</td>
<td>92 ± 7 *</td>
<td>89 ± 6 *</td>
</tr>
<tr>
<td>thalamus</td>
<td>123 ± 9</td>
<td>74 ± 7 *</td>
<td>70 ± 5 *</td>
</tr>
</tbody>
</table>

* Statistically significant compared to control group (p ≤ 0.01)
RESULTS

Four hours after poisoning, the mean optical density per microscopic field significantly decreased by 16, 11, 47, 36, 32, 25, and 40% in amygdaloid body, head of caudate nucleus, somatosensory cortex, Amon’s horn of hippocampus, hypothalamus, brain stem ventral respiratory group, and medial nuclei of thalamus, respectively (all $p < 0.001$, table 1, figure 2). HI-6 therapy did not affect the outcome with values being decreased by 16, 10, 44, 27, 36, 27, and 43%, respectively (table 1, figure 3).

![Figure 2](image1.png)

**Figure 2.** Microphotography of 20 µm of the selected areas – control group.
1 – amygdaloid body, 2 – head of caudate nucleus, 3 – Amon’s horn of hippocampal formation, 4 – hypothalamus, 5 – ventral respiratory group; Magnification: 20x, Staining: AChE histochemistry according to Karnovsky – Roots.

DISCUSSION

In this study, we evaluated the ability of HI-6 to reactivate sarin inhibited AChE in different parts of dog brain 30 min after poisoning. Our model does not correspond to military or any other situations, in which autoinjectors might be available, but rather reflects terrorist attack victims, to whom atropine and diazepam could be administered soon after the arrival of emergency medical personnel but reactivators could be delivered with a significant delay.

After sarin intoxication, we found significantly decreased AChE activity in all selected brain areas. The inhibition showed non-uniform pattern with somatosensory cortex being the most and caudate nucleus being the least affected. Similar results were found in rats [5, 8]. The results show that structures with primarily low AChE activity are more sensitive to AChE inhibition than structures with high AChE activity.

HI-6 is a bisquaternary compound with poor blood–brain barrier penetration [4, 12]. Although it has been reported to modulate brain AChE and to counteract acute neurotoxicity in sarin poisoned animals in several studies [13 - 17], according to our results, it did not affect sarin-inhibited AChE activity in dog brain when administered in a human therapeutic dose (11.4 mg/kg) 30 min after poisoning. Two major differences can be found if we compare above mentioned studies with our model. Firstly, HI-6 was applied in a significantly higher dose (39.5 – 50 mg/kg), which may increase the blood–brain barrier transport. Secondly, HI-6 was used as a protective agent 30 min before or injected no later
than 1 min after intramuscularly or subcutaneously induced poisoning. Such timing may significantly support catalytic scavenging of blood cholinesterases and affect sarin distribution kinetics rather than re-activate inhibited AChE in brain [18 - 20]. Delayed administration of HI-6 may still have its rationale in the management of sarin victims to facilitate peripheral recovery; nevertheless, causal effects in CNS cannot be expected. For that, compounds with better blood–brain barrier penetration should be used.

CONCLUSION

HI-6 does not reactivate brain AChE in dogs if administered 30 min after sarin poisoning.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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