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Original Research Article

Evaluation of soman-induced extracranial histopathology in the context of clinical biochemistry, mitotic and apoptotic activity and morphometric analysis

Jaroslav Pejchal*, Jiří Kassa, Jan Misík, Jana Hatlapatková

Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic

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ABSTRACT

We aimed this study to evaluate histopathology of heart, lung, liver, kidney, small and large intestine in the context of clinical biochemistry, mitotic (intestine only) and apoptotic activity and morphometric analysis (intestine and lung only). Male Wistar rats were poisoned by soman (*i.m.*, 52 μg kg^{-1}; 70% LD_{50}). Samples were taken 1, 3 and 7 days following soman exposure. Biochemistry was evaluated in blood. Hematoxylin-eosin staining, Mallory's PTAH method, immunohistochemical detection of activated-caspase-3, TUNEL, and morphometric computer analysis were performed. We found increased AST and CK in blood, areas ranging from acute myolysis and necrosis to areas undergoing resolution in heart, a biphasic response consisting of hyperaemia, edema and bleeding leading to decreased airiness (day 1) and delayed inflammatory response with increased apoptosis (day 7) in lung, lymphangiectasis in small intestine (day 1) and subepithelial edema in large intestine (day 3) after soman intoxication. In intestine, mitotic activity decreased in crypts 1 and 7 days after the intoxication. Apoptotic activity decreased in the superficial compartment on the day 1, whereas it increased in the same compartment 3 days after the poisoning. Soman intoxication at the sublethal dose induced significant biochemical changes in blood and histopathological changes in heart, lung and intestine.

Introduction

Chemical warfare nerve agents (CWNA), such as soman, sarin, tabun and VX, are potent and irreversible inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7), the enzyme that breaks down the neurotransmitter acetylcholine. The effect of CWNA leads to the accumulation of acetylcholine at cholinergic synapses and neuromuscular junctions resulting in prolonged stimulation of muscarinic and nicotinic receptors (Bajgar 2004). Symptoms of poisoning include muscle weakness, increased secretions, respiratory depression, seizures, coma, or death due to respiratory and/or cardiovascular failure (Romano et al., 2008). In surviving victims, subacute and chronic neuropsychiatric/neurological impairments could be observed probably due to initial brain damage and long-term alteration of brain physiology and cellular signalling (Brown and Brix, 1998; Collombet et al., 2011; Kadar et al., 1995; Pejchal et al., 2008, 2009; RamaRao et al., 2011).

In addition to CWNA-induced neuropathology, the compounds have been reported to induce changes in extracranial tissues such as heart, skeletal muscles and lung. Heart lesions (*e.g.* hemorrhage, myofiber degeneration and necrosis, myocarditis) occur at higher frequency than neuropathological changes and they are associated with seizures in rat model (McDonough et al., 1995; Singer et al., 1987). Such findings, however, were not confirmed in non-human primates after exposure to soman (Britt et al., 2000). The same study also compared the characteristic features and extent of heart and skeletal muscle damage and showed that fewer myofibres were affected (*e.g.* myofiber degeneration) in skeletal muscle than in heart. Additionally, no significant correlation

Abbreviations: AChE, acetylcholinesterase; ALT, alanine amino transferase; AST, asparatate amino transferase; b.w., body weight; CK, creatine kinase; CWNA, chemical warfare nerve agents; DNA, deoxyribonucleic acid; GD, soman; GGT, gamma glutamyl transferase; JNK, c-Jun N-terminal kinase; LD_{50}, 50% lethal dose; LDH, lactate dehydrogenase; ROS, reactive oxygen species; SEM, standard error of mean; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

* Corresponding author.
E-mail address: jaroslav.pejchal@seznam.cz (J. Pejchal).

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between convulsions and skeletal muscle lesions was found although the changes were more extensive in animals with convusions (Britt et al., 2000). Finally, in lung, histopathological changes (*e.g.* perturbation of epithelial mesenchymal interface, edema, inflammation, progressive diffuse interstitial thickening) correlating with alterations in various respiratory dynamic parameters have been observed after exposure to nerve agents (Gundavarapu et al., 2014; Pant et al., 1993; Perkins et al., 2011).

Majority of studies investigating CWNA is focused on their cholinergic effects and data concerning other toxicologic mechanisms are rather sporadic. So, the aim of presented study is to revise histopathology of heart, lung, liver, kidney, small and large intestine in the context of clinical biochemistry and tissue apoptotic activity in rats 1, 3 and 7 days after soman (pinacolyl phosphonofluoridate, GD) poisoning, which may disclose effects of CWNA on cellular level. Additionally, mitotic activity was evaluated in intestine and computer morphometric analysis was performed in intestine and lung.

Material and methods

Animals

Male albino Wistar rats aged 12–16 weeks (b.w. 250–300 g, Velaz, Unetice, Czech Republic) were housed in accredited animal facility (accreditation number: c.j. 25895/2010-17210) in an air-conditioned breeding unit (22 ± 2 °C and 50 ± 10% relative humidity, with 12/12 h light cycle) and allowed to access to standard rodent diet (Velaz) and tap water *ad libitum*. The acclimatization period was 14 days before starting the experiments. On the day of the experiment, rats were randomly divided into groups of 6 or 8 individuals). All procedures involving animals were approved by the Ethics Committee (Faculty of Military Health Sciences, Hradec Kralove, Czech Republic).

Chemicals

Soman was obtained from Military Technical Institute (Brno, Czech Republic). Its purity (97.5%) was assayed by acidimetric titration. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification.

Experimental setup

Animals were divided into 3 control groups (6 rats each) intramuscularly adminstered with saline (0.9% natrium chloratum; Sigma-Aldrich Company, Prague, Czech Republic) and 3 groups (8 animals each) intramuscularly administered with soman at the dose of 52 μg/kg (70% LD_{50}). Surviving rats were anesthetized by ether vapor and blood and organs were collected 1, 3 and 7 days after the poisoning.

Blood biochemical paramters

Venous blood (2 ml) was taken from right heart ventricle into heparinized tubes (Scanlab Systems, Prague, Czech Republic) and kept on ice untill the analysis. The biochemical paramters including concentration of glucose, urea, creatinin, total and direct bilirubin, cholesterol, triglycerides, total protein, and albumin and activity of alanine amino transferase (ALT), asparatate amino transferase (AST), alkaline phosphatase, gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), and creatine kinase (CK) were measured at the Institute of Clinical Biochemistry and Diagnostics, University Hospital Hradec Kralove according to standard protocol validated by the quality manager.

Histopathology

Samples of heart, lung, liver, kidney, ileum (1–2 cm from ileocecal valve) and colon ascendens (1–2 cm from ileocecal valve) were fixed with 10% neutral buffered formalin (Chemapol, Prague, Czech Republic), embedded into paraffin (Paramix, Holice, Czech Republic), and tissue sections 5 μm thick were cut (Microtome model SM2000 R, Leica, Heidelberg, Germany). Histopathological changes were evaluated in sections stained with hematoxylin and eosin (both Merck, Kenilworth, NJ, USA).

Mallory's phosphotungstic acid hematoxylin method

In heart, degenerative/necrotic changes were visualized in 5 μm thick sections stained with Mallory's phosphotungstic acid hematoxylin method according to the manufacturer's instructions using detection kit (Bamed, České Budějice, Czech Republic). Number of degenerative/necrotic cells was scored per microscopic field under 400fold magnification.

Morphometric computer analysis

For morphometric analysis, 5 μm thick sections of lung, ileum and colon ascendens stained with haematoxylin-eosin were evaluated using the BX-51 microscope (Olympus Czech Group, Prague, Czech Republic) and ImagePro 5.1 computer image analysis system (Media Cybernetics, Bethesda, MD, USA).

In lung, tissue airiness (percentual air area) was assessed in the red/green/blue scale in the ranges: red 185–255, green 185–255, and blue 185–255, where 0 is black and 255 is white.

In ileum and colon ascendens, the length and width of 20 randomly selected villi (ileum) and crypts (both ileum and colon ascendens) were measured under 200fold magnification.

Evaluation of apoptotic activity in lung, kidney and liver

To detect apoptosis in lung, kidney and liver, immunohistochemical detection of activated caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method were done in 5 μm thick sections.

Immunohistochemical detection of activated caspase-3 (cleaved at aspartic acid-175) was performed using rabbit monoclonal antibody (1:200; Biotech, Prague, Czech Republic) by a standard peroxidase technique published previously (Pejchal et al., 2012a).

TUNEL method was carried out strictly according to the manufacturer's instructions using *in situ* cell death detection kits (Roche, Mannheim, Germany). Briefly, paraffin sections were dewaxed and rehydrated through xylene and an alcohol series (Dr. Kulich Pharma, Hradec Kralove, Czech Republic). The permeability of cell membranes was increased by incubating the sections in 0.1% Triton X-100 (Sigma-Aldrich Company) with 0.1% sodium citrate (Sigma-Aldrich Company) at 37 °C for 8 min. After permeabilization, tissue samples were incubated with 50 μl of TUNEL reactive mixture at 37 °C for 60 min in a moist chamber (Bamed, Ceske Budejovice, Czech Republic) to incorporate fluorescein (in TUNEL mixture) into fragmented DNA. Subsequently, anti-fluorescein antibody conjugated with horseradish peroxidase (from the TUNEL kit) was added for 30 min at 37 °C and 3,3′-diaminobenzidinetetrahydrochloride-chromogen solution was used to visualize DNA fragments.

Both caspase-3 and TUNEL positive cells were scored per microscopis field at 400fold magnification, except for renal glomeruli, in which number of positive cells per glomerulus was counted.

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