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ORIGINAL ARTICLE

The therapeutic efficacy of oxime treatment in cyclosarin-poisoned mice pretreated with a combination of pyridostigmine benactyzine and trihexyphenidyl

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Summary
The present study was performed to assess and compare the therapeutic efficacy of various oximes (methoxime, BI-6, HI-6) combined with benactyzine (BNZ) in cyclosarin (GF-agent)-poisoned mice and to evaluate the influence of pretreatment with PANPAL (pyridostigmine, benactyzine and trihexyphenidyl) on the effect of antidotal treatment in mice poisoned with the GF-agent. In the first part of our experiment, methoxime, BI-6 or HI-6 in combination with benactyzine were used for the treatment of GF-poisoned mice. In the second part of the experiment the animals were pretreated with PANPAL 60 min before the GF-agent challenge and then the oxime therapy was applied in the same time scheme as before. All the therapeutic regimens showed a protective ratio higher than 2 and significantly increased the LD_{50} of GF-agent. The most efficacious oxime in decreasing of GF-agent toxicity was HI-6. PANPAL increased the protective ratios of all therapeutic regimens in comparison with administration of the oxime and BNZ alone. From the results obtained, the combination of pretreatment with PANPAL and following therapy with BNZ and HI-6 seems to be the most efficacious therapeutic regimen for treatment of GF-agent-poisoned mice.

Keywords: GF-agent – cyclosarin – benactyzine – pyridostigmine – PANPAL – oxime

INTRODUCTION

Organophosphate (OP) nerve agents (sarin, soman, cyclosarin and tabun) are extremely toxic chemicals that were first developed by the German chemist Gerhard Schrader before and during World War II. They pose potential neurotoxic threat to both the military and civilian population as evidenced in recent terrorist attacks (Ohtomi et al. 1996). Some OP compounds are employed as insecticides and acute insecticide poisoning caused by occupational exposures or non-occupational accidents or intentional suicides, is still a major public health problem (Stefanidou et al. 2003).

New interest in cyclosarin (GF-agent; O-cyclohexyl methylphosphonofluoridate) arose shortly after the Gulf War in 1991, when it was recognized that cyclosarin was stockpiled by Iraq (Lundy et al. 1992).

The acute toxicity of OP compounds in mammals is generally believed to be due to their irreversible inhibition of the enzyme

acetylcholinesterase (AChE, EC 3.1.1.7) and subsequent accumulation of the neurotransmitter acetylcholine (ACh) in synapses of the central and peripheral nervous systems and over-stimulation of post-synaptic cholinergic receptors (Marrs 1993).

The current standard antidotal treatment usually includes a muscarinic ACh receptor antagonist to block the over-stimulation of cholinergic receptors by ACh, and an oxime to reactivate OP-inhibited AChE (Kassa 2002). Pralidoxime (2-PAM; 2-hydroxyiminomethyl-l-methylpyridiniumchloride), obidoxime (Toxogonin; 1,3-bis (4-hydroxyimino methylpyridinium)-2-oxa-propane dichloride) and HI-6 (1-(2-hydroxyiminomethyl pyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) are the most widely used representatives of the oximes. These compounds, with quaternary nitrogen that promotes binding in the catalytic site of the AChE, are now a mainstream of treatment for organophosphate exposure. Unfortunately, none from the above mentioned oximes can be regarded as a broad spectrum antidote, because of their inability to reactivate AChE inhibited by all nerve agents (Worek et al. 2002). For example, H-oxime HI-6, currently considered as the best known AChE reactivator, is not able to reactivate satisfactorily tabun-inhibited AChE (Kuča et al. 2003).

The relatively unsatisfactory treatment available for acute soman, tabun and cyclosarin poisoning has resulted in study of pretreatment possibilities that allow survival and increase the resistance of organisms exposed to nerve agents. Pyridostigmine, that is usually administered as pretreatment, "reversibly" inhibits the AChE and is used to protect a portion of the enzyme from irreversible phosphonylation by the OP. Spontaneous decarbamylation of pyridostigmine inhibited AChE in parallel with the rapid removal of the OP from the body provides a source of free enzyme to sustain the cholinergic function (Berry and Davis 1970). However, pyridostigmine-induced increase in the level of ACh can itself cause symptoms of poisoning. Therefore, it would be useful to counteract the effects of the accumulated ACh using anticholinergic drugs such as benactyzine (BNZ) or trihexyphenidyl (THP). In addition, the combination of pyridostigmine with anticholinergic drugs, allows the dose of pyridostigmine that is otherwise limited by symptoms caused by an elevated level of ACh, to be increased and results in a higher prophylactic efficacy than that observed for pyridostigmine alone (Kassa and Vachek 2002; Bajgar et al. 1994). One of these mixtures, pyridostigmine in combination with BNZ and THP, designated PANPAL has been developed in the Czech Republic and introduced to the Czech Army (Vachek et al. 1993).

Therefore, the present study was performed to assess and compare the therapeutic efficacy of various oximes combined with BNZ in GF-poisoned mice and to evaluate the influence of pretreatment with PANPAL on the effect of antidotal treatment in mice poisoned with GF-agent.

MATERIAL AND METHODS

Animals

Female mice, weighing 21–27 g from Velaz Prague (Czech Republic) were kept in an air-conditioned room with light from 07:00 to 19:00 h and were allowed free access to standard chow and tap water. The mice were divided into groups of six animals each. The handling of the experimental animals was under the supervision of the Ethics Committee of the Purkyně Military Medical Academy and the Medical Faculty of Charles University (Hradec Králové, Czech Republic).

Material

GF-agent (cyclohexyl methylphosphonofluoridate; cyclosarin) of 99.9% purity was obtained from the Military Technical Institute in Zemianské Kostolany (Slovak Republic). Its purity was determined by acidimetric titration. The oximes of at least 98.0 % purity were synthetized earlier in the Department of Toxicology of Purkyne Military Medical Academy in Hradec Králové (Czech Republic). All other chemicals and drugs of analytical grade were obtained commercially and used without further purification.

Animal experiment

In the first part of our experiment, methoxime (1,1-bis(pyridinium-4-aldoxime)methane dichloride) (13.78 mg/kg), BI-6 (1-(2-hydroxyiminomethyl pyridinium)-4-(4-carbamoylpyridinium)-butene dibromide) (5.33 mg/kg) or HI-6 (1-(pyridinium-2-aldoxime)-3-(pyridinium-4-carbamoyl)-2-oxapropane dichloride) (13.78 mg/kg) in combination with BNZ (3.39 mg/kg) were used for the treatment of GF-poisoned mice. This oxime therapy combined with an anticholinergic drug was administered intramusculary (i.m.) 1 min after i.m. GF-agent challenge. In the second part of experiment the animals were pretreated with PANPAL – a combination of pyridostigmine (5.82 mg/kg), BNZ (70.0 mg/kg) and THP (16.0 mg/kg) 60 min before the GF-agent challenge and then the oxime therapy was applied in the same time scheme as before. All the drugs were applied in a dose of 2% LD_{50}. The

doses of GF-agent applied during the experiment were in the range 0.3–1.0 mg/kg (1.67–5.56 LD_{50}).

Data analysis

GF-agent-induced toxicity was evaluated by the assessment of LD_{50} values and their 95% confidence limits within 1 week after the administration of GF-agent at five different doses with six mice per dose (Tallarida and Murray 1987). The efficacy of the tested treatment was expressed as a protective ratio (LD_{50} value of GF-agent in treated mice/LD_{50} value of GF-agent in non-treated mice). The statistical differences between LD_{50} values were considered to be significant using t-test at the significance level=2α (Roth et al. 1962).

RESULTS AND DISCUSSION

The therapeutic efficacies of the oxime therapy applied 1 min after GF-agent challenge are presented in Table 1. All the therapeutic regimens showed a protective ratio higher than 2 and significantly increased the LD_{50} of GF-agent. The most efficacious oxime in decreasing the GF-agent toxicity was HI-6. PANPAL increased the protective ratio in all cases but it was only statistically significant for methoxime (Table 2).

Table 1. **The therapeutic effect of various oximes in combination with benactyzine in GF-poisoned mice**

Treatment	LD_{50} (μg/kg) with 95% confidence limits	Protective ratio
–	190 (171–210)	–
Methoxime, Benactyzine	406(362–455)*	2.14
BI-6, Benactyzine	533(506–561)*	2.80
HI-6, Benactyzine	641(543–757)*	3.37

Oxime therapy in combination with benactyzine administered 1 min following GF-agent challenge.
* statistically significant as compared with the non-treated animals

From the results obtained, the combination of pretreatment with PANPAL and following therapy with BNZ and HI-6 seems to be the most efficacious therapeutic regimen for treatment of GF-agent-poisoned mice.

A comparison of the therapeutical efficacy of the oxime HI-6 and methoxime or BI-6 showed that the protective effects of HI-6 were a little higher than methoxime or BI-6 in GF-poisoned mice although the difference between the protective effects of HI-6 and methoxime or BI-6 was not significant. The *in vitro* reactivation of rat brain AChE showed that HI-6 was a more powerful reactivator of GF-inhibited AChE than methoxime or BI-6 (oxime concentration 10^{-3} M) (Kuča and Patočka 2004), a result in good agreement with our *in vivo* experiment. Furthermore, HI-6 produced significant *in* vivo reactivation of GF-inhibited ChE as well as AChE in the blood, brain and diaphragm when compared to obidoxime (Kassa and Bajgar 1995). In addition, the safety factor (LD_{50}/ED_{50}) of HI-6 is considerably greater than the conventional oximes (PAM and obidoxime) and the other oximes tested (methoxime, BI-6) (Kassa 2001), demonstrating that the choice of this oxime as replacement therapy for GF-agent poisoning is justified.

The addition of anticholinergic drugs to pyridostigmine is useful for eliminating the side effects of pyridostigmine, especially the effects of accumulated ACh. Generally, pyridostigmine at a commonly used dose (30 mg pyridostigmine tablet three times a day) is thought to be without significant side effects, but when it was taken by 10 asthmatic soldiers during Operation Desert Storm the exacerbation of asthma symptoms in seven of the asthmatics was observed (Gouge et al. 1994, Wenger et al. 1993). It was demonstrated that exposure to physiologically relevant doses of pyridostigmine leads to neurobehavioral deficits and region specific alterations in AChE and

Ach receptors (Abou-donia et al. 2001). On the other hand, a peripherally acting carbamate with centrally acting anticholinergics may also result in severe side effects. Neverthless, the combination of pyridostigmine with two centrally acting anticholinergics – BNZ and THP designated as PANPAL – was clinically examined at doses recommended for humans and no health problems or side effects were found during clinical and laboratory observation of the volunteers following usage of PANPAL (Kassa et al. 2001).

In conclusion, the data suggest that all the oximes tested in combination with BNZ are effective antidotes against GF-agent poisoning and pretreatment with PANPAL increases the therapeutic efficacy of antidotal treatment in mice poisoned with the GF-agent. The results of this study once again point to the benefit of employing HI-6 as a therapy for nerve agent poisoning, because both tharapeutic regimens including HI-6, the one with BNZ as well as the one with PANPAL pretreatment, were the most effective from those tested.

Table 2. **The therapeutic effect of pretreatment with PANPAL on the therapeutic efficacy of antidotal treatment in GF-poisoned mice**

Pretreatment	Treatment	LD_{50} (μg/kg) with 95% confidence limits	Protective ratio
–	–	190 (171–210)	
PANPAL	Methoxime, Benactyzine	670 (583–771)*	3.53
PANPAL	BI-6, Benactyzine	610 (484–767)	3.20
PANPAL	HI-6, Benactyzine	766 (641–916)	4.03

Mice were pretreated with PANPAL 60 min before GF-agent challenge. Oxime therapy in combination with benactyzine was administered 1 min following GF-agent challenge

* statistically significant as compared with animals after oxime and benactyzine administration

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REFERENCES

Abou-donia M.B., L.B. Goldstein, K.H. Jones, A.A. Abdel-Rahman, T.V. Damodaran, A.M. Dechkovskaia, S.L. Bullman, B.E. Amir, W.A. Khan: Locomotor and sensimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET, and permethrin, alone and in combination. Toxicol. Sci. 60: 305–314, 2001.

Bajgar J., J. Fusek, J. Vachek: Treatment and prophylaxis against nerve agent poisoning. ASA Newsl. 94: 10–11, 1994.

Berry W.K. and D.R. Davis: The use of carbamates and atropine in the protection of animals against poisoning with 1,2,2-trimethylpropylmethyl-phosphonofluoridate. Biochem. Pharmacol. 19: 927–934, 1970.

Gouge S.F, D.J. Daniels, C.E. Smith: Exacerbation of asthma after pyridostigmine during Operation Desert Storm. Mil. Med. 159: 108–111, 1994.

Kassa J.: Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J. Toxicol. 40: 803–816, 2002.

Kassa J.: The influence of anticholinergic drug selection on the effectiveness of oximes against soman-induced supralethal poisoning in mice. Acta Medica (Hradec Králové) 44: 77–79, 2001.

Kassa J. and J. Bajgar: Comparison of the efficacy of HI-6 and obidoxime against cyclohexyl methylphosphonofluoridate (GF) in rats. Hum. Exp. Toxicol. 14: 923–928, 1995.

Kassa J. and J. Vachek: A comparison of the efficacy of pyridostigmine alone and the combination of pyridostigmine with anticholinergic drugs as pharmacological

pretreatment of tabun-poisoned mice and rats. Toxicology 177: 179–185, 2002.

Kassa J., J. Vachek, J. Bajgar, J. Fusek: A combination of pyridostigmine with anticholinergic drugs:_effective pharmacological pretreatment of soman-poisoned mice. ASA Newslett. 16: 1–3, 2001.

Kuča K., J. Bielavský, J. Cabal, J. Kassa: Synthesis of a new reactivator of tabun inhibited acetylcholinesterase. Bioorg. Med. Chem Lett. 13: 3545–3547, 2003.

Kuča K. and J. Patočka: Reactivation of cyclosarin-inhibited rat brain acetylcholinesterase by pyridinium oximes. J. Enzyme Inhib. Med. Chem. 19: 39–43, 2004.

Lundy P.M., A.S. Hansen., B.T. Hand, C.A.Boulet: Comparison of several oximes against poisoning by soman, tabun and GF. Toxicology 72: 99–105, 1992.

Marrs T.C.: Organophosphate poisoning. Pharmacol. Ther. 58: 51–66, 1993.

Ohtomi S., S. Takase, F. Kumagai: Sarin poisoning in Japan. A clinical experience in Japan Self Defense Force (JSDF) Central Hospital. Int. Rev. Arm. 69: 97–102, 1996.

Roth Z., M. Josífko, V. Trčka: Statistické metody v experimentální medicíně. Státní zdravotnické nakladatelství, Praha 1962, pp. 405–413.

Stefanidou M., S. Athanaselis, M. Velonakis, F. Pappas, A. Koutselinis: Occupational exposure to cholinesterase inhibiting pesticides: a Greek case. Int. J. Environ. Health Res. 13: 23–29, 2003.

Tallarida R. and R. Murray: Manual of pharmacological calculation with computer programs. Springer Verlag, New York 1987, pp.145.

Vachek J., J. Kassa, J. Fusek, J. Bajgar: Present possibilities of treatment of organophosphate poisoning. Sborník vědeckých prací VLA JEP Hradec Králové 116: 67, 1993.

Wenger B., M.D. Quigley, M.A. Kokla: Seven-day pyridostigmine administration and thermoregulation during rest and exercise in dry heat. Aviat. Space Environ. Med. 64: 905–911, 1993.

Worek F., G. Reiter, P. Eyer, L. Szinicz: Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch. Toxicol. 76: 523–529, 2002.

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