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Analytical Note

Elemental changes in hemolymph and urine of *Rhodnius prolixus* induced by in-vivo exposure to mercury: A study using synchrotron radiation total reflection X-ray fluorescence

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ABSTRACT

In recent years, the effects of pollution on the health of humans and other vertebrates were extensively studied. However, the effects on some invertebrates are comparatively unknown. Recent studies have demonstrated that toxic metals interfere with the reproduction, development and immune defenses of some terrestrial and marine invertebrates. Some environmental conditions including pollution produce chronic and acute effects on different animal's organs and systems. In this work, we investigated changes in the concentrations of Cl, K, Ca, Fe and Zn in *Rhodnius prolixus* as insect model. The elements were quantified using urine and hemolymph samples collected on different days after feeding the insects with blood containing $HgCl_2$. The synchrotron radiation total reflection X-ray fluorescence measurements were carried at the X-ray fluorescence beamline facility in Brazilian Synchrotron Light Laboratory. The observation reveals that the calcium level was higher in the hemolymph than in urine. On the other hand, the urine collected from insects treated with $HgCl_2$ showed higher level of Cl than hemolymph samples. Ca, Fe and Zn concentrations decrease drastically in urine samples collected after 2 days of $HgCl_2$ treatment. The regulation of triatomines excretion was discussed pointing out the importance of trace elements.

1. Introduction

Rhodnius prolixus is strictly hematophagous and belongs to the order Hemiptera subfamily Triatominae, family Reduviidae and is one of the most important vectors of the hemoflagellates *Trypanosoma cruzi* and *Trypanosoma rangeli* in Central America. Chagas' disease, also called American trypanosomiasis, is a human tropical disease, which is endemic in large areas of South and Central America. Among the parasitic diseases, Chagas' disease is ranked as one of the most important in Latin America in terms of social and economic impact, affecting about 18 million people, with about 100 million people living in what are considered to be high risk zones, and approximately 300,000 new cases occurring every year with around 21,000 deaths annually [1,2].

R. prolixus removes from its mammalian host a volume of blood enough to increase its own weight by about tenfold in larval stage and two or three times in the adult insect stage. In this engorged state, the insect is vulnerable to predation and must eliminate the excess of water and salt to reduce its own volume. To reduce the bulk and concentrate the nutritious part of the meal and, in addition, prevent dilution of its hemolymph, the insect rapidly excretes a fluid of high-sodium content [3,4].

Water and ion metabolism in insects is regulated in large part by both Malpighian tubules and hindgut. The Malpighian tubules filter hemolymph and secrete a liquid that is often compared with the primary urine in vertebrates. The uptake of fluids, ions and waste materials is named as primary urine and it is transported into the tubule lumen [5]. Insect neuropeptides, called diuretic or antidiuretic hormones, stimulate fluid secretion by the Malpighian tubules or fluid reabsorption by the tubules or hindgut, respectively [6]. The transport regulation of Malpighiam tubules is one of the key points for insect homeostasis and, certainly, it is also important for *Trypanosoma cruzi* transmission [7].

The use of faster and accurate analytical methods for biological fluids analysis might shed some light on a better understanding of *R. prolixus* trace elements excretion. In the last decade, synchrotron radiation total reflection X-ray fluorescence (SR-TXRF) became a highly useful multielement technique for the determination of trace elements in environmental and biological samples.

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Modern instrumental techniques, such as inductively coupled plasma optical emission spectrometry, inductively coupled plasma mass spectrometry, and atomic absorption spectrometry, have been used for the determination of trace metals in various media. Among the instrumental analytical techniques, total reflection X-ray fluorescence (TXRF) is highly attractive because it allows the quantification of trace and ultra-trace elements in various matrices [8]. This technique presents an advantage when compared with other analytical techniques, in the multi-elemental determination of various inorganic elements in small sample volumes [9].

TXRF analysis is a powerful analytical tool with respect to detectable elemental range, simplicity of quantification and detection limits [10]. This includes the capacity to detect almost all elements of the periodic system, namely from B to U [11]. The benefits of TXRF are that it is essentially unaffected by the matrix, is very sensitive, requires small amounts of sample (5–10 μL) of most elements from samples analyzed for few seconds, and requires very little sample preparation time. When considering sample preparation procedures, TXRF could also have the advantage of being more economical in technique. That there is no need for any chemical preparation is an additional advantage of TXRF in such cases [12].

The aim of this work is to investigate the elemental changes observed in hemolymph and urine of *R. prolixus* fed on blood containing $HgCl_2$ using the analytical approach of SR-TXRF technique. The SR-TXRF method, as applied in this work, has proved to be rather simple and fast. We emphasize here that in this kind of systematic, usually large-scale studies, the number of samples investigated can be of the order of hundreds, the advantages of TXRF technique are really predominant. A general description of the use and advantages of SR-TXRF was given by Streli et al. [13].

2. Materials and methods

2.1. Sample preparation

2.1.1. Insects feeding, hemolymph and urine collection

R. prolixus were reared and maintained as previously described [14]. Fifth-instar larvae were collected from a colony in the Laboratory of Biochemistry and Physiology of Insects, Oswaldo Cruz Foundation, Brazil. After molting, insects were randomly chosen, starved for 15–20 days and then fed through a membrane feeder as described by Azambuja et al. [14]. $HgCl_2$ previously dissolved in distilled water was added in a final concentration of 1 μg mL^{-1} to defibrinated rabbit blood. At different days after feeding the larvae were separated for both hemolymph and urine collection. The hemolymph was collected using 5 μL calibrated micropipettes after insect leg cutting. The urine samples were collected in days one and two after feeding according to Garcia et al. [15]. Basically each insect was put inside an Eppendorf tube, which had a small hole on the bottom. For improving urine collection, this tube (containing insect) was kept in vertical position with another empty tube fitted underneath one for 24 hours at room temperature. Control groups were fed with defibrinated rabbit blood without addition of mercury chloride. The hemolymph and urine samples were kept at −20 °C until analysis. Three replicates were prepared for each sample in order to evaluate the reproducibility of measurement.

2.1.2. Sample preparation procedure for SR-TXRF analysis

Samples were prepared taking aliquots of hemolymph and urine from different insects groups, which were then homogenized and pooled with volumetric pipettes and transferred to calibrated flasks. To perform quantitative analysis, an internal standard was added to the sample to correct the system instability and operational errors. In this work, Ga (Aldrich 84378) was used as an internal standard at a concentration of 8.27 μg mL^{-1}. The end concentration of Ga into hemolymph sample was 1:10 (1 part of Ga and 9 parts of sample). Small samples (5 μL) of the final solution (Ga + sample) were pipetted on to the Perspex® support ((poly(methyl methacrylate))—PMMA) for later evaporation under an infrared lamp.

2.2. Measurements

The SR-TXRF measurements were performed at the XRF beamline at Brazilian Synchrotron Light Laboratory, Campinas, Brazil [16]. All measurements were carried out under normal pressure and temperature. All samples were analyzed by exciting with a white beam with maximum energy of 20 keV. Fluorescent photons were detected with a Ge detector of 165 eV at 5.9 keV of resolution with 8 mm beryllium window thickness, 30 mm^2 active area, coupled to an amplifier module and a multi channel analyzer.

The samples were excited for 100 s and the X-ray spectra obtained were evaluated by the software QXAS Quantitative X-ray Analysis System distributed by the International Atomic Energy Agency in order to obtain the X-ray intensities for each element and the associated uncertainty [17]. Intensities were corrected to account for the dead time and the incident beam.

One of the main advantages of TXRF with respect to other XRF techniques is the easy way of quantification. A quantitative multi-element analysis becomes possible after recording simultaneously X-ray intensities [18,19]. The accuracy of the results and the reliability of the analytical procedures were checked with the aid of two different reference materials: Sigma S2263 Sheep serum (Sigma-Aldrich Co., USA origin, sterile-filtered, cell culture tested) and Sigma H4522 Human serum (Sigma-Aldrich Co., from human male AB plasma, sterile-filtered) with certified value of 1.81 μg mL^{-1} and 0.77 μg mL^{-1}, respectively, for Fe concentration. Sample handling and preparation procedure was the same for the blood samples and the reference materials. The results obtained are in good agreement with the certified values. The standard deviation for Fe concentration among independent replicates ($n = 5$) was less than 10% for both reference materials.

3. Results and discussion

Preliminary results showed that for *R. prolixus* fifth-instar larvae fed with blood containing $HgCl_2$ in concentration up to 1 mg mL^{-1}, the mortality and urine volume excretion was similar to a control group which received only blood (data not shown).

Changes in elemental contents present in hemolymph and urine samples were monitored throughout this particular study. Quantitative analysis was performed for Cl, K, Ca, Fe and Zn for all analyzed samples.

Mean values ± standard deviation in replicates for hemolymph and urine samples are shown in Tables 1 to 4. The results were analyzed using independent-samples *t*-test. Difference between samplings statistically non-significant was set at $P > 0.05$.

3.1. Hemolymph

Table 1 shows the elemental contents (mean values ± standard deviation) obtained from the hemolymph samples collected 2 days

Table 1
Comparison of elemental contents (μg mL^{-1}) for hemolymph samples collected 2 days after HgCl2 oral treatment and control ones.

Element	Concentration (μg mL^{-1}) (mean value ± standard deviation)	
	Control ($n = 20$)	Fed with $HgCl_2$ ($n = 20$)
Cl	18.7 ± 4.8[a]	19.8 ± 4.8[a]
K	2.1 ± 0.4[a]	1.9 ± 0.4[a]
Ca	2.1 ± 0.6[a]	1.9 ± 0.3[a]
Fe	37.1 ± 10.9	34.8 ± 8.3
Zn	22.8 ± 7.9	22.8 ± 4.1

[a] In mg mL^{-1}.