Human serum butyrylcholinesterase (BChE, EC 3.1.1.8) is currently under advanced development as a pretreatment for organophosphorus (OP) poisoning in human. It was shown to protect mice, rats, guinea pigs, and monkeys against multiple LD$_{50}$ challenges of OPs nerve agents intoxications. The aim of this study was to verify the efficacy of the pretreatment by the human BChE in blood and brain after intramuscular intoxication by Russian VX agent (RVX). Purified human BChE was administered intraperitoneally (500 U/kg) 30 minutes before a single dose of RVX corresponding to 1 LD$_{50}$ (15 µg/kg). Changes in cholinesterases activities were assessed by standard Ellman’s method.

In conclusion, BChE was not able to absolutely protect acetylcholinesterase against inhibition in blood. On the other hand it was able to reduce toxic effect of RVX in brain. The protection of cholinesterases in brain is important in prophylaxis, because brain damage is inconsistent with survival of intoxicated organisms.

**Key words:** Bioscavenger; Butyrylcholinesterase; In vivo; Nerve Agent; Organophosphate; Prophylaxis; Russian VX.

**INTRODUCTION**

Organophosphorus inhibitors (OPs) are spread around the world as many preparations. Of them, pesticides and nerve agents are well discussed in the current literature. The first nerve agent, tabun, was discovered in 1936 and subsequent modification of its structure led to the more toxic analogues - sarin, soman and cyclosarin [1]. Several agents with higher toxicity, VX (1952) and Russian VX (RVX, Figure 1) (1963); were developed during the Cold War.

OPs acute toxicity is usually attributed to irreversible inhibition of acetylcholinesterase (AChE; EC 3.1.1.7). AChE hydrolyses neurotransmitter acetylcholine at cholinergic clefts. [2]. Subsequent accumulation of acetylcholine (ACh) over-stimulates the cholinergic path-ways in both central and peripheral nervous system and also in neuromuscular junctions. The strong AChE inhibition leads to general cholinergic crisis and death of intoxicated organism [3].

The RVX (O-isobutyl-S-[2-(diethyl)ethyl]methylyphosphonothioate), is the main V-type nerve agent, was developed for the chemical warfare ar-
senal in the Former Soviet Union. Among the known nerve agents, RVX belongs to the most toxic OPs ever prepared. Its high toxicity may be explained by the disabling of blood hydrolases that are able to detoxify this nerve agent in the blood stream [4].

Current antidotes for OPs poisoning consist of a combination of pretreatment and post-exposure therapy. The carbamates (reversible AChE inhibitors) are commonly used as a pretreatment (usually pyridostigmine) that should protect AChE against the OPs irreversible inhibition. The post-exposure therapy includes anticholinergic drugs (mainly atropine) to counteract the ACh effects in peripheral compartment in combination with AChE reactivator (e.g. 2-PAM, obidoxime, HI-6). AChE reactivators (called oximes) are able to partially restore AChE activity. Anticonvulsive supportive therapy (e.g. diazepam, avizafon) may be also successfully used in OPs poisoning treatment [5].

The other way to increase the survival of intoxicated person is the use of bioscavengers. The bioscavengers are mainly enzymes (cholinesterases, paraoxonase, etc.) with hydrolytic activity that inactivate OPs before they reach main targets (AChE in peripheral and central nervous system or in neuromuscular junctions). Cholinesterases (AChE, butrylcholinesterase – BChE; EC 3.1.1.8) are considered as the potential scavengers without significant harmful effects. Especially purified human plasma BChE has been shown to protect targets against strong inhibitors such as soman, sarin, tabun and VX [6].

Biological monitoring is an essential component of any comprehensive assessment of exposure by highly toxic OPs. The biological effect of nerve agents is usually estimated by measurement of blood or tissues cholinesterases activity [7]. The aim of this study was to evaluate the prophylactic efficacy of human BChE in RVX-poisoned rats by the monitoring of blood and brain cholinesterase activities.

**Figure 1.** Formula of RVX

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**MATERIAL AND METHODS**

**Chemicals**

The RVX (O-isobutyl-S-[2-(diethyl)ethyl]-methylphosphonothioate) of 98% purity was obtained from Military Technical Institute of Protection (Brno, Czech Republic) and stored at -20°C until the assay.

The experimental solution of RVX for experiment was prepared before its use. Orientation toxicity determination was done to prove that the dose that is administered to animals really corresponds to the 1 LD₅₀. All other chemicals were bought from Sigma–Aldrich (branch Prague, Czech Republic).

**Animals**

Female Wistar rats were purchased from BioTest (Konarovice, Czech Republic). The animals were selected by body weight (180 - 200 g). Rats were maintained in air-conditioned room under standard condition (temperature 22 ± 2°C, humidity 50 ± 5%, and standard light/dark cycles). Standard laboratory food and tap water were available ad libitum. The experiment was performed under permission and supervision of the Ethic Committee of the Faculty of Military Health Sciences, University of Defence; Hradec Kralove, Czech Republic. All experiments were conducted in agreement with the Animal Protection Law of the Czech Republic (311/1997).

**Animal treatment and sample collection**

Although the main route of administration of V-agents is percutaneous, intramuscular administration was used in our experiments due to better comparability with literature data and due to best control over absorbed dose of inhibitor (Bajgar 2006).
After seven days of rats' acclimatization, purified human BChE was administered (i.p.; 500 U/kg). After 30 minutes, a single dose of 1 LD_{50} of RVX (15 µg/kg) was injected intramuscularly (i.m.; hind limb). Control rats were injected by saline solution in both intervals.

Animals were narcotized with carbon dioxide in four different time intervals; 6, 10, 20 and 60 min after intoxication (6 animals in group). After that, the thoracic cavity was opened. The blood was drawn directly from the heart and was collected in heparin-coated tubes and gently mixed.

Whole brains were also harvested. Because the blood in brain vessels could also contain BChE, it is not suitable for direct measurement of real cholinesterase levels in brain tissue. To solve this issue, experimental animals were perfused transcardially by saline solution (0.9% NaCl) for 10 min (50 ml/min). After perfusion, the skull was opened and the brain was carefully removed [8]. The brains were stored at –80°C until the Ellman's assay.

Methods

After thawing, brains were homogenized (Ultra-Turrax homogenizer; Janke & Kunkel) with distilled water in the ratio 1:10 (w/v) for 60 seconds. Appropriate volume of homogenate was used for enzymatic assay.

The activities of both AChE and BChE were assessed by standard spectrophotometric Ellman’s method. Acetylthiocholine or butyrylthiocholine iodides were used as an appropriate substrates and 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) was used as a chromogen [9]. Our method was modified (when compared with original method) in wavelength, which was 436 nm [10]. The spectrophotometer UVIKON 752 (Kontron Instruments; Germany) was used for determination of absorbance. The activities were expressed as µcat/g of wet weight tissue.

Statistical analysis

The number of animal per group was minimally 6. Statistical analysis was performed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, California). The mean and SEM were calculated.

RESULTS

Intoxication by RVX demonstrated its strong inhibition potency towards AChE. The main mechanism of the action for this nerve agent type seems to be predominantly inhibition of cholinesterases in the peripheral compartment.

Figure 2. : Changes of cholinesterase activities in whole blood after pretreatment with human BChE (500 u/kg) and experimental intoxication with RVX (15 µg/kg) in rats.
The inhibition of AChE in whole blood (model representing the peripheral tissues) was very strong and fast (Figure 2). Although cholinesterase activity in blood significantly increased with the pre-application of human BChE, its blood level was unable to protect cholinesterases against RVX inhibition in the first minutes after intoxication. The reduction of cholinesterase activities was approximately 50% in 6 min. This strong inhibition of AChE occurred at the 6th minute after intoxication and was subsequently repaired in the 10th minute by about 10% and in 20 minute about 20%.

Figure 3. Changes of AChE activity in brain after pretreatment with human BChE (500 u/kg) and experimental intoxication with RVX (15 µg/kg) in rats.

The RVX alone caused inhibition of brain AChE activity around 20% in 6 minutes (Figure 3). Human BChE applied in this dose retained without significant AChE activity changes in brain for whole time of the study (60 minutes after intoxication). The penetration of BChE through the blood-brain barrier was first recorded after RVX application (45 minutes after its administration) (Figure 4).

Human BChE pretreatment provided almost 100% survival effect against 1 LD50 of RVX. Only one animal died in 33 minutes after intoxication.

Figure 4. Changes of BChE activity in brain after pretreatment with human BChE (500 u/kg) and experimental intoxication with RVX (15 µg/kg) in rats.
DISCUSSION

For more than 50 years the scientists have been searching for effective medical countermeasures against OPs poisoning. The concept of bioscavengers has emerged as an alternative approach to pharmacological pre- and post-exposure treatments. Human BChE have not serious pharmacological side effects for human and has been shown to protect against up to 5 LD$_{50}$ of nerve agent (soman, VX) in guinea pigs and non-human primates [11]. Protection against single LD$_{50}$ dose of sarin was also clearly demonstrated [12]. From the study, it is evident that human BChE in dose 500 U/kg is able to partially protect rats against RVX in dose corresponded with 1 LD$_{50}$.

In conducted series of assays was demonstrated a linear correlation between blood cholinesterase activity and the level of protection against poisoning [13]. In our study, reduction of cholinesterases activity in whole blood was found during the first minutes after intoxication. Based on these results, it is evident that this application scheme (500 U/kg BChE 30 minutes before intoxication) is not sufficient in case of RVX intoxications. Depression of blood cholinesterase activities revealed relatively strong inhibition AChE in peripheral compartment. Subsequent increasing of cholinesterases level may be explained by pharmacokinetic of BChE absorption.

Despite formerly described data, the protection of central compartment was sufficient during this study. The brain AChE activity level was not changed after administration of BChE and also after RVX intoxication. The permeability of this nerve agent through the blood-brain barrier is not so high in comparison to other nerve agents such as sarin and soman [11, 12]. The dose corresponding to 1 LD$_{50}$ of RVX is able to decrease cholinesterase level in central nervous system about 20 % during the first 3 minutes [14]. The protection of cholinesterases in central compartment is very important, because the major damage of brain is inconsistent with survival of intoxicated organisms [15].

The activity of BChE in brain was not significantly elevated before RVX application. Results indicated that the presence of RVX in organism may change the blood-brain barrier permeation because the brain BChE level significantly increased after RVX application.

In conclusion, subsequent progress in central nervous system confirms the hypothesis: Bioscavenger present in blood before exposure is able to reduce nerve agent concentration to toxicologically irrelevant level. RVX does not affect its main target in organism - brain cholinesterases.

ACKNOWLEDGEMENTS

Authors would like to thank to the Ministry of Defence (Czech Republic) – Grant No. FVZ0000604. Thanks are due to Mrs. J. Uhlírova and Mrs. E. Reslova for skilled technical assistance.

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