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Japanese quail acute exposure to methamidophos: Experimental design, lethal, sub-lethal effects and cholinesterase biochemical and histochemical expression

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HIGHLIGHTS

- We examined the effects of methamidophos to Japanese quail in an acute oral test.
- We estimated ChE activity in various tissues using various substrate-inhibitors.
- Changes concerning the activity of plasma, brain and liver ChEs, were reversible.
- Cyto-architectural and histochemical changes were persistent.
- We assessed enteric neuronal function in a major absorption site, the duodenum.

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ABSTRACT

We exposed the Japanese quail (*Coturnix coturnix japonica*) to the organophosphate methamidophos using acute oral test. Mortality and sub-lethal effects were recorded in accordance to internationally accepted protocols. In addition cholinesterases were biochemically estimated in tissues of the quail: brain, liver and plasma. Furthermore, brain, liver and duodenum cryostat sections were processed for cholinesterase histochemistry using various substrates and inhibitors. Mortalities occurred mainly in the first 1–2 h following application. Sub-lethal effects, such as ataxia, ruffled feathers, tremor, salivation and reduced or no reaction to external stimuli were observed. Biochemical analysis in the brain, liver and plasma indicates a strong cholinesterase dependent inhibition with respect to mortality and sub-lethal effects of the quail. The histochemical staining also indicated a strong cholinesterase inhibition in the organs examined and the analysis of the stained sections allowed for an estimation and interpretation of the intoxication effects of methamidophos, in combination with tissue morphology visible by Haematoxylin and Eosin staining.
We conclude that the use of biochemistry and histochemistry for the biomarker cholinesterase, may constitute a significantly novel approach for understanding the results obtained by the acute oral test employed in order to assess the effects of methamidophos and other chemicals known to inhibit this very important nervous system enzyme.

1. Introduction

Various studies carried out in both terrestrial and aquatic organisms have demonstrated (WHO, 1993) that exposure to organophosphorus pesticides (OPs), known for their anti-cholinesterase (ChE) properties, poses a significant occupational hazard in agriculture, and in addition exerts toxic effects to non-target organisms. The activity of ChEs, inclusive of both acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, also called pseudo-cholinesterase or non-specific cholinesterase, EC 3.1.1.8), has been commonly used as a biomarker for pollution evaluation and risk assessment (Fossi et al., 1992; Thompson and Walker, 1994; Soler-Rodriguez et al., 1998; Storm et al., 2000).

BuChE is mainly found in plasma but is also present in brain, liver, muscle, and other tissues (Geula et al., 1995; Monteiro et al., 2005). Being found in both the developing and mature brain, it suggests that BuChE may play important roles in neurogenesis, neuronal development and cell proliferation (Geula and Nagykery, 2006; Mack and Robitzki, 2000). Moreover, its physiological role seems also related to detoxification processes and lipid metabolism (Mack and Robitzki, 2000; Darvesh et al., 2003).

The mechanism of toxic action of OPs insecticides, or their metabolites, is based on the irreversible inhibition of the enzyme AChE, which hydrolyzes the neurotransmitter acetylcholine (ACh) to end the cholinergic neural transmission in both the central and peripheral nervous

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systems of vertebrates (Carr and Chambers, 2001). Accumulation of ACh in synapses, due to AChE inhibition, results in overstimulation followed by depression or paralysis and eventual death (Abou-Donia, 1992). OPs are also capable to induce apoptosis by multifunctional pathways in tissues such as brain (Caughlan et al., 2004).

In addition to cellular changes, the use of OPs is known to result in physiological changes in various organs (liver, kidney) and systems (nervous, immune and reproductive system) (Aly and El-Gend, 2000; Gomes et al., 1999; Nagymajtenyi et al., 1998; Rawlings et al., 1998), inclusive of behavioral and/or even psychological dysfunction (Beauvais et al., 2000). Signs of sub-lethal ChE inhibition in birds include nausea, lethargy, nutation, wing-drop, loss of righting-reflex, paralysis, opisthotonos and coma (Somers et al., 1991). In the end, death, secondary to the acute cholinergic syndrome by OPs, is usually due to a combination of excessive bronchial secretion, bronchospasm and paralysis of the respiratory center (Klaassen, 2008).

Japanese quail (*Coturnix coturnix japonica*) is a well established bio-indicator in accordance with the OECD guidelines and the European legislation for the risk assessment of pesticides, biocides and chemicals (OECD 223, 2002; OECD 205, 1984; OECD 206, 1984).

Methamidophos (O,S-Dimethyl phosphoramidothioate) was selected as a model compound given its well-established AChE inhibitory action (Brimijoin, 2005; Sheets et al., 1997) and one of the most widely used OPs in agriculture (Suresh et al., 2007; Tacal and Lockridge, 2010).

The aim of this study was to evaluate acute lethal and sub-lethal effects of methamidophos, by means of ChE biochemistry and histochemistry in a variety of organs (brain, liver and duodenum), as well as plasma of Japanese quail.

2. Materials and methods

2.1. Chemicals

Methamidophos technical (purity 98.2%) was supplied by Bayer CropScience [batch no. 2377 (M01585)].

2.2. Animals

This study was designed to assess the acute oral toxicity (LD_{50}) of methamidophos to the Japanese quail (*Coturnix coturnix japonica*). The study design was in compliance with the OECD guidelines draft 223 (2002), and the principles of the US EPA (1996). The study described in this report was conducted in compliance with Good Laboratory Practice standards.

Japanese quails (3.5 months old) were bred in the Ecotoxicological Laboratory, Fácáncert, Hungary. The birds were weighed individually shortly before the beginning of the test and were allocated to the test groups by a randomization plan on the basis of their body weights (bw). This was done with the aim, all treatment groups to have similar mean bw and bw distribution at the beginning of the trial. The birds were marked individually by numbered leg bands. The quails were offered a commercial poultry diet ad libitum throughout maintenance before the study and during the test with the exception of a fasting period of about 16 h prior to dosing. Relative humidity was 30–75%, temperature 18–22 °C and illumination on an 8:16 LD cycle under natural intensity. Before intoxication, and one week before the beginning of the test, to allow acclimatization, the birds were assigned to individual cages (103 × 51 × 37 cm) with a floor area for 10 birds (in the control 8 birds) which contained an automatic drinker and food hopper.

The birds received a single dose of the test material in an aqueous solution by oral intubation using a disposable syringe and a catheter. Birds were randomly assigned to a control group (not treated) or groups exposed to methamidophos at nominal concentrations of 1, 2.2, 5, 11.2 and 25 mg/kg bw. The observation period lasted 14 days.

Given the fact that for the middle and high doses high mortality was observed soon after dosing, we re-dosed 5 new birds for each of the above-mentioned treatments in order to take samples 30 min and 1 h after.

2.3. Biological observations and measurements

The birds were monitored for sub-lethal effects of toxicity every 2 h during daylight for the 14-day duration of the study. In particular, only for day 0 the birds were also monitored at 30 min, 1, 2 and 3 h after the administration of the gavage doses.

Body weights were determined at initiation of the study (day 0) and on days 3, 7 and 14. Feed consumption was estimated for each treatment and control group every day.

2.4. Sampling and preparation of tissues

Birds were euthanized by decapitation. Tissue sampling for both biochemical and histochemical analysis was performed on day 0/1, and on days 3/4 and 14; evidently dead and alive quails were used. Blood was collected into heparinized tubes for plasma preparation using a syringe inserted in the heart, immediately after decapitation, and whole brain, liver and duodenum samples were removed and immediately placed at −80 °C until the time of the biochemical assay (not for duodenum), and for histological evaluation.

Notably, no control quails' were sampled at 30 min and 1 h after the administration of the gavage dose. This did not pose a statistical problem given that the biochemical and histochemical observations for the control did not differ between samplings, as logically expected for the whole duration of the trial (Tables 2–7).

2.4.1. Preparation for biochemical analysis

2.4.1.1. Plasma. For the enzyme assay blood was collected from sacrificed animals. Plasma was separated by centrifugation at 4000 *g* for 5 min, aspirated and stored in aliquots at −80 °C.

2.4.1.2. Brain and liver. For the enzyme assay the left hemisphere of the brain inclusive of the left part of the cerebellum and liver samples were weighed and homogenized in 0.05 M Tris–HCl (pH 7.6, T-3253 Sigma). To solubilize membrane bound AChE, Triton X-100 (3% v/v in distilled water, T8787 Sigma) was also added. Homogenates were then decanted into an Eppendorf tube and centrifuged at 9000 *g* for 30 min at 4 °C and the supernatant stored in aliquots at −80 °C.

2.4.2. Preparation for histochemical analysis

2.4.2.1. Fixation. Pieces of the removed tissues, the right hemisphere of the brain and the right part of the cerebellum, liver and duodenum were immersed in 4% paraformaldeyde (P6148 Sigma) in 0.1 M phosphate-buffered saline (PBS, pH 7.4, 79,382 Fluka), at a volume of at least 20 times the volume of the tissue, for 1 h at room temperature. Tissues were washed two times for 30 min in PBS before storage overnight at 4 °C in PBS containing 7% sucrose and 0.1% sodium azide.

2.4.2.2. Cryostat sections. The fixed tissue was attached to a piece of cork (1 × 1 cm) and embedded in cryoprotection medium (Tissue-Tek, JUNG), and rapidly frozen in liquid-nitrogen-cooled isopentane (AC126470010 Acros). Isopentane acts as a cryoprotective agent against the liquid nitrogen. 10–30 μm thick transverse sections were cut using Leica CM1500 cryostat. A number of frozen sections (4 to 5) from different layers of the brain, liver and duodenum were thaw-mounted on poly-L-lysine-coated glass slides and finally stored at −4 °C.