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The evaluation of oxidative damage of DNA after poisoning with nerve agents

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Abstract
The potency of three nerve agents (sarin, soman, tabun) to induce oxidative damage of DNA in lymphocytes, liver and brain during lethal or sublethal poisoning was investigated. The single strand breaks or oxidative base DNA damage was evaluated with the help of Comet assay and a specific enzyme able to detect oxidative bases of DNA (endonuclease III). While sarin and soman administered at sublethal doses corresponding to 50% of their LD_{50} values were not able to induce oxidative damage of DNA, their lethal dose (LD_{50}) induced the significant increase of the number of oxidative bases in DNA of hepatocytes. In addition, tabun administered at lethal dose (LD_{50}) induced significant increase of the number of single strand breaks and oxidative bases of DNA in glial cells isolated from pontomedullar brain region. Thus, some nerve agents were able to induce oxidative damage in the peripheral as well as central compartment but only in the case of severe poisoning caused by lethal doses of nerve agents. This non-cholinergic effect of nerve agents has probably consequences with nerve agents-induced hypoxic status during acute cholinergic crisis and it can contribute to their long-term toxic effects.

Keywords: Comet assay; DNA; Oxidative damage; Rats; Sarin; Soman; Tabun

Highlights:
- Oxidative DNA damage was evaluated by Comet assay and a specific enzyme able to detect oxidative bases of DNA.
- Lethal dose of sarin and soman induced the significant increase of the number of oxidative bases in DNA of hepatocytes.
- Lethal dose of tabun induced significant increase of the number of oxidative bases of DNA in glial cells.
- This non-cholinergic effect of nerve agents has probably consequences with nerve agents-induced hypoxic status.

Introduction

Highly toxic organophosphorus compounds (OPC) have been developed as chemical warfare agents called nerve agents. They are considered to be the most dangerous chemical warfare agents and represent potential threats to both military and civilian populations. The most important representatives of nerve agents are tabun, sarin, soman, cyclosarin and VX. The acute toxic effects of OPC are based on the phosphonylation of acetylcholinesterase (AChE, EC 3.1.1.7), leading to the irreversible inhibition of its active site and subsequent overstimulation of postsynaptic cholinergic receptors due to the accumulation of the neurotransmitter acetylcholine in synapses of the central and peripheral nervous system (Čolović et al., 2013; Marrs, 2007). The overstimulation of cholinergic receptors results in muscarinic and nicotinic signs and symptoms including excitotoxicity, seizures and brain damage. The death is usually caused by respiratory failure resulting from bronchospasm, excessive bronchial secretion, paralysis of respiratory muscles, and depression of brain respiratory centers (Bajgar, 2004; Delfino et al., 2009).

However, the mechanism of acute and chronic toxicity of OPC is much more complex. There are adverse health effects connected with OPC toxicity that are not related to AChE inhibition and direct cholinergic receptors overstimulation. The mechanisms of these adverse effects of OPC, called non-cholinergic or non-specific effects, are still under extensive research. OPC have many non-specific effects involving the activation of multiple non-cholinergic neurotransmitter systems in the central nervous system, mutagenic, stressogenic, immunotoxic, hepatotoxic, membrane and hematotoxic effects (Bajgar, 2004). OPC-induced glutamate-mediated excitotoxicity (O'Donnell et al., 2011), calcium overload (Choi, 1988), ATP depletion and cellular energy loss (Gupta et al., 2001), alteration of immune functions (Kassa et al., 2000a) and DNA damage (Kassa et al., 2000b; Mehta et al., 2008; Ojha et al., 2013) were also described in the literature. The non-specific effects of OPC are considered to contribute to their long-term toxic effects, therefore, it is important to find the mechanism of the non-specific effects of OPC at the cellular and molecular level. Understanding of the mechanism of non-specific effects of OPC might contribute to the early diagnosis and complex treatment of OPC poisoning (Čolović et al., 2013).

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One of the most important non-specific effects of OPC is the development of oxidative stress that is simply defined as a serious imbalance between enhanced generation of reactive oxygen species (ROS), nitrogen species (RNS) and antioxidant defence, results in disruption of a cellular redox state and associated macromolecular damage. Oxidatively modified biomolecules formed during nitration and carbonylation of proteins, peroxidation of lipids and oxidation of DNA cause deterioration of cellular structural architecture and disturbances in physiological processes and intracellular signaling (Ischiropoulos and Beckman, 2003).

OPC-induced oxidative stress is connected with OPC-induced excitotoxicity, mitochondrial dysfunction and inhibition of endogenous antioxidant capacity. Calcium homeostasis (sustained elevation of intracellular calcium), cellular respiration, the production of ROS, and control of apoptotic cell death have all been reported to be altered in OPC models, even at low concentrations of OPC (Pearson and Patel, 2016). Mitochondrial dysfunction in the central nervous system and other tissues, which was found to occur in OP pesticide poisoning (Karami-Mohajeri and Abdollahi, 2013) and in nerve agent exposure (Collombet et al., 2009), has a causal role in the elicitation of oxidative imbalance and apoptosis. *In vitro* studies of the effects of OP pesticides have shown that their apoptotic effects are mediated via mitochondrial signaling pathway and driven by oxidative rather than cholinergic processes (Karami-Mohajeri and Abdollahi, 2013). The mitochondrial dysfunction may be a causal factor in the generation of OPC-induced seizures (Folbergova and Kunz, 2012). Specifically, a dose of chlorpyrifos, not sufficient to inhibit AChE, significantly increased mitochondria fusion versus fission events and decreased axonal mitochondrial transport (Middlemore-Risher et al., 2011). It was found that both acute and subacute exposure to OPC are sufficient to decrease activities of key antioxidants (glutathione peroxidase, glutathione reductase, GSH, SOD and calalase) that is connected with OPC-induced oxidative stress (Brocardo et al., 2005; Kaur et al., 2007).

One of the symptoms of OPC-induced oxidative stress is considered to be the oxidative damage of DNA. The aim of the study is to evaluate the presence and intensity of oxidative damage of DNA in lymphocytes, hepatocytes and brain cells after sublethal or lethal poisoning of rats with nerve agents.

Materials and methods

Animals

Male Wistar albino rats weighing 180–200 g were purchased from VELAZ (Prague, Czech Republic). They were kept in an air-conditioned room (22 ± 2 °C and 50 ± 10% relative humidity, with lights from 7 a.m. to 7 p.m.) and allowed access to standard food and tap water *ad libitum*. The rats were divided into groups of six to sixteen animals (N = 6–16). Handling of experimental animals was done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Králové (Czech Republic).

Cells

Human embryonic lung fibroblasts were obtained from the Institute of Sera and Vaccines (Prague, Czech Republic). Cells were incubated in medium MEM (PAA) supplemented with foetal bovine serum (Biotech, Prague, Czech Republic). They were passaged once a week in the ratio 1:2 by trypsinisation.

Chemicals

Nerve agents (sarin, soman, tabun) were obtained from the Military Technical Institute in Brno (Czech Republic) and were 90–95% pure as assayed by acidimetric titration. The basic solution of nerve agents (1 mg/ml) was prepared in propyleneglycol three days before starting the experiments. Actual solution of tested nerve agent was prepared from its basic solution with the help of saline immediately before administration. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification. The saline solution (0.9% NaCl) was used as a vehicle. All substances were administered intramuscularly (i.m.) at a volume of 1 ml/kg body weight (b.w.).

Comet assay

The Comet Assay, also called single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analyzing DNA damage including oxidative DNA damage in individual cells (Kucharova et al., 2019). The alkaline comet assay was basically performed following the Singh's protocol (Singh et al., 1988). Thirty five µl of the suspension was mixed with 85 µl of 1% low melting point agarose and 85 µl of this suspension was pipetted onto slides pre-coated with high melting point agarose (Serva Electrophoresis GmbH). After solidifying of the agarose, the slides with gels were placed in a lysis buffer [pH 10.4 °C; 100 mM EDTA, 2.5 M NaCl, 10 mM Tris (all from Penta, Prague, Czech Republic) and 1% Triton X-100 (Serva Electrophoresis GmbH)] for 1 h. A modification of the comet assay according to Collins et al. (1996) was used further. At this stage, 50 µl of the endonuclease III solution were added to parallel slides and slides were incubated for 30 min at 37 °C. Then, all slides were placed in a electrophoresis tank containing electrophoresis buffer [pH ≥ 13; 300 mM NaOH, 1 mM EDTA (both from Penta, Prague, Czech Republic)] and after 40 min of incubaton at 4 °C for alkali DNA unwinding, the electrophoresis was performed at 25 V and 300 mA (0.75 V/cm) for 30 min. After that, the slides were placed in neutralization buffer (pH 7.5; 0.4 M Tris) (Penta, Prague, Czech Republic) three times for 5 min and washed with distilled water. Air dryed slides were rehydrated in deionized water and stained with 20 µl of ethidium bromide (20 µg/ml; Sigma-Aldrich) before image analysis using Nikon epifluorescence microscope with LUCIA® comet assay software (Laboratory Imaging, Ltd., Prague, Czech Republic) for image analysis of the comets. Fifty cells per slide were scored and %tail DNA value was used as a parameter of DNA damage. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage (Collins, 2004).

In vitro *experiments*

Monolayers of human embryonic lung fibroblasts were seeded into 75 cm^2 culture flask and grown to confluence. Soman was added to the culture medium at the concentrations 1000, 500, 250, 125 or 62.5 µg/ml, respectively, for 1 h at 37 °C. After the treatment, the medium was exhausted, monolayers were washed with PBS and cells resuspended by trypsin in PBS. The suspension was used for the comet assay. Fifty cells were analysed per one estimation. The effect of soman was compared with the effect of hydrogen peroxide.

In vivo *experiments*

To evaluate the potency of nerve agents studied to induce oxidative damage of DNA, the rats were administered i. m. with sarin, soman or tabun at sublethal doses corresponding to

50% of their LD_{50} values or at lethal doses corresponding to their LD_{50} values. The rats were decapitated and exsanguinated to obtain the blood 30 min after nerve agent poisoning. The blood was centrifuged to obtain isolated lymphocytes. The tissues (liver, pontomedullar region of the brain) were removed and trypsinated to obtain hepatocytes and glial cells, as it was described in detail earlier (Richterova et al., 2018). DNA damage (direct single strand breaks) was evalutated with the help of Comet assay (single cell gel electrophoresis, SCGE). To detect oxidised bases of DNA, the enzyme endonuclease III was used (obtained from Dr. K. Angelis, Inst. of Eptl. Botany, Czech Acad Sci., Prague, Czech Republic) which is known to cleave oxidised pyrimidines. The evaluation of DNA damage was done in fluorescent microscope by image analysis Lucia G software involving the comet module (Laboratory Imaging, Prague, Czech Republic). For each experimental point, 50 cells were evaluated.

Statistical analysis

The differences among groups were calculated using Kruskal–Wallis test and the statistical significance between control and experimental groups was tested by Mann–Whitney test. The differences were considered significant when $2\alpha = 0.05$.

Results

Contrary to hydrogen peroxide, soman did not induce any significant DNA damage in human fibroblasts *in vitro* within the broad range of concentration (62.5–1000 μg/ml) used (Table 1). As the *in vitro* results with soman were negative (without any effects), *in vitro* experiments with other nerve agents studied were not done.

Table 1. *In vitro* soman-induced DNA damage in human embryonic lung fibroblasts (% DNA in tail of comet represents the amount of single strand breaks)

Concentration of soman (μg/ml)	% DNA in tail of comet
0	2.5 ± 1.3[a]
62.5	2.9 ± 1.8
125	2.1 ± 0.2
250	3.5 ± 1.4
500	2.9 ± 1.2
1000	3.8 ± 1.9
Concentration of H_2O_2 (μM)	
20	12.5 ± 3.9
100	38 ± 7.2

[a] means ± SD

In vivo experiments showed that lower dose of sarin or soman corresponding to 50% of LD_{50} did not induce any significant amount of oxidative DNA damage. When the lymphocytes or hepatocytes were incubated with the enzyme endonuclease III that is able to cleave oxidised pyrimidine bases, the percentage of DNA in the tail was usually higher compared to the cells without incubation with endonuclease III in experimental as well as control cells. The differences of the oxidative damage of DNA between cells from sarin or soman-poisoned rats and control rats were minimal (Tables 2–5).

Table 2. Sarin-induced DNA damage in peripheral lymphocytes (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	0.095 ± 0.038[a]	6
	yes	2.399 ± 1.849	6
Sarin – 0.5 LD_{50}	no	0.782 ± 1.351	10
	yes	1.209 ± 1.003	10
Saline controls	no	0.253 ± 0.162	6
	yes	3.090 ± 1.497	6
Sarin – LD_{50}	no	0.198 ± 0.314	11
	yes	7.456 ± 9.982	11

[a] means ± SD

Table 3. Sarin-induced DNA damage in hepatocytes (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	13.389 ± 4.688[a]	6
	yes	12.238 ± 7.801	6
Sarin – 0.5 LD_{50}	no	15.112 ± 4.815	10
	yes	18.974 ± 6.163	10
Saline controls	no	3.540 ± 1.844	6
	yes	7.209 ± 4.022	6
Sarin – LD_{50}	no	6.517 ± 4.070	11
	yes	**15.281 ± 6.519***	11

[a] means ± SD
* significantly different from saline controls

Table 4. Soman-induced DNA damage in peripheral lymphocytes (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	1.017 ± 0.336[a]	6
	yes	0.666 ± 0.467	6
Soman – 0.5 LD_{50}	no	0.094 ± 0.018	12
	yes	0.168 ± 0.507	12
Saline controls	no	1.128 ± 1.694	6
	yes	5.756 ± 2.650	6
Soman – LD_{50}	no	0.814 ± 0.937	11
	yes	4.803 ± 1.662	11

[a] means ± SD

Table 5. Soman-induced DNA damage in hepatocytes (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	4.838 ± 2.510[a]	6
	yes	3.573 ± 2.181	6
Soman – 0.5 LD_{50}	no	1.348 ± 1.696	12
	yes	3.064 ± 2.077	12
Saline controls	no	5.891 ± 3.409	6
	yes	5.368 ± 2.809	6
Soman – LD_{50}	no	4.360 ± 1.953	11
	yes	**8.307 ± 3.453***	11

[a] means ± SD
* significantly different from saline controls

Table 6. Soman-induced DNA damage in glial cells (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	5.133 ± 4.304[a]	6
	yes	8.112 ± 2.476	6
Soman – LD_{50}	no	4.682± 3.532	6
	yes	11.735 ± 4.151	6

[a] means ± SD

Table 7. Tabun-induced DNA damage in peripheral lymphocytes (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	0.126 ± 0.082[a]	12
	yes	1.819 ± 1.752	12
Tabun – LD_{50}	no	0.367 ± 0.677	16
	yes	1.820 ± 3.228	16

[a] means ± SD

Table 8. Tabun-induced DNA damage in hepatocytes (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	7.708 ± 5.458[a]	12
	yes	8.684 ± 5.839	12
Tabun – LD_{50}	no	4.903 ± 3.794	16
	yes	6.012 ± 2.379	16

[a] means ± SD

Table 9. Tabun-induced DNA damage in glial cells (% DNA in tail of comet represents the amount of single strand breaks)

Treatment	Presence of endonuclease III	Percentage of DNA in tail of comet	Number of animals
Saline controls	no	8.030 ± 2.728[a]	6
	yes	6.824 ± 2.487	6
Tabun – LD_{50}	no	**12.292 ± 3.491***	7
	yes	**11.923 ± 4.644***	7

[a] means ± SD
* significantly different from saline controls

In the case of higher doses of sarin or soman coresponding to LD_{50}, a marked increase of oxidative DNA damage in peripheral lymphocytes and hepatocytes incubated with endonuclease III was found. However, the statistically significant differences between DNA damage in cells from control rats and cells from nerve agent-poisoned rats were observed in hepatocytes only. The difference between the amount of DNA oxidised bases in lymphocytes found in sarin-poisoned and control rats was not significant because of their high variability. The levels of DNA damage in hepatocytes was increased approximately two times in the case of sarin administration (Tables 2–5). On the other hand, marked DNA damage in glial cells from rats poisoned with soman was not observed (Table 6). Tabun administered at lethal dose was not able to markedly increase of DNA damage in lymphocytes as well as hepatocytes regardless of the incubation of cells with endonuclease III (Tables 7–8). However, it induced significant oxidative damage of DNA in glial cells from pontomedullar region of the brain (Table 9). As only peripheral compartment (lymphocytes and hepatocytes) was chosen at the beginning of our study (experiments with sarin), the ability of sarin to induce oxidative damage of DNA in glial cells was not investigated. Generally, the DNA damage was relatively low regardless of nerve agent used and type of cells investigated.

Discussion

Exposure to OPC produces indices of oxidative stress both *in vitro* and *in vivo*. *In vitro* incubation of OPC in cell models has been shown to increase the production of ROS and induce changes in endogenous antioxidant enzymes leading to free radical-mediated lipid peroxidation (Glutekin et al., 2000). *In vivo* studies have further confirmed that oxidative stress occurs in animals exposed to a wide variety of OPC in varying exposure paradigm, including subacute, acute and chronic exposures (Soltaninejad and Abdollahi, 2009). Humans exposed either acutely or chronically to OPC develop similar indices of oxidative stress, which include decreased antioxidant capacity, free radical-mediated DNA damage and free radical-mediated lipid peroxidation (Ranjbar et al., 2002, 2005; Shadnia et al., 2005). Evidence of elevated oxidative stress has been reported in cases of low-dose and long term poisoning with various OPC (Binukumar et al., 2010; Delgado et al., 2006; Elsharkawy et al, 2013; Possamai et al., 2007).

As described in above mentioned *in vivo* studies, in the case of low-level poisoning with OPC, no cholinergic signs and symptoms were observed when oxidative stress occured. It means that other mechanisms than AChE irreversible inhibition with subsequent overstimulation of muscarinic and

nicotinic cholinergic receptors seem to be able to generate oxidative stress after OPC poisoning. The studies dealing with the occupational workers exposed chronically to low levels of OPC show alterations in antioxidant function, increased lipid peroxidation and increased free radical-mediated DNA damage (Muniz et al., 2008; Ranjbar et al., 2002). On the other hand, it was found that the degree of oxidative damage correlated with the degree of AChE inhibition (Kazi and Oommen, 2012; Ranjbar et al., 2002, 2005).

Exogenous and endogenous oxidants frequently cause oxidative damage of DNA (Halliwell, 1999). Therefore, one of the type of DNA damage after exposure to OPC is oxidative damage of DNA based on the development of OPC-induced oxidative stress. It is speculated that OPC phosphorylate proteins/low molecule antioxidants, creating a pro-oxidative status. In addition, several studies have demonstrated that OPC bind to DNA (Zeljezic et al., 2016). DNA damage activates response machinery, including p53, NF-κB, and MAPK pathways which regulate genes involved in ROS metabolism as well as Ca^{2+} homeostasis and apoptosis (Lee et al., 2014; Pejchal et al., 2009, RamaRao and Bhattacharya, 2012). Unrepaired DNA damage might induce cytokine cascade activation with subsequent pro-apoptotic mechanisms at the early time intervals (Kokkinakis et al., 2004) which might be followed by anti-apoptotic mechanisms in longer time intervals as observed in the study with soman-poisoned rats (Pejchal et al., 2008).

Our results demonstrate that DNA damage can be induced by nerve agents at high, lethal doses only. In the case of poisoning with sublethal doses of nerve agents studied, no significant DNA damage was observed. According to our knowledge, there is only one paper decribing the induction of the DNA damage by sarin in the blood leucocytes and parietal cortex *in vivo* in guinea pigs, which describes an increase in DNA fragmentation at 0.2 and 0.4 LD_{50}, but not at 0.1 LD_{50} (Dave et al., 2007). However, sarin was administered repeatedly for 10 days in above mentioned study. No significant increase in DNA fragmentation in any experimental group at 17 days after sarin exposure was found (Dave et al., 2007). Our results presented in this paper confirm the ability of sarin, soman and tabun to induce the DNA damage *in vivo*. In addition, the increase of DNA damage by incubating of the analysed cells with endonuclease III highly suggests that nerve agents-induced DNA damage is not caused by direct interaction of nerve agents with DNA bases but it rather represents the oxidation of DNA bases. It is in accordance with our results *in vitro*, where any significant DNA damage after the treatment of human fibroblasts with soman was not observed. Gross with co-authors did not find any DNA damage in human small airway epithelial cells (SAEC) and normal human epidermal keratinocytes treated with soman or VX *in vitro*, either (Gross et al., 2010).

Conclusions

DNA damage caused by oxidation of DNA bases seems to be secondary toxic effect of nerve agents connected with general hypoxia and it is developed during severe poisoning with nerve agents. This non-cholinergic effect of nerve agents has probably consequences with nerve agents-induced hypoxic status during acute cholinergic crisis and it can contribute to their long-term toxic effects.

Conflict of interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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