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Downregulation of nicotinic and muscarinic receptor function in rats after subchronic exposure to diazinon

Saša R. Ivanović[a,*], Blagoje Dimitrijević[b], Vitomir Ćupić[a], Milanka Jezdimirović[a], Sunčica Borozan[c], Mila Savić[d], Djordje Savić[e]

[a] Department for Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Belgrade, Bul. Oslobođenja 18, 11000 Belgrade, Serbia
[b] Clinic for Ruminants and Swine, Faculty of Veterinary Medicine, University of Belgrade, Bul. Oslobođenja 18, 11000 Belgrade, Serbia
[c] Department for Chemistry, Faculty of Veterinary Medicine, University of Belgrade, Bul. Oslobođenja 18, 11000 Belgrade, Serbia
[d] Department for Animal Breeding and Genetics, Faculty of Veterinary Medicine, University of Belgrade, Bul. Oslobođenja 18, 11000 Belgrade, Serbia
[e] Department for Paediatric Surgery, Faculty of Medicine, University of Belgrade, Serbia

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ABSTRACT

Diazinon (DZN) is an organophosphate insecticide which exerts its effect through the inhibition of acetylcholinesterase enzyme (AChE). In this work, we studied the development of tolerance to subchronic p.o. administration of DZN in rats, under both *in vivo* and *in vitro* conditions. A group of 20 rats (2 groups, n = 10) was administered p.o. the 1/10 of established LD_{50} DZN (namely 55.87 mg/kg bw) for 28 days. On the 14th and 28th day of study with isolated diaphragm and ileum, we examined the downregulation of nicotinic and muscarinic receptor function through Electrical Field Stimulation (EFS). Maximum contractility of the diaphragm was recorded on the 14th day of the study (25% higher compared to the non-treated rats), while on the 28th day the contractions almost did not differ from the values found in non-treated rats. EFS of isolated ileum on the 14th day of study caused significantly higher contractions compared to the non-treated rats, but after 28 days, ileum contractions decreased approximately to the level of contractions in non-treated rats. On the 14th study day, we also recorded increased amplitude of spontaneous ileum contractions, compared to non-treated rats. The application of increasing ACh concentrations caused dose-dependent ileum contractions, without statistically significant differences of median effective concentration (EC_{50}) values in non-treated and treated rats. Tolerance to subchronic DZN administration develops due to various adaptation mechanisms, including the most important one—downregulation of nicotinic and muscarinic receptor function.

1. Introduction

Diazinon (*O,O*-diethyl *O*-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate) is an organophosphate insecticide with a broad spectrum of activity. Therefore, in the past it was used in agriculture and horticulture worldwide for controlling insects in crops, fruit and vegetables and as a pesticide in domestic, agricultural and public buildings. Within Veterinary Medicine, DZN has been used in ectoparasiticide formulations for sheep and cattle, and in collars and washes for external parasite control in companion animals [13].

Poisoning of people and animals with DZN typically occurs through the ingestion of contaminated food or water. When diazinon gets into the body, through metabolic oxidative desulphurization and mediation of cytochrome P450 (CYP 450), the sulphur is being replaced with oxygen, thus creating phosphoryl compound—diazoxon, characterized by anticholinesterase activity [22,29,5], leading to the accumulation of acetylcholine at nerve endings, resulting in overstimulation of the cholinergic system (nicotinic and muscarinic receptors) [4].

Overstimulation of nicotinic receptors results in the paralysis of principal respiratory muscle-diaphragm, which is a life-threatening condition. Taking this into account, the phrenic nerve-diaphragm preparation [19] has been adopted as a common model in the study of toxic effects of organophosphates, as well as

* Corresponding author.
E-mail address: si34826@gmail.com (S.R. Ivanović).

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in the studies related to mice and rat diaphragm neuromuscular synapse [2,24,38,41,34].

However, a significant limitation of this method is that phrenic nerve must be intact; and therefore it is not applicable to diaphragms of large animal species or to diaphragm biopsies. The alternative method relies on EFS, with diaphragm samples containing only intramuscular branches of phrenic nerve [42].

The EFS reevaluation has been described by Seeger et al. [36]. EFS method may be used for two stimulation types of diaphragm samples: indirect stimulation – induced by the activation of post-synaptic nicotinic acetylcholine receptors (nAChR) through the release of ACh neurotransmitter, and direct stimulation – directly activating the contractile machinery of the muscle.

The overstimulation of muscarinic receptors (M_3 subtype) causes bronchoconstriction, airway hyper-reactivity and bronchial mucus overproduction [11]), resulting in severe clinical picture of poisoning with organophosphate compounds. It is well known that M_3 subtype of muscarinic receptors is also found in smooth airway and ileum muscles [7] and a primary role of the receptors is to contract smooth muscles [26], as well as to regulate the activity of many glands, both endocrine and exocrine. Therefore, an isolated rat ileum may be used as a muscarinic receptor model for the study on toxic effects of organophosphates.

Having in mind relatively short DZN half-time and a risk of misinterpretation of laboratory data related to AChE activity (still a "gold" standard in diagnosing organophosphate poisoning), as well as nonspecific clinical manifestations of DZN poisoning, the aim of this study was to examine the EFS method as a potential tool for the diagnostic of DZN poisoning.

2. Material and methods

2.1. Animals

All animal procedures were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for study and other scientific purposes and approved by the Ethical Committee of the Faculty of Veterinary Medicine of Belgrade University.

In vivo tests (determination of LD_{50} values) and *in vitro* tests (on isolated diaphragm and ileum) were conducted on a total of 110 white male Wistar rats, weighing 200 ± 20 g. The rats were housed under standard conditions for laboratory animals, on a 12 h light/dark cycle, at room temperature 21–24 °C, and *ad libitum* access to standard diet and water.

2.2. Substances and methods of administration

For *in vivo* tests, we used technical DZN (Makhteshim Chemical Works Ltd., Israel) minimum purity 95%, and corn oil as a solvent of DZN (cold-pressed oil from corn germs) (Uvita, Serbia). During *in vivo* tests, DZN was orally administered to rats, using a stiff gastric sonde (75 mm length) (Hauptner, Switzerland). Maximum volume administered p.o. did not exceed 0.1 ml/100 g of rat bw.

For *in vitro* tests on isolated rat diaphragm and ileum we used: mecamylamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), pancuronium bromide (Sigma-Aldrich, Germany), atropine sulphate (Sigma-Aldrich, St. Louis, MO, USA), acetylcholine (Sigma-Aldrich, St. Louis, MO, USA) and distilled water as a solvent. The organ bath was filled with aerated Tyrode's solution (125 mM NaCl, 24 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Glucose, 95% O_2, 5% CO_2, pH 7.4), and temperature was maintained at 37 °C. The substances were applied to isolated organ bath through 1 ml syringe marked in hundredths.

2.3. Procedures

2.3.1. Determination of acute oral toxicity (LD_{50}) of DZN in rats

The testing was conducted on 30 rats, divided into 4 equal experimental groups of 6 rats each, and the control one. DZN was administered p.o., in single doses ranging from 400 to 700 mg/kg of rat bw. Control rats were dosed once with 0.1 ml/100 g of bw DZN solvent (corn oil). The mortality of treated rats was monitored on daily basis for 7 days after the administration.

2.3.2. Determination of acute oral toxicity (LD_{50}) of DZN in rats treated for 28 days with 1/10 LD_{50}

The study included 50 rats, divided into 4 equal experimental groups (of 10 rats each) and the control one. DZN was administered p.o. for 28 consecutive days at 1/10 LD_{50}. Control rats were orally administered only DZN solvent (corn oil) at 0.1 ml/100 g bw. After 28 days of treatment, survived rats were randomized into four new experimental groups of 6 rats each, in order to determine the value of LD_{50}. DZN was then administered in doses ranging from 500 to 800 mg/kg bw. The mortality of all treated rats was monitored on daily basis for 7 days after the administration.

Two determined LD_{50} DZN values (LD_{50} in non-treated rats and LD_{50} in rats treated for 28 days with 1/10 LD_{50}) were analysed and compared in the Results and the Discussion section.

2.3.3. Study of DZN effects on isolated rat diaphragm and ileum

The study was conducted on 30 rats, divided into two experimental groups (n = 10) and the control one. DZN was administered p.o. at 1/10 of acute LD_{50}, for 28 days. Control rats were orally administered DZN solvent (corn oil) at 0.1 ml/100 g bw. At the end of the treatment period, on the 14th day (group 1) and the 28th day (group 2), 6 rats were randomly selected from both groups and euthanized with an overdose of pentobarbitone. Diaphragms and appropriate ileum segments from sacrificed rats were removed immediately, for further *in vitro* study.

The diaphragm preparation for *in vitro* study was arranged as described by Trailović et al. [40]. Diaphragm hemispheres were quickly excised and cut into strips of 1 × 0.5 cm, with incisions parallel to the direction of muscle fibres. Strips were mounted horizontally in an organ bath (with aerated Tyrode's solution), such that one end was fixed to the bath base, and the other end attached to isometric force transducer, connected to SmartPlus 50 software (El Unit, Serbia). With a pair of platinum electrodes placed parallel to the muscles, EFS was performed by applying tetanic pulses (50 Hz frequency, 25 V voltage, width 15 μs, and 2 s duration) in trains of five pulses every 30 s, with rest interval of 3 min in between.

The preparation from isolated rat ileum was made of preterminal part of ileum. A sample of 4–5 cm length was taken at approximately 10 cm from ileocecal valve, and placed into Tyrode's solution. After removal of fatty tissue, an intestine segment (2 cm length) was placed into organ bath filled with Tyrode's solution, warmed to the temperature of 37 °C, pH value 7.4. Oxygenation of Tyrode's solution in organ bath was performed by mixing oxygen and carbon dioxide (95% + 5%, respectively). Similar as with the diaphragm, one end of ileum was fixed to the bath base, and the other end attached to isometric force transducer, so that the ileum segment was located centrally between the two electrodes. Platinum electrodes were placed on the sides of the preparation at a distance of 3–4 mm and connected with stimulator BioSmart 150 (El Unit, Serbia). EFS was performed by applying pulses (50 Hz, 35 V, 2 μs and 2 s) in trains of five pulses every 60 s. After rest interval of 3 min, the same ileum segment was injected with increasing doses of acetylcholine (0.1, 0.3, 1, 3 and 10 μM), in order to determine the dose-response effect. Spontaneous ileum activity, its contractions