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Original article

The effect of formalin on acetylcholinesterase and catalase activities, and on the concentration of oximes, in the earthworm species *Eisenia andrei*

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ABSTRACT

Formalin, the aqueous solution of formaldehyde, is used as a standard earthworm expellant. Since the possible biochemical effects of formalin to earthworms were not investigated, in the present study adult individuals of the earthworm species *Eisenia andrei* were exposed to sub-lethal concentrations of formalin in order to determine whether its usage as an expellant will influence the physiological status of the earthworms. In all experiments filter paper contact test was used and experiments were conducted under controlled laboratory conditions. Earthworms were exposed to 0.005, 0.01, 0.05, 0.1 and 0.2 mg ml^{-1} of formalin for 5 min, 15 min, 30 min and 2 h, and the acetylcholinesterase (AChE) activity, catalase (CAT) activity and concentration of oximes were measured. As expected, the lowest AChE activity was measured at the highest concentration of formalin applied (0.2 mg ml^{-1}). However, following a 2 h exposure to concentration of 0.01 mg ml^{-1}, the AChE activity increased up to 1.12 times the activity in the control. Similar results were obtained when concentration of oximes was measured: the lowest concentration of oximes occurred following 2 h exposure to the highest concentration (0.2 mg ml^{-1}); and the highest concentration of oximes—equating to 1.18 times increase relative to the control—occurred after a 2 h exposure at 0.01 mg ml^{-1}. Dose–response curves for AChE activity showed an inverted U-shape characteristic for hormesis and concentration of oximes indicates a role in maintaining the normal AChE activity in the organism. Measurement of CAT activity measurement showed dose and time dependent induction, indicating the occurrence of oxidative stress. The obtained results showed formalin causes measurable effects on the metabolism of *E. andrei*, therefore the usage of formalin as an earthworm expellant is unsuitable for ecotoxicological research or biomonitoring.

1. Introduction

Earthworms have been broadly used as terrestrial model organisms for assessing the environmental pollution because of their importance in the structure and function of soil ecosystems. Formalin, the aqueous solution of formaldehyde (concentrations of 0.1–0.5%), is used in the field as standard earthworm expellant [1] and its use is recommended in ISO standard ISO/DIS 23611-1 [2]. Formalin is poured on to the soil and it drives earthworms to the surface as it acts as a skin irritant when contacted in their burrows. Formaldehyde reacts with monoamines or amides to form methylene bridges and produces covalently cross-linked complexes with proteins and DNA [3]. Formaldehyde toxicity is thought to be mediated by the activation of free radical producing enzymes, and also by the inhibition of free radical scavenging systems, thereby enhancing the production of reactive oxygen species (ROS) [4]. Several studies have shown that formalin affects biomarkers of oxidative stress in rats [5–7]. Catalase (CAT) is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to water and molecular oxygen, thereby protecting cells from the toxic effects of hydrogen peroxide. CAT also uses hydrogen peroxide to break down potentially harmful toxins in the body, including phenols, formic acid, formaldehyde and alcohols. It has also been shown that formaldehyde is a substrate for human cytochrome P-450 monooxygenase system II E1 isozyme (CYP2E1) and can be oxidized by the endoplasmic reticulum by peroxidase, aldehyde oxidase and xanthine oxidase [8].

In order to evaluate the incurred stress in the organism many biomarkers have been identified. Among the numerous biomarkers available the measurement of acetylcholinesterase (AChE) activity is routinely used as a biomarker of the exposure to organophosphate and carbamate compounds [9,10]. Enzyme inhibition in contaminated organisms often persists for an extended period of time, while

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organophosphate and carbamate insecticide detection in the environment may prove unsuccessful due to their rapid degradation [11]. Measurement of the AChE activity is therefore a useful tool for detecting contamination by readily degradable AChE inhibiting compounds. Several other studies have shown that AChE inhibition can be caused by other compounds such as polycyclic aromatic hydrocarbons [12], heavy metals [13] and herbicides [14]; and various authors [15–17] have found, using biochemical and histochemical methods, that formalin inhibits cholinesterase activity to varying extents. Besides the xenobiotics and different types of pollutants, it has been also demonstrated that some substances, which naturally occur within the organism, can also affect the AChE activity. For example, studies have shown that arginine, phenylalanine, proline and aspartic acid can cause inhibition of AChE [18–21].

Oximes are chemical compounds belonging to the imines, with the general formula $R^1R^2C{=}NOH$, where R^1 is an organic side chain and R^2 may be hydrogen, forming an aldoxime, or another organic group, forming a ketoxime. Because of their unique α-effect nucleophilic reactivity [22], oximes (especially oximate anions) have long been used as potential reactivators of organophosphate-inhibited AChE [23]. Although many studies investigated the possibility of oximes to reactivate phosphorylated AChE, only a few used earthworms as a model organism [24,25]. However, several studies have revealed that oximes occur within the organisms during normal metabolic and detoxication processes [26–28] and these naturally occurring oximes could therefore play a role in maintaining the steady state in the organism.

Studies about effects of formalin application have been conducted, but only a few with earthworms as test organisms [29,30]. The endpoints in these studies were various—effects on metabolic and antioxidative enzymes, apoptosis, neurological functions, histopathological and morphometric changes, etc.—and did not include the effects of formalin on AChE activity. Although formalin has been recognized as a cancer-producing chemical [31] and toxic to earthworms, plants and their environment [30,32] it is still the most commonly used chemical expellant. Extraction of earthworms with formalin usually lasts about 15 min to 2 h. It is well known that earthworms avoid unfavourable conditions in soil (e.g., a chemical stressor) and since formalin acts as a skin irritant when earthworms come into contact with it they will tend to avoid it, so it is more likely that earthworms will be exposed to formalin for shorter periods. Based on the duration of the extraction and possible avoidance response, the exposure periods of 5 min, 15 min, 30 min and 2 h were chosen for the experiment.

Although the application of formalin to soil is a commonly used method for earthworm extraction the possible biochemical effects of formalin to earthworms were still not investigated. Given the characteristics of formalin it was assumed that its usage as an expellant would influence the physiological status of earthworms. Therefore the main focus of this research was to investigate the dose and time dependent biochemical effects of formalin that could occur as a result of the extraction of earthworms. For that purpose, the AChE activity, CAT activity and concentration of oximes in the earthworm species *Eisenia andrei* were measured.

2. Material and methods

2.1. Earthworms

Adult individuals of the earthworm species *E. andrei* [33] (Oligochaeta, Lumbricidae) were obtained from the culture maintained in our laboratory (the culture was established from earthworms purchased from earthworm farm OPG Škrljak, Sv. Ivan Zelina-Biškupec, Croatia). The worm culture was maintained at $20 \pm 2\,^{\circ}C$ in complete darkness with cow dung as a substrate and food. They were removed from culture, rinsed with tap water and stored in Petri dishes on damp filter paper for 24 h (in dark at $20 \pm 1\,^{\circ}C$) to void the gut content. All individuals used in this assay had well-developed clitella (0.31 ± 0.09 g after voiding the gut content).

2.2. Chemicals

All reagents used in the study were of analytical grade. 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide (AcSChI), Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 4-Nitrobenzaldehyde and hydroxylamine hydrochloride were purchased from Alta Aesar (Johnson Matthey, Ward Hill, MA). Formaldehyde (36%, stabilized with methanol max. 10%) and hydrogen peroxide (30%) were purchased from Kemika d.d. (Zagreb, Croatia).

2.3. Filter paper contact tests

All experiments were conducted by the filter paper contact toxicity method [34]. The sides and bottom of flat-bottom glass vials (the height of 4.4 cm and the circular bottom of 5 cm in diameter) were lined with Whatman No. 1 filter papers without overlapping. The test chemical, formalin, was suspended in distilled water and loaded onto the filter paper (2 ml of solution per vial). Controls were also run in parallel with distilled water only. One earthworm per vial was added and each vial was closed with a cap with a small ventilation hole and placed in dark at 20 °C. In a preliminary mortality test earthworms were exposed to eight different concentrations of formalin for a period of 1, 2, 3 and 24 h. From the number of organisms that died during the exposure the values of lethal concentrations 50% (LC50) were calculated. Based on the results from the preliminary test, the sub-lethal concentrations of formalin for the final experiment were chosen. In the final experiment earthworms were exposed for 5 min, 15 min, 30 min and 2 h to 0.005, 0.01, 0.05, 0.1 and 0.2 mg ml^{-1} of formalin. Formalin concentrations of 0.005, 0.01, 0.05, 0.1 and 0.2 mg ml^{-1} correspond to 0.11, 0.21, 1.07, 2.13 and 4.26 μg cm^{-2} of filter paper. As expected, no mortality occurred after exposure to any of the applied formalin concentrations in the final experiments. After the time of exposure had ended activities of AChE and CAT, as well as concentration of oximes were measured in homogenized earthworm tissue. The experiments were repeated 3 times in order to prove the reproducibility and 5 earthworms per each concentration and per each exposure period were used.

2.4. Sample preparation

Whole earthworms with addition of cold sodium phosphate buffer (0.1 M, pH 7.2) were homogenized on ice using an Ultra-Turrax T18 homogenizer (IKA, Labortechnik, Germany). Since homogenization ratio was 1:4/w:v for all earthworms, the amount of protein was similar in all samples ($\sim$15 $mg_{protein}/ml_{homogenate}$). The homogenates were centrifuged (Hettich MIKRO 22R Microcentrifuge) for 30 min at 9000 × *g* and 4 °C to obtain the post-mitochondrial fraction (supernatant: S9). Aliquots of the supernatant were stored at −80 °C until use, and from each sample 7 replicates were used for measurements of biomarkers.

2.5. Enzyme activities (biochemical assay)

Activity of AChE was determined according to the method of Ellman et al. [35], using a Shimadzu UV-1601 spectrophotometer. Kinetic measurements were performed with AcSChI as substrate.