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ORIGINAL ARTICLE

Inhibition of blood and tissue cholinesterases by soman in guinea pigs *in vivo*

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Summary
Guinea pigs were intoxicated intramuscularly with different doses of soman, and cholinesterase activities were determined in the blood, diaphragm and parts of the brain – the pontomedullar area, the frontal cortex and the basal ganglia. The time course of poisoning following low doses (1,3, 5 μg/kg) and a dose equal to $1\times LD_{50}$ (28.5 μg/kg) were studied. The dose having a negligible effect on cholinesterases in the tissues studied was assessed at 1–3 μg/kg, and, following administration of a dose of 5 μg/kg, statistically significant blood cholinesterase inhibition was demonstrated.

Key words: blood; brain parts; cholinesterases; guinea pig; soman; inhibition *in vivo*

INTRODUCTION

The most important chemical warfare agents are sarin (O-isopropyl methylphosphonofluoridate), soman (O-pinacolyl methylphosphonofluoridate) (these two compounds belong to so called G-compounds) and VX (O-ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate) (V-compounds) (Bajgar 2004, Patočka 2004). Many organophosphorus compounds are produced in civilian facilities and evaluated in industry, agriculture, human and veterinary medicine etc. The threat of their use not only in military conflicts but also therefore in civilian life cannot be excluded as is evident from the terroristic attacks in Japan (Morita et al. 1995, Okumura et al. 1996, Nozaki et al. 1997) and extensive knowledge of their effects is a necessary basis for studies searching for the effective treatment of intoxication following their use.

From the pharmacodynamics perspective, soman is the most serious poison: its toxicity and inhibition potency to AChE is relatively high and comparable with that of VX (Clement et al. 1981, Clement 1989, Shih et al. 1990, Bajgar 1991, 1992, Patočka et al. 2005, Fawcett et al. 2009). Soman is quickly resorbed at all routes of administration (Bajgar 1991) and inhibits cholinesterases (particularly acetyl-cholinesterase, AChE, EC 3.1.1.7) in the central and peripheral nervous system. The high lipophility of soman leads to a higher affinity to the central nervous system with subsequent strong inhibition of the brain AChE *in vivo* (Bajgar 1991, 1992). The inhibition of the blood and brain AChE by soman is very fast, attaining 50% activity within minutes (Bajgar 2004).

Soman and sarin are detoxified in the liver plasma (Jokanović 1990, Bajgar 1991, Skopec and Bajgar

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1993) and, therefore, this part is excluded from the toxic effect. Also losses of G-compounds in the organism are caused by their binding to non specific esterases, cholinesterases and detoxification, so this part of soman is not able to produce a toxic effect. It has been estimated that only 1–3% of the dose administered inhibited AChE in the brain; i.e. 1–3% of the dose administered produced the basic toxic effect (Kadar et al. 1985, Bajgar 1991). Sterri and Fonnum (1981) have reported that only about 5% of the given dose of soman reacts with AChE, while the remainder is detoxified. On the other hand, V compounds are not detoxified (by direct decomposition) in the organism (Bajgar 1991), but VX is detoxified by other routes, e.g. a decrease in the effective level of VX in the organism can be caused via its binding to the enzymes mentioned. Soman is also bound to other proteins and a decrease in its level can also be produced by tissue depots (Jokanović 2009).

The mechanism of AChE inhibition is practically the same for all nerve agent compounds: phosphorylation or phosphonylation of serin in the catalytic triad – the so-called esteratic site (Ser_{200}-His_{440}-Glu_{327}) at the bottom of a deep and narrow cavity of the enzyme. The rate of spontaneous dephosphorylation is very low and it can be omitted in most cases. However, it can be improved/increased using cholinesterase reactivators (oximes) able to reactivate nerve agent-inhibited AChE (Kuča et al. 2004, Patočka et al. 2005, Musílek et al. 2007). Depending on the structure of the inhibitor, inhibited AChE is dealkylated (aged) and the complex formed is resistant to reactivation. This reaction is very fast for soman-inhibited AChE (the half-life is about 10 min) (Bajgar 1991, 2004). Therefore it can be concluded that AChE activity inhibited by soman cannot be changed spontaneously after a short period of exposure. Thus, soman can be used as a model of stable inhibition of AChE. Data are available on cholinesterase inhibition following relatively high doses of soman applied to experimental animals (Bajgar 1992, Patočka et al. 2005, Shih et al. 2005, 2009a, b, Žďárová Karasová et al. 2009a), but there are no detailed data dealing with the effect of low doses of soman by parenteral administration. Some attempts have been made using soman inhalation exposure, to assess the minimal dose of soman causing negligible changes of AChE activity (Bajgar et al. 2004).

The question of choice of experimental animal is of great interest. Most experiments have been performed on rats (Clement 1989, Patočka et al. 2005, Bajgar et al. 2007, 2009, Novotný et al. 2009, Žďárová Karasová et al. 2009a, b and others) and guinea pigs have been used in a few studies (Shih et al. 2005, 2009a, b, Fawcett et al. 2009, Mamczarz et al. 2010) but the presence of carboxylesterases in rats influences toxicity and therapeutic studies (Kadar et al. 1985). Different levels of organophosphate metabolizing carboxylesterases in the blood of various species contribute to their differential sensitivity to these agents. Primates (including humans) have almost none, whereas guinea pigs have low levels, and rats and mice have high levels of blood carboxylesterases. Thus, to standardize the development of antidotes to nerve agents, it was recommended that these studies should be carried out on guinea pigs (Koplovitz et al. 1992, Fawcett et al. 2009). Soman toxicity is not dependent on the sex or age of the guinea pigs (Fawcett et al. 2009).

This study is focused on the dose dependent and time dependent changes in AChE activities in different tissues of guinea pig, and an estimation of the minimal dose of soman causing negligible changes in AChE activity. To compare our results more precisely with data from relevant literature (time course of AChE inhibition following s.c. soman administration; Shih et al. 2005), the same dose ($1\times LD_{50}$) was also used.

MATERIAL AND METHODS

Animals

Female guinea pigs (Tricolor, BIO.TEST, s.r.o., Konárovice, Czech Republic) weighing 350±30 g were used in groups of 6 animals. The animals were housed in the Central Vivarium of the Faculty of Military Health Sciences under veterinary control. All the experiments were performed with the permission of and under the supervision of the Ethics Committee of the Faculty of Military Health Sciences, Hradec Králové (permission No 153/06) according to § 17 of Czech law No 207/2004, permission of responsible person 0001/94 – M 699.

Chemicals

Soman was obtained from the Military Technical Institute of Protection (Brno, Czech Republic). It was of minimally 98% purity and stored in glass ampullas (0.33 ml). The solutions for the experiments were prepared immediately before use.

Intoxication – dose dependence

The control group received an i.m. injection of saline (0.1 ml/100 g body weight) whereas the study groups were given soman i.m. in doses of 1, 3, 5, 20, 24, 27, 28.5 and 30 μg/kg; the LD_{50} of soman determined in

previous experiments was 28.5 μg/kg. The animals were killed following aether anaesthesia 30 min after intoxication.This procedure does not influence cholinesterase activity (Novotný et al. 2009). The blood and organs were removed and haemolysates or homogenates were prepared. The animals injected with saline served as the control group.

Intoxication – time dependence
The animals in groups were intoxicated with soman in doses of 1, 3, 5 and 28.5 μg/kg (i.m.) and blood and organs were removed at different time intervals (1, 5, 10, 30, 60 and 120 min) after intoxication except for the last dose used, where the sampling was made 30 min after intoxication or immediately after death.

Preparation of samples
The blood was haemolysed with distilled water (1:10) and AChE activity was determined immediately in haemolysates. The tissues were frozen at –40 °C. After thawing, the frontal cortex (FC), basal ganglia (BG) and pontomedullar part (PM) of the brain were prepared. Then the brain parts and diaphragm were homogenised (Janke and Kunkel homogeniser, Germany) 1:10 with distilled water. AChE activity was determined in homogenates.

Determination of cholinesterase activity
AChE activity was determined according to Ellman et al. (1961) as follows: 100 μl of the haemolysate was mixed with 1700 μl DTNB solution (1 mM solution of DTNB in 0.1 M TRIS-HCl buffer, pH 7.6) and the enzymatic reaction was started by adding 200 ul of substrate solution (1 mM acetylthiocholine in distilled water). The mixture was stabilized for 3 min and then the absorbancy at 436 nm/min was monitored (this delay is necessary for exclusion of false positive results by titration of free SH-groups interfering with the activity determination). The activity was expressed as μkat/l or g wet weight tissue or as % of control values.

Statistical evaluation
The homogeneity of the experimental groups was tested by Bartlett's test. The differences between groups were calculated using means + SD and differences were tested by Student's test at the significance level $2\alpha=0.05$.

RESULTS

Absolute values of cholinesterase activity in the guinea pig blood varied from 77.0 to 98.5 μkat/l, with an average of 83.3±12.5 μkat/l. It is of interest that the activity determined in the diaphragm is very low. In the brain, AChE activity varied from the highest of 435.3±47.2 μkat/g, BGto the lowest of 90.2±10.9 μkat/g, FC. For better comparison following soman intoxication, all the data were expressed as a % of the control values.

Changes in cholinesterase activity following different doses of soman are shown in Fig. 1. It is clear that the decrease of activity is dose dependent. The sensitivity of various tissues is different: cholinesterase activity in the blood is the most sensitive, followed by the diaphragm, and the relative resistance of AChE activity in the basal ganglia is evident. The dose causing 50% of inhibition in particular tissues can be assessed to be about 15 μg/kg in the blood, while the dose causing 50% of inhibition in the basal ganglia is 25 μg/kg. At doses approaching LD_{50} value, all activities decrease to 0–10% of control values for all tissues studied. Statistically significant inhibition was observed for cholinesterases in the blood from the 5 μg/kg dose; for other tissues studied, this inhibition was registered from the 10 μg/kg dose except cholinesterases in the basal ganglia. Following administration of soman at the highest dose (20 μg/kg), all activities were significantly different from the control values. As for the time course of cholinesterase inhibition, very fast changes for all tissues studied were found. The changes in blood cholinesterase inhibition following administration of three low doses of soman is shown in Fig. 2; the time course of cholinesterase activity decrease following administration of the dose equal to LD_{50} is also demonstrated. The activity reaches a steady state within 5–10 min and it remains practically without changes for 2 h after soman intoxication. The changes in cholinesterase activities following three low doses of soman (120 min after intoxication) are statistically significant at the dose of 5 μg/kg for the blood cholinesterases only. A summary of the changes in cholinesterase activities following low doses of soman 120 min after the intoxication is given in Fig. 3.

DISCUSSION

A comparison of AChE activity in various tissues as reported in the literature is difficult due to the different methodical details adopted in the determination of enzyme activity (e.g. different expression of activity per ml of wet weight tissue or mg of protein). However, when the activity of the structure having the highest activity is compared

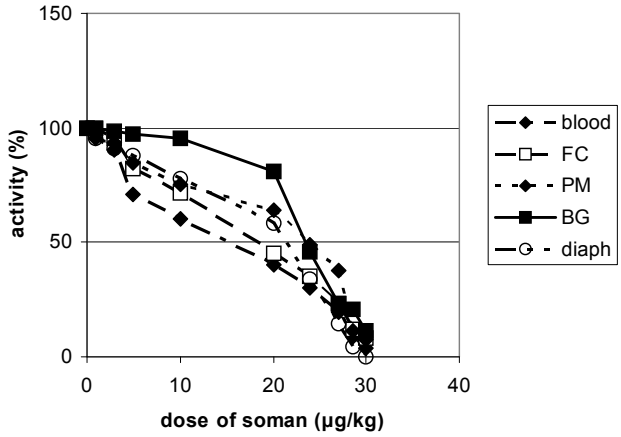

Fig. 1. **Cholinesterase activities in the blood and different organs following soman intoxication** (i.m.) **in different doses.** The results are means only; SD were not higher than ± 15%; example of SD. Cholinesterase determination was made 30 min after the soman injection or immediately after death. Animals injected with saline served as the control group; diaph – diaphragm; FC – frontal cortex; PM – pontomedullar area; BG – basal ganglia.

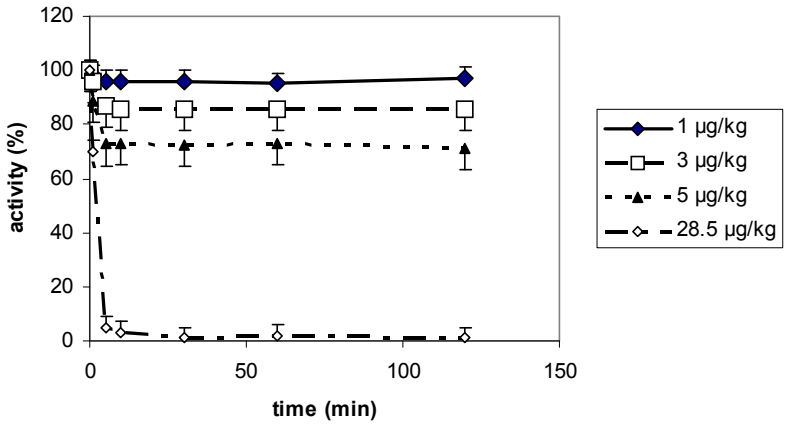

Fig. 2. **Time-course of changes in cholinesterase activities in the blood following soman intoxication** (i.m.) **in different doses.** The results are means with their SD. The animals in groups were intoxicated with soman in doses of 1, 3, 5 and 28.5 µg/kg (i.m.), and blood and organs were removed in different time intervals after the intoxication except for the last dose used (the sampling was made 30 min after the intoxication or immediately after death).

relatively (in %, the highest activity relative to 100%), then comparisons show a good relationship (Fig. 4).

The toxicity of soman in guinea pigs given subcutaneously has been reported variously as 28 µg/kg (Shih et al. 2005) or from 25.1 to 27 µg/kg (Fawcett et al. 2009); very close to the toxicity at i.m. administration. Our results showed toxicity from i.m. administration (for female guinea pigs) as 28.5 µg/kg. In contrast, percutaneous administration showed approximately 400 times higher toxicity (DeMar et al. 2010). It has been demonstrated recently in guinea pigs, that the lethal potencies of nerve agents VX and sarin are sex-and age dependent, whereas the lethal potency of soman is not so dependent (Fawcett et al. 2009).

The minimal dose of soman causing negligible changes in blood cholinesterase activity can be considered to be 1–3 µg/kg; very roughly, this dose is equivalent to the dose of 1.2 µg/l as demonstrated in

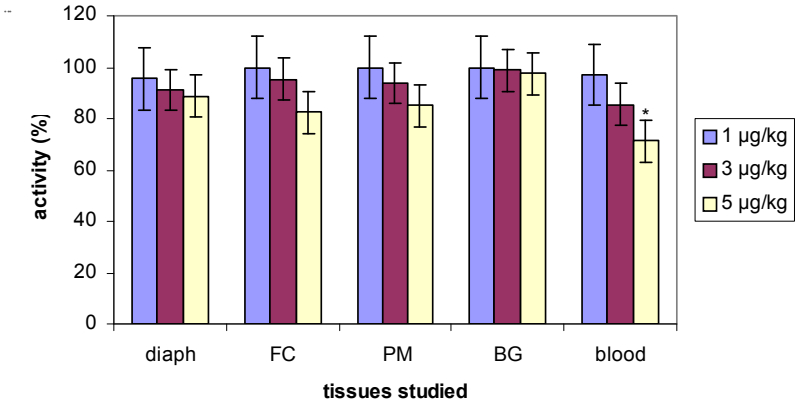

Fig. 3. **Changes in cholinesterase activities (%) 120 min following intramuscular soman administration at low doses.** The results are means with their SD. Statistically significant difference was observed for the blood and highest dose, 5 µg/kg; indicated by asterisk; abbreviations as in Fig. 1.

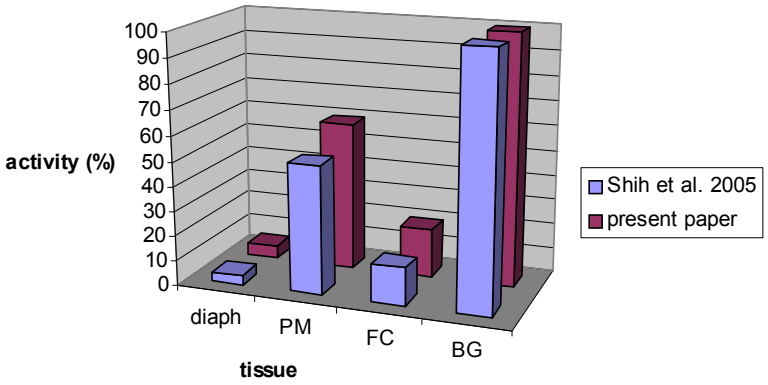

Fig. 4. **Comparison of our present results and results described** by Shih et al. (2005). AChE activity in the basal ganglia was expressed as 100%, the activities of other structures was expressed in % (highest activity in each paper related to 100 %); abbreviations as in Fig. 1.

our previous studies of the exposure of guinea pigs to soman inhalation (Bajgar et al. 2004), and corresponds to $0.3\times LCt_{50}$ or approximately $0.1\times LD_{50}$ for i.m. administration as in our present experiments. The dose most significantly influencing the activity is 5 µg/kg, corresponding to 0.2×LD50. For inhalation exposure, this concentration was 1.5 µg/l i.e. $0.4\times LCt_{50}$, respectively. Thus, a small increase in the dose of soman can be followed by a fast decrease in cholinesterase activity in the brain; it is of interest that small changes in AChE activities in the brain have been described as critical for survival or non-survival of experimental animals – in this case rats (Bajgar et al. 2008) and thus for treatment. Partial reactivation caused by different oximes in reactivation experiments was found to be important to their therapeutic effectiveness (Bajgar 2004, Kuča et al. 2004, Patočka et al. 2005, Musílek et al. 2007). These fine changes are of great interest for further studies. Moreover, this approach could lead to improvement in our knowledge of the mechanisms of the action of organophosphates and soman poisoning. At the same time, it could contribute to a better understanding of cholinergic nerve transmission and thus to pharmacology and neuropharmacology in general.

CONCLUSIONS

Low doses of soman (1,3 and 5 μg/kg) administered i.m. caused statistically significant ($p<0.05$) inhibition of cholinesterases at a dose of 5 μg/kg in the blood only but not in the diaphragm and brain parts.

Following a dose of soman close to LD_{50} (28.5 μg/kg), changes in cholinesterase activity were very fast attaining a steady rate 10 min after soman injection.

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