ORIGINAL ARTICLE

PROTECTIVE EFFECT OF REVERSIBLE CHOLINESTERASE INHIBITORS (TACRINE, PYRIDOSTIGMINE) AND EQBUCHE AGAINST VX POISONING AND BRAIN ACETYLCHOLINESTERASE INHIBITION IN RATS

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Summary: The protective effect of the reversible cholinesterase inhibitors tacrine and pyridostigmine alone or in combination with different drugs against acetylcholinesterase inhibition in the pontomedullar area and cerebellum of rats caused by VX agent (O-ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate) in vivo (2xLD50) was studied along with survival of animals pretreated with different combinations of the drugs used. The best prophylactic effect was observed in a combination of pyridostigmine with benactyzine, trihexyphenidyle and HI-6. Tacrine alone or in other combinations has had no better prophylactic effect in comparison with these combinations containing pyridostigmine. Equine butyrylcholinesterase, also protected against VX poisoning very effectively.

Key words: *Benactyzine; Trihexyphenidyle; HI-6; Tacrine; VX; Rat; Prophylaxis; Acetylcholinesterase; Cerebellum; Pontomedullar area*

Introduction

Nerve agents are among the most dangereous chemical warfare agents (3, 29, 40, 45). They can be (and have been) misused by terrorists (4, 31). Moreover, the whole spectrum of these agents of the same basic chemical structure covers organophosphorus insecticides (OP) – chemicals easily available, frequently used over the world and causing professional, suicidal or accidental intoxications (4). All these facts underline the necessity to study these agents to achieve better diagnosis, prophylaxis and treatment.

Sarin, soman, tabun, and VX etc. are the most important representatives of the nerve agents. Keeping acetylcholinesterase (AChE, EC 3.1.1.7) – the main target for nerve agent action – intact is a basic requirement for effective prophylaxis. It can be achieved using reversible inhibitors which are able to inhibit AChE reverzíbly, and after spontaneous recovery of the activity (decarbamylation), normal AChE serves as a source of the active enzyme. Pyridostigmine and other carbamates were tested as the most promising prophylactic drugs (e.g. 3, 14, 16, 33). Pyridostigmine was introduced into some armies as a prophylactic antidote against nerve agents. Its prophylactic effect (like the effects of other carbamates) is limited by its dose. With a higher dose, a higher efficacy was observed, but the side effects were more expressed too (for a review, see 3, 5, 33). This problem can be solved by adding pyridostigmine antagonizing drugs – anticholinergics. The prophylactic combination of pyridostigmine with trihexyphenidyle and benactyzine called PANPAL was introduced into the Czech Army (3, 14). The presence of these two anticholinergics allowed us to increase the pyridostigmine dose and to increase its prophylactic efficacy. Structurally different drugs/inhibitors were also studied. Petroianu et al. (34, 37) compared pretreatment with pyridostigmine and tiapride against paraoxon intoxication. The best results were achieved with pyridostigmine pretreatment only. Some protective effects against paraoxon were achieved by pretreatment with ranitidine (35) or metoclopramide (21). From other studies of compounds preferably binding to the AChE anionic site, tacrine, 7-methoxytacrine (7-MEOTA) and huperzine A were considered and experimentally studied with respect to prophylaxis against nerve agents in vitro and in vivo (3, 6, 17, 27, 33).

Diminishing the level of OP using enzymes which hydrolyze these agents or enzymes which bind the agents (to specific proteins or to antibodies) and thus reducing the OP level and inhibition of cholinesterases – AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8) (scavenger effect) – in the organism can be described as detoxification (11, 12, 41, 42). Another approach to prophylaxis is based on using present antidotes, especially reactivators such as HI-6 and other drugs (3, 5, 15).

New reversible inhibitors of acridine type or naturally occurring agents are being studied (3, 5, 32, 33).

The aim of this present study was to compare the prophylactic effect of reversible acridine inhibitor – tacrine (1,2,3,4-tetrahydroaminoacridine) and its combinations with presently used prophylactics and equine butyrylcho-

linesterase (EqBuChE) against VX in female laboratory rats. Due to the high toxicity of VX, the differences in LD50 values for male and female rats are negligible (3, 15). Simultaneously, the changes in the brain AChE activity following in vivo administration of these drugs were studied. Two different parts of the brain were used: the cerebellum – roughly representing AChE activity in the whole brain, and the pontomedullar area; AChE activity in this structure is considered to be the most sensitive to OP and antidotal/prophylactic countermeasures (1, 3, 7, 19).

Material and Methods

Chemicals

VX (O-ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate) was obtained from the Military Technical Institute of Protection (Brno, Czech Republic). It was of minimally 95% purity and stored in glass ampullas (1 ml). The solutions of the agents for experiments were prepared before use. HI-6 and tacrine were synthesized at the Department of Toxicology of the Faculty of Military Health Sciences (Hradec Kralove, Czech Republic) Their purities were analyzed using HPLC technique. Benactyzine a trihexyphenidyle were products of Leciva Prague (Czech Republic). Equine BuChE (original activity 20 000 mU/ml) was a kind gift from Dr. Bhupendra Doctor, Walter Reed Army Institute of Research, Washington, USA. All other chemicals of analytical purity were obtained commercially and used without further purification. All substances were administered i.m. (i.p.) at a volume of 0.1 ml/kg body weight.

Tab. 1: Schematic representation of drug administration in different groups used in the experiments. The doses are given in the text.

Designation of group	Administration	
	first	second
CONT SAL	saline	saline
CONT TAC	tacrine	saline
CONT VX	saline	VX
CONT H	HI-6	saline
CONT Tr	trasentine	saline
CONT B	benactyzine	saline
CONT PYR	pyridostigmine	saline
H-VX	HI-6	VX
B-VX	benactyzine	VX
Tr-VX	trasentine	VX
H+Tr-VX	HI-6 + trasentine	VX
H+B-VX	HI-6 + benactyzine	VX
B+Tr-VX	benactyzine + trasentine	VX
TAC-VX (1_{TAC})	tacrine	VX
TAC+B-VX (2_{TAC})	tacrine + benactyzine	VX
TAC+H-VX (3_{TAC})	tacrine + HI-6	VX
TAC+B+H-VX (4_{TAC})	tacrine + benactyzine + HI-6	VX
TAC+B+Tr-VX(5_{TAC})	tacrine + benactyzine + trasentine	VX
PYR-VX (1_{PYR})	pyridostigmine	VX
PYR+B-VX (2_{PYR})	pyridostigmine + benactyzine	VX
PYR+H-VX (3_{PYR})	pyridostigmine + HI-6	VX
PYR+B+H-VX (4_{PYR})	pyridostigmine + HI-6	VX
PYR+B+Tr-VX (5_{PYR})	pyridostigmine + benactyzine + trasentine	VX
TAC+B+Tr+H-VX (6_{TAC})	tacrine + benactyzine + trasentine + HI-6	VX
PYR+B+Tr+H-VX (6_{PYR})	pyridostigmine + benactyzine + trasentine + HI-6	VX
BuChE-VX	EqBuChE	VX

Animals

Female Wistar rats, weighing from 200 to 220 g, were purchased from BioTest (Konarovice, Czech Republic). The animals were maintained in an air-conditioned room (22° ± 2 °C and 50 ± 10% relative humidity, with light from 7 a.m. to 7 p.m.), and were allowed free access to standard chow and tap water. Housing of animals took place in the Central Vivarium of the Faculty of Military Health Sciences under veterinary control. All the experiments were performed under permission and supervision of the Ethic Committee of the Faculty of Military Health Sciences, Hradec Kralove.

Prophylaxis and intoxication

The general scheme of prophylaxis and intoxication was as follows:

Administration of prophylactic agent (in control group saline) – first administration, and 20 min. later, administration of VX (in control group saline) – second administration. The animals were decapitated and the brains were removed 60 min. after the second administration or after death. Groups of 10 animals were used. Designation of groups and drugs administered are summarized in Tab. 1. For the doses of drugs used – see below.

Doses of drugs used

The doses and timing of drug administration were used on the basis of our previous results with tacrine (2), reactivators including HI-6 (3, 15), pyridostigmine, benactyzine and trihexyphenidyle (3, 23, 24), and EqBuChE (8, 12), respectively. The doses (except VX) represent the doses approximately to be used for human prophylaxis (2, 3, 8,15, 23). They were as follows:

- Tacrine 10 mg/kg, i.m.;
- HI-6 15 mg/kg, i.m.;
- Benactyzine 9 mg/kg, i.m.;
- Trihexyphenidyle 6 mg/kg, i.m.;
- Pyridostigmine 1 mg/kg, i.m.;
- BuChE 250 mU/kg, i.p.;
- VX 28 μg/kg, i.m., i.e. 2xLD50 dose;
- saline 0.1 ml/kg; all doses are related to body weight.

Determination of AChE activity

After decapitation, the brain parts (pontomedullar area containing i.a. ncl. gigantocellularis /PM/ and cerebellum)

were prepared and frozen. After thawing, tissue was homogenized (1:10, distilled water, Ultra-Turrax homogenizer, Janke and Kunkel, Germany) and homogenates were used for enzymatic analysis.

AChE activity was determined using the method of Ellman et al. (13) as described elsewhere (9). Acetylthiocholine was used as a substrate (TRIS-HCl buffer pH 7.6). The results were expressed as μkat/g wet weight tissue, UVIKON 752 (Germany) spectrophotometer was used for determination of absorbancy at 412 nm. The activity was also expressed as a relative % of control values.

Statistical evaluation

The results were evaluated by the usual statistical methods (mean, SD, Student's t-test) according to relevant PC programmes.

Results

The mortality of experimental animals varied according to the drug/combination used. In control groups all animals survived after two administrations of saline (CONT SAL), and following administration of tacrine and saline (CONT TAC). All animals survived in groups with administration of HI-6 and two parasympatholytics or pyridostigmine (CONT H, CONT B, CONT Tr, CONT PYR. Following saline administration and VX intoxication (CONT VX), all animals died within 20–55 min (Tab. 2).

In experimental groups, the mortality was different depending on the administration of prophylactic drugs. Administration of oxime HI-6 alone, benactyzine or trihexyphenidyle (H-VX; B-VX; Tr-VX) had no high prophylactic effect – 60 % of the animals in the group died (Tab. 2). Combinations of these substances (B+Tr-VX) was not able to protect all animals; when oxime HI-6 was combined with both anticholinergics (H+Tr-VX, H+B-VX), 50 % of the animals survived. Tacrine (Ta-VX) or pyridostigmine (PYR-VX) alone had a prophylactic effect, more expressed for pyridostigmine. The best prophylactic effectiveness was observed for combinations of benactyzine and trihexyphenidyle with pyridostigmine (PYR+B+Tr-VX).

When HI-6 was added (PYR+B+Tr+H-VX), survival of all experimental animals was also observed. Administration of butyrylcholinesterase (BuChE-VX) had the same effect (100% survival). If we compare the prophylactic effectivity of combinations of reversible cholinesterase inhibitors (ta-

Tab. 2: AChE activity and mortality in rats following administration of different prophylactic mixtures.

Group	**Pontomedullar part** (μkat/g)	**Cerebellum** (μkat/g)	**Mortality**	**Comment**
CONT SAL	283.1±23.3	61.5±5.5	0/10	
CONT TAC	214.3±20.2*	54.5±5.4	0/10	
CONT VX	51.5±4.2*	30.4±3.1*	10/10	
CONT H	286.2±20.1	63.8±3.8	0/10	
CONT Tr	278.8±20.8	63.1±3.4	0/10	
CONT B	276.9±21.9	64.4±4.1	0/10	
CONT PYR	289.6±19.7	59.9±3.3	0/10	
H-VX	94.8±9.0*	32.3±3.1*	6/10	
B-VX	59.2±5.2*	31.8±2.9*	6/10	
Tr-VX	59.5±5.3*	30.9±2.9*	6/10	
H+Tr-VX	92.8±8.1*	33.8±3.0*	5/10	
H+B-VX	96.5±8.3*	33.3±2.8*	5/10	
B+Tr-VX	56.1±6.0*	30.8±2.5*	6/10	
TAC-VX (1_{TAC})	121.2±9.9*	36.0±2.8*	4/10	
TAC+B-VX (2_{TAC})	120.9±10.6*	34.9±3.1*	4/10	
TAC+H-VX (3_{TAC})	125.4±11.4*	35.9±3.2*	2/10	
TAC+B+H-VX (4_{TAC})	128.0±11.1*	37.6±3.2*	1/10	
TAC+B+Tr-VX(5_{TAC})	126.3±11.9*	36.6±3.5*	1/10	
PYR-VX (1_{PYR})	123.4±7.8*	34.4±3.4*	3/10	Pyridostigmine is prophylactic drug in many Armies
PYR+B-VX (2_{PYR})	126.0±10.3*	36.1±3.7*	2/10	
PYR+H-VX (3_{PYR})	128.5±10.2*	35.6±3.2*	1/10	
PYR+B+H-VX (4_{PYR})	132.3±12.1*	35.7±3.3*	0/10	
PYR+B+Tr-VX (5_{PYR})	131.1±11.5*	36.2±3.4*	0/10	PANPAL in the Czech Army
TAC+B+Tr+H-VX (6_{TAC})	132.8±11.7*	37.1±3.6*	1/10	
PYR+B+Tr+H-VX (6_{PYR})	136.7±12.0*	40.1±3.7*	0/10	It represents simultaneous administration of PANPAL and TRANSANT
BuChE-VX	255.1±20.6	57.4±4.9	0/10	

The results are means ± SD; *statistically significant difference ($p< 0,05$) in AChE activity compared with control group (CONT SAL)

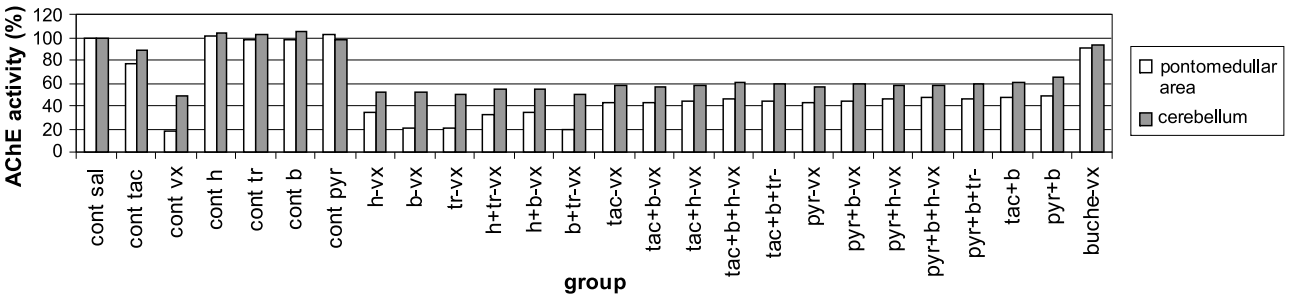

Fig. 1: Changes of AChE activity in the pontomedullar part and cerebellum of the rat brain following different prophylactic treatment. The results are expressed as means – % of control values (group CONT SAL – 100 %). Absolute values (means ± SD) see Tab. 1.

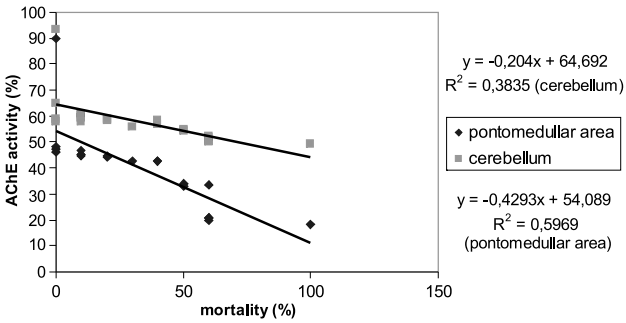

Fig. 2: Correlation between AChE activity (pontomedullar area and cerebellum) expressed as activity per cent and mortality (%) of experimental animals. Excell programme for PC was used.

crine and pyridostigmine) and other drugs used (groups $1\text{-}5_{TAC}$, $1\text{-}5_{PYR}$, Tab. 2), it is clear that all combinations containing pyridostigmine are more effective. AChE activity in the pontomedullar area and cerebellum in control group (CONT SAL) was different with higher activity in the pontomedullar area. Following VX administration, AChE activity in the pontomedullar area was more inhibited (about 20 %) than that observed in the cerebellum (about 50 % of control values) (Fig. 1). This „relative resistance" of AChE activity was expressed in experimental groups too. Comparing absolute and relative values of AChE activity, significant differences ($p< 0.05$) were demonstrated, indicating higher AChE sensitivity in the pontomedullar part of the brain (Tab. 2, Fig. 1).

Administration of parasympatholytics (benactyzine, trihexyphenidyle) or HI-6 before VX intoxication did not affect AChE activity either in the pontomedulllar area or the cerebellum. Following prophylactic administration of HI-6, AChE activity in the pontomedullar area was increased ($p< 0.05$) in comparison with VX intoxication only. This increase was not statistically significant in the cerebellum.

When the AChE activity in the pontomedullar area and cerebellum is correlated with the survival/death of the experimental animals (expressed as percentage of the mortality, see Tab. 2), a better relationship for the pontomedullar area was demonstrated (Fig. 2). The slopes of the curves indicate a closer and significantly different ($p<0.05$) relationship between mortality and AChE activity in the pontomedullar area than that in the cerebellum, as is indicated by R^2 values.

Discussion

A comparison of normal AChE activity was possible for the whole brain homogenate. Kassa et al. (22) described normal AChE activity in the whole brain homogenate (54 μkat/kg) slightly lower than our activity in the cerebellum (61.5 μkat/kg). The AChE inhibition in the pontomedullar area following tacrine administration was very similar to our former results (2). It was also proved that the central effect of tacrine is higher than that of pyridostigmine – pyridostigmine practically does not cause AChE inhibition in the brain, i.e. it does not penetrate through the blood brain barrier (BBB) (20, 26). However, in extremaly high doses, pyridostigmine penetrates the BBB and causes AChE inhibition in the whole brain homogenate (38). The protection of the brain AChE observed in our experiments can be explained by diminishing the VX dose at the periphery and thus decreasing the VX level penetrating into the brain. However, penetration of VX into the rat brain was demonstrated (3, 7, 43). The toxicokinetics of VX is known for guinea-pigs. Greater in vivo persistence of VX in comparison with sarin and soman showing shorter half lives in the plasma (minutes) was demonstrated and summarized (3). Agent VX (and other V compounds) penetrate the BBB and inhibit differentially AChE activity in various brain structures. Different sensitivity of AChE in the cerebellum and brainstem was observed for guinea pigs in lower doses with VX (1xLD50); the activity was decreased to approximately 40 % of control values (43). It corresponds to a more expressed AChE activity decrease following doses of 2x LD50 (rats) observed in our experiments. It was demonstrated that the drugs in the doses used did not affect AChE activity either in the pontomedullar area or cerebellum. This is in agreement with literature data (3) but no paper was found where this „non-influence" was directly described. Only data with atropine provided as a supple-

ment to other (reactivation) experiments have been published (22–24). Moreover, these doses represent doses close to the doses planned for human use (14, 28).

A good prophylactic effect of pyridostigmine was also confirmed in combination with trihexyphenidyle and benactyzine. This combination is a model for prophylactic antidote PANPAL (3, 14). When this mixture is combined with oxime HI-6, it would be described as a model for simultaneous use of PANPAL and TRANSANT (3, 15). On the other hand, pretreatment with pyridostigmine and pralidoxime did not support their combined use against paraoxon poisoning (36).

The prophylactic effect of another reversible inhibitor, tacrine, was similar but not so high – the effect was caused more by parasympatholytics or HI-6. It suggests that tacrine is not a prophylactic drug comparable with pyridostigmine, demonstrated as survival of experimental animals.

These differences were marked when AChE activity was monitored, and AChE activity was more sensitive in the pontomedullar area in comparison with cerebellum. AChE activity in the pontomedullar part of the brain is sensitive to nerve agents including VX as demonstrated previously (7). It is known that sublethal concentrations of VX, induce changes in respiratory dynamics and ventilation in guinea pigs (39) treatable with pulmonary therapeuticals (30).

A very important brain part connected with ventilation is the pontomedullar area (10, 18, 44). When the AChE activity is determined in the whole brain, the fine changes are not expressed, as was demonstrated in our experiments for this area and the cerebellum. It is explanable by different AChE activities in the brain parts – Gupta (19) detected AChE activity in the brainstem about 4.5 times higher than that in the cerebellum (our results – 4.6). The activity detected in the whole brain homogenate (as well as cerebellum) can then be considered as a "mean" of all activities. The pontomedullar area was more sensitive than the rest of the brain, represented by the cerebellum. In connection with this, a correlation between residual AChE activity in this brain part of rats and survival/death following intoxication with nerve agents was demonstrated (1).

A very good prophylactic effect of EqBuChE against VX intoxication was confirmed as well as protection of AChE in the brain parts, similarly as demonstrated for sarin earlier (8). This prophylactic effect can be comparable with protection caused by one of our most effective combinations simulating prophylactic antidotes in the Czech Army (PANPAL and TRANSANT). This observation supports the need for further study of scavengers based on an enzymatic basis.

Conclusions

1. Intramusculary administered drugs trihexyphenidyle, benactyzine and HI-6 in doses used did not affect AChE activity in the rat brain.
2. AChE activity in the cerebellum of VX intoxicated rats is not as sensitive to the effect of the drugs used as that in the pontomedullar area.
3. Tacrine is a more effective central AChE inhibitor in vivo than pyridostigmine.
4. The prophylactic effect of pyridostigmine against VX is higher in comparison with tacrine.
5. EqBuChE administered prophylactically has a very good protective effect (survival of 100 % of animals).
6. The best prophylactic effect was observed for the following combinations:
   - pyridostigmine with benactyzine and trihexyphenidyle;
   - pyridostigmine with benactyzine and HI-6;
   - pyridostigmine with benactyzine, trihexyphenidyle and HI-6, i.e. the same prophylactic efficacy as that observed for administration of EqBuChE.

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