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ORIGINAL ARTICLE

Acute poisoning with sarin causes alteration in oxidative homeostasis and biochemical markers in Wistar rats

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Summary
Sarin is a potent inhibitor of acetylcholinesterase (AChE). It is known as an agent of chemical warfare and is one of a number of nerve agents misused for chemical terrorism, e.g. on the Tokyo subway attacks. Though effect of sarin on the cholinergic system is well-known, long-term adverse effects and the role of oxidative stress in sarin toxicity remain unknown. The experiment reported here was carried out on laboratory Wistar rats intramuscularly exposed to 0.5–50% of sarin LD_{50} for one hour. A complex biochemical examination of plasma samples and an assessment of oxidative stress in the liver, kidney, spleen, cerebellum and frontal lobe were performed after euthanasia of the animals. By means of these biochemical markers, we were able to observe the induction of hyperglycaemia in a dose-dependent manner. Other biochemical markers such as transaminases were influenced in a non-standard manner as sarin probably acted as an inhibitor of these markers. Oxidative stress markers and an assessment of AChE activity showed an unequal impact of sarin on different tissues. Significant inhibition of AChE was found in the cerebellum and frontal lobe. Besides this, alterations in reduced glutathione, ferric reducing antioxidant power (FRAP) and thiobarbituric acid reactive substances (TBARS) were proven. In particular, an accumulation occurred of reduced glutathione in the frontal lobe, whereas depletion of FRAP was found in the kidney and spleen, and a strong increase in TBARS occurred in the spleen in a dose-dependent manner. We infer that sarin extensively influences oxidative homeostasis. Surprisingly, the central nervous system seems to be more resistant than the other organs.

Key words: sarin; nerve agents; chemical warfare; acetylcholinesterase; oxidative stress; antioxidant; biochemistry

Abbreviations:
Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; Glu, glucose; IP, inorganic phosphate; T-Bil, total bilirubin; TP, total protein.

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INTRODUCTION

Sarin (IUPAC name: propan-2-yl methylphosphonofluoridate) is a highly toxic nerve agent with the reported median lethal dose (LD_{50}) 103 μg/kg for subcutaneously exposed rats (Brimblecomb et al. 1970). Like the other nerve agents, it is an irreversible inhibitor of enzymes acetylcholinesterase (AChE) and

butyrylcholinesterase (BuChE). The irreversible inhibition of AChE causes hyper-stimulation of nicotinic as well as muscarinic acetylcholine receptors, as the neurotransmitter acetylcholine is not degraded (Marrs 1993). After exposure, symptoms of acute toxicity such as tremors, seizures and hypothermia follow (Abu-Qare and Abou-Donia 2002). Exposure to sarin can be resolved by oxime reactivators such as obidoxime or HI-6. Application of the reactivators results in a reverse of cholinesterase activities (Kassa and Cabal 1999). Sarin has a prominent role as one of the most important nerve agents. In the 1991 Gulf War, soldiers were accidentally exposed to sarin and cyclosarin during the disposal of military stockpiles at Khamisiyah (Proctor et al. 2006). Some years later, sarin was used for terrorist purposes by Aum Shinrikyo in Japan in 1994 and 1995 (Yanagisawa et al. 2006).

The complex effects of nerve agents on the nervous system remain unknown. Nerve agents have been reported as inductors of neuronal cell apoptosis. Surprisingly, N-methyl-D-aspartate receptor pathways are also involved in the pathological process after poisoning by sarin (Wang et al. 2008). Sarin has been shown to be an initiator of oxidative stress, and as a marker of DNA oxidative degradation; 8-hydroxy-2´-deoxyguanosine, was significantly elevated after intramuscular administration of the toxin into rats (Abu-Qare and Abou-Donia 2001). Our primary intention was aimed at identification of the adverse effects of sarin in a Wistar rat model. For experimental purposes, we chose a complex set of tests suitable for oxidative stress scoring in tissues. Additionally, we applied a standard assessment of biochemical plasma markers in order to estimate organ-specific pathologies. We aimed the experiment to answer the question whether the toxic effects of sarin can also be followed by oxidative stress and imbalance in antioxidants. Answering this question would be an initial step in research into a non causative therapy in nerve agents poisoning.

MATERIAL AND METHODS

Animal model and samples collection

40 male Wistar rats were purchased from the Velaz Company (Prague, Czech Republic). The rats were six weeks old at the beginning of the experiment and weighed 192±5 g. For the entire experiment, the rats were kept in an air conditioned room at temperature 22±2 °C, humidity 50±10% and light period 12 hours per a day. Feed and drinking water were provided ad libitum. The entire experiment was approved and supervised by the Ethical Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic.

Sarin was obtained from the Military Technical Institute of the former Czechoslovakia. The Faculty of Military Health Sciences has permission for the manipulation of nerve agents from the government institution SUJB, the official representative of the Organisation for the Prohibition of Chemical Weapons (OPCW) in the Czech Republic. The LD_{50} was assessed in a separate experiment to be 0.472 mg/kg (mortality followed up to 24 hours).

Animals were exposed intramuscularly in the pelvic area of the hindlimb to sarin at doses 0 (controls; saline only), 0.5, 2, 10 and 50% of LD_{50}. After one hour, animals were sacrificed by carbon dioxide narcosis. Blood, kidney, liver, brain and spleen were collected from the sacrificed animals. The frontal lobe and cerebellum were collected from the brain. Blood was kept in heparinised tubes (Dialab, Prague, Czech Republic) and plasma was collected after blood centrifugation at 3,000G for 15 minutes. The collected tissues were homogenised using an Ultra-Turrax mill (Ika Werke, Staufen, Germany). A total of 100 mg of freshly collected tissue was mixed with 1 ml of saline solution for one minute.

Ex vivo assay

Ferric reducing antioxidant power (FRAP), glutathione reductase (GR), reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) were assessed as oxidative stress markers. Measurement procedures were in compliance with reported papers (Pohanka et al. 2010, 2011a). AChE activity in tissues was measured in compliance with another paper (Pohanka et al. 2011b). The following plasma biochemistry markers were assayed by SPOTCHEM TM EZ SP-4430 (Arkray, Japan).

Statistics

A group size was eight specimens. The Bonferroni test (Origin 8 SR2, OriginLab Corporation, Northampton, MA, USA) was used at the significance level $2\alpha=0.05$.

RESULTS

The poisoned animals had no conspicuous manifestation when poisoned by doses 0.5 and 2% LD_{50}. The animals exposed to sarin in doses 10 and

50% exerted tonic-clonic seizures starting five to ten minutes after poisoning and lasting up to the sacrificing.

Biochemistry markers in plasma

The assayed biochemistry markers are depicted as Table 1. Albumin was significantly elevated at 10 and 50% of the LD_{50} of sarin. Alanine aminotransferase and aspartate aminotransferase were significantly decreased when sarin was administered at a dose 0.5% of LD_{50} and more. Sarin had no significant effect on total protein levels and calcium in plasma. Blood urea nitrogen was significantly decreased at 10 and 50% of the LD_{50} of sarin, while glucose was significantly elevated at 50% of LD_{50}. Creatinine was significantly decreased at doses above 0.5% of LD_{50} and at 2% of LD_{50}. Inorganic phosphate was significantly decreased when the sarin dose exceeded 2% of LD_{50}.

Table 1. **Selected biochemical markers.**

Sarin dose (% LD_{50})	0 (controls)	0.5	2	10	50
Alb (g/l)	26.5±5.0	31.5±2.0	32.0±5.7	34.5±3.7 (*)	33.4±3.9 (*)
ALT (μkat/l)	1.03±0.19	0.688±0.086 (*)	0.475±0.12 (*)	0.455±0.12 (*)	0.329±119 (*)
AST (μkat/l)	5.51±1.48	3.54±0.70 (*)	2.09±0.55 (*)	2.18±0.46 (*)	2.27±0.46 (*)
BUN (mmol/l)	9.40±1.14	8.83±1.59	7.71±0.68	6.98±1.61 (*)	7.11±0.70 (*)
Ca (mmol/l)	2.53±0.21	2.59±0.13	2.58±0.22	2.53±0.17	2.61±0.23
CRE (μmol/l)	77.5±19.6	58.3±10.4 (*)	45.5±7.3 (*)	45.6±13.6 (*)	34.9±5.5 (*)
Glu (mmol/l)	7.15±1.17	7.50±0.84	7.23±0.78	7.86±0.67	11.3±2.0 (*)
IP (mmol/l)	2.56±0,29	2.27±0.17	2.04±0.27 (*)	1.78±0.19 (*)	1.57±0.32 (*)
T-Bil (μmol/l)	30.9±6.7	18.8±3.8 (*)	17.8±1.5 (*)	16.4±4.4 (*)	11.9±1.9 (*)
TP (g/l)	57.9±2.9	56.5±3.0	57.1±7.4	56.6±5.0	63.1±8.4

Mean ± standard deviation. (*) Significant as compared with control.

Acetylcholinesterase

The activity of AChE was tested in all collected organs. The liver, kidney and spleen had low AChE activity. The effect of sarin was not recognisable due to the low activity and high relative standard deviations. The assessed activity of AChE in the frontal lobe and cerebellum is depicted in Fig. 1. No significant effect was seen at the lowest dose of sarin (0.5% of LD_{50}), i.e. no inhibition of AChE was observed in either the cerebellum or frontal lobe. An increased dose of sarin caused significant inhibition of AChE. In the frontal lobe, between 70–80% inhibition was observed and the alteration was significant. The sarin effect on the cerebellum was greater than that observed in the frontal lobe. Inhibition reached 87.9% at the highest dose of sarin. In the cerebellum, the effect was significant at sarin doses 2% of LD_{50} and higher. Higher doses caused a significant decrease in AChE activity in the cerebellum.

Markers of oxidative stress

According to the statistical test used, sarin significantly influenced the balance between antioxidants and oxidative stress in regard to TBARS, GSH and FRAP. However, GR activity was not significantly altered by sarin in any of the examined organs. The GSH level was significantly increased in a dose-dependent manner in the frontal lobe and liver (Fig. 2). The increase was up 40% in the liver and

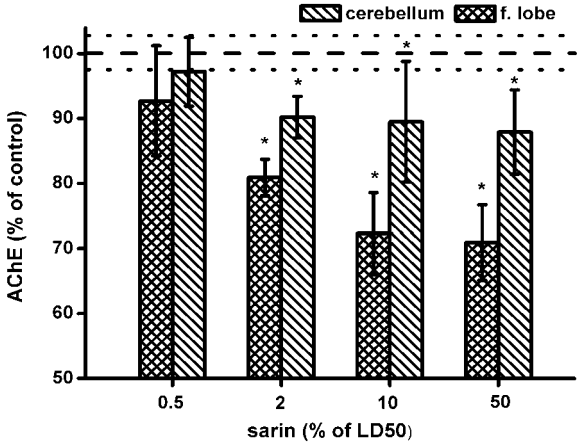

Fig. 1. **Alteration in the acetylcholinesterase (AChE) activity in exposed animals.** Dashed line lay in the control value (100% of AChE activity), dotted lines determinate standard deviation for controls. Error bars represent the standard deviation for n = 8 specimens. * Significant compared with control.

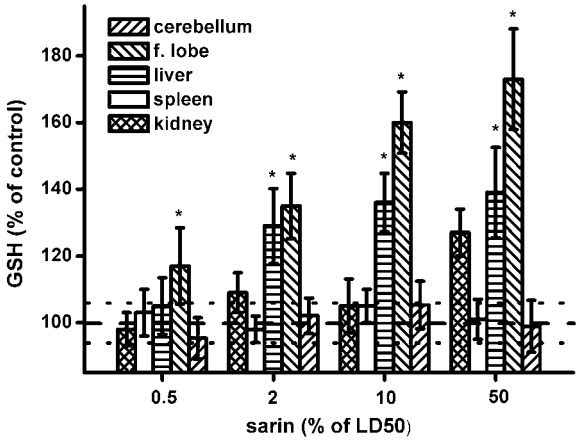

Fig. 2. **Alteration in the reduced glutathione (GSH) level in exposed animals.** Dashed line lay in the control value (100% of GSH level), dotted lines determinate standard deviation for controls. Other symbols as in Fig. 1.

70% in the frontal lobe. The increase in the kidney was insignificant. The livers and cerebellum did not show evidence of sarin on the GSH level. In contrast to GSH, the FRAP level was constant in the frontal lobe, cerebellum and liver in exposed animals (Fig. 3). The kidney and spleen were extensively influenced at the highest dose of sarin. Low molecular weight antioxidants were depleted in the kidney and spleen with the highest sarin dose. Nearly 90% of low molecular weight antioxidants disappeared in the kidney and spleen after the administration of 50% of the sarin LD_{50}.

TBARS (depicted in Fig. 4) were increased in a dose-dependent manner in all organs after the application of sarin. However, the increase was the greatest and significant only in the spleen. TBARS in the spleen were significantly increased after the administration 10% of the sarin LD_{50} and (an approximate increase of 30% against controls) and very significantly increased after the administration of 50% of the sarin LD_{50} (an approximate increase of 50% against the controls).

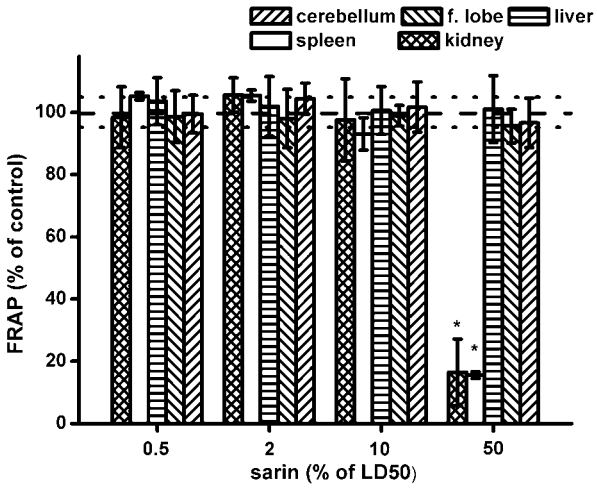

Fig. 3. **Alteration in the ferric reducing antioxidant power (FRAP).** Dashed line lay in the control value (100% of FRAP level), dotted lines determinate standard deviation for controls. Error bars represent the standard deviation for n = 8 specimens. Other symbols as in Fig. 1.

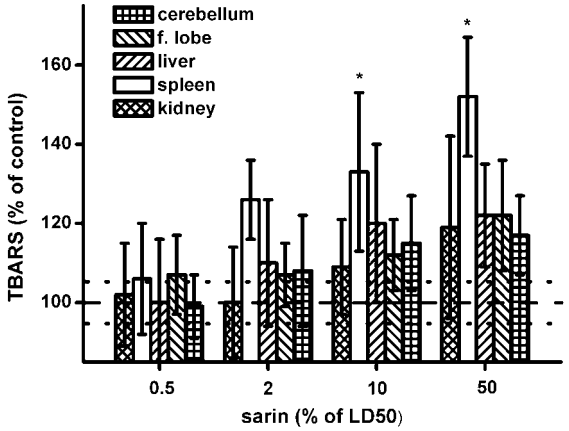

Fig. 4. **Alteration in the thiobarbituric acid reactive substances (TBARS) level in exposed animals.** Dashed line lay in the control value (100% of TBARS level), dotted lines determinate standard deviation for controls. Error bars represent the standard deviation for n = 8 specimens. Other symbols as in Fig. 1.

DISCUSSION

Sarin is a representative nerve agent. As reported by many authors, the toxicity of sarin is based on the inhibition of AChE followed by cholinergic crisis (Niven and Roop, 2004). We decided to investigate AChE activity in the organs rather than blood while establishing the impact of sarin on the endangered organs. The decrease of AChE activity in organs was expected when considering the known mechanism of sarin molecular mechanism of toxicity (Pohanka 2011a). However, the other assessed markers had not been investigated previously and the findings were unpredictable., We were especially surprised by the alterations in plasma biochemical markers. The strong decreases in alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine and total bilirubin have no common biochemical cause as most of the pathologies diagnosed by the markers are distinguished by an increase in their plasmatic values. However, the molecular mechanism of sarin action on the markers can be inferred. Sarin is a reactive

compound, and esterification of tyrosine residues in peptides has been reported (Schopfer et al. 2010). The biological effect of esterification was hypothesised but unproven by Li et al. (2009), e.g. aspartate aminotransferase has a tyrosine residue in its active site (McPhalen et al. 1992). We infer that sarin influenced the assessed markers by an inhibitory mechanism. This should be taken into account when treating human subjects accidentally exposed to sarin. On the other hand, the unexpected decrease in some biochemical markers can serve as a novel marker for clinical toxicologists due to the fact that the decrease is specific for the poisoning. However, experiment focusing on diagnosis should be carried out before considering it as a marker. Unlike the other biochemical markers, glucose and albumin levels increased in plasma. A description of the effect of sarin on the albumin level has not been found in several searched databases. An increase in glucose can be attributed to stress conditions. This phenomenon is in compliance with searched literature (Tiruvoipati et al. 2011). Hyperglycaemia and glycosuria were also reported to be pathological consequences of accidental exposure to organophosphorus and carbamate pesticides (Shobha and Prakash 2000).

Alterations in oxidative homeostasis were found to extensively overweigh the inhibition of AChE. The TBARS level indicates lipid peroxidation by the detection of low molecular weight antioxidants as they respond to a degradation product of lipids, malondialdehyde and can be interpreted as a lack of antioxidants for oxidative stress covering (Lykkesfeldt 2007, Pohanka 2011b). In the spleen, the significant accumulation of TBARS was accompanied by a depletion in low molecular weight antioxidants represented by FRAP. This means that the organ was more vulnerable to oxidative stress induced by sarin. The central nervous system, a part of the body extensively influenced by sarin, seems to be resistant to oxidative insult as no extensive increase in TBARS or depletion in low molecular weight antioxidants was found. On the contrary, reduced glutathione was found to be accumulated in the frontal lobe, even though no significant increase in glutathione reductase, the enzyme which reduces glutathione from the oxidised state (Lowes and Galley 2011), was seen.

Our findings show that sarin alone has only a limited effect on oxidative stress and correspond with the study by Abu-Qare and Abou-Donia (2001) where a small effect of sarin on the oxidation of DNA represented by 8-hydroxy-2′-deoxyguanosine was proven. It seems to be more plausible that sarin induces alterations in oxidative homeostasis and regulation rather than a simple implication in reactive oxygen species generation. The long-term adverse effects of sarin, e.g. reported in a rat model by Allon et al. (2011), are a pathological consequence of exposure. The long-term effects remain even after the reconstitution of new cholinesterase molecules, as the approximate half-life of butyrylcholinesterase is 12 days (Ostergaard et al. 1988). In the central nervous system of rats, new mRNA for AChE is created shortly after exposure to 0.5% of the LD_{50} of sarin (Damodaran et al. 2003). We have shown that alterations in oxidative homeostasis are seen simultaneously with AChE inhibition, and this starts early after exposure. Owing to the results of this study, we infer the changes in oxidative homeostasis to be a relevant consequence following sarin exposure. On the other hand, direct induction of oxidative stress was proven only in the spleen, and the central nervous system seems to be resistant to sarin-induced oxidative insult. When considering the present findings, sarin causes the body to be more vulnerable to other adverse effects once oxidative homeostasis is impaired. This conclusion is in compliance with the results presented by Abu-Qare and Abou-Donia (2001).

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