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5-Aminolevulinic acid and the hepatic oxidative stress in the early phase of experimental hexachlorobenzene intoxication

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This work evaluated the levels of 5-aminolevulinic acid (ALA) in the liver of rats exposed to different doses of HCB (25,50, and 100 mg/kg b.w. for 4 weeks) and correlated them with lipid peroxidation parameters. Levels of ALA were determined by high-pressure liquid chromatography after derivatization with acetylacetone and formaldehyde, followed by fluorescence detection. The methodology was carefully validated, nonetheless hepatic levels of ALA in all animals treated or not were below the detection limit of the method (2.27mg of ALA/g liver). On the other hand, lipid peroxidation, evaluated as thiobarbituric acid reactants production and chemiluminescence was found significantly increased in the livers of all treated rats, in comparison with control values ($p<0.05$). These results suggest that the hepatic oxidative stress observed in animals following HCB treatment may not necessarily be associated with increased levels of ALA in the liver. Another possibility is that increased levels of ALA in the liver, even below the detection limit of the method are sufficient to induce hepatic oxidative stress.

Uniterms
- Hexachlorobenzene
- Oxidative stress
- 5-aminolevulinic acid

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INTRODUCTION

Hexachlorobenzene (HCB) has been used in the past as a fungicide for crop protection. Nowadays, it can be released in the environment as a by-product of the chemical industry and it may be present as impurity in the formulation of several widely used pesticides (Jacoff *et al.,* 1986).

Between 1955 and 1959 many people in southeastern Turkey developed chronic porphyria after consuming wheat that had been treated with HCB (Schmid, 1960). HCB was shown to be hepatotoxic; effects in the liver range from hepatocellular enlargement to severe disturbance of the haem synthesis, resulting in accumulation of porphyrins in the liver and their massive excretion in the urine, as consequence of the inhibition of hepatic uroporphyrinogen-decarboxylase activity (URO-D) (Elder *et al*., 1976; Börge *et al.,* 1979).

Reactive oxygen species (ROS) have been proposed to play an important role in the pathogenesis of the porphyria caused by polyhalogenated aromatic hydrocarbons, such as HCB (Ferioli *et al.,* 1984; Almeida *et al*., 1997; Billi de Catabbi *et al.,* 1997). Several authors

have shown an increase of lipid peroxidation in the liver of rats treated with different doses of HCB. According to Billi de Catabbi *et al*. (1997), the increase in lipid peroxidation found in the liver of rats treated with 1g/kg b.w. of HCB reflects rises in ROS levels. Almeida *et al*. (1997) postulated this higher lipid peroxidation could be a consequence of increased levels of superoxide anion produced by cytochrome P450 (Urquhart, Elder, 1987; Visser *et al*., 1989; Almeida *et al.,* 1997). Additionally, ROS could also play some role in the inhibitory process of URO-D, by formation of an URO-D inhibitor (De Matteis, 1988).

Uroporphyrinogen-decarboxylase is the key enzyme of the heme metabolic pathway, which is blocked in cases of porphyria, not only in humans but also in the experimental HCB-induced model. This blockage impairs the regulation of the heme pathway as observed in lead intoxication (Elder *et al.,* 1976; Hindmarsh, 1986) when ALA-dehydratase is inhibited, leading to the increase of ALA excretion in urine (Wainstok De Calmanovici *et al.,* 1986).

ALA has been proposed as an endogenous prooxidant in these pathological conditions (Bechara *et al.,* 1996). Some authors reported the involvement of ROS produced during autooxidation of enolic ALA in rat liver mitochondria as an explanation to the pathophysiology of acute intermittent porphyria (Hermes-Lima *et al*., 1991; Monteiro *et al*., 1986; Vercesi *et al*., 1994). This work sought to evaluate lipid peroxidation parameters in the liver of rats exposed to different doses of HCB and their correlation with the levels of ALA, considering this molecule as a potent prooxidant with capacity to induce oxidative stress.

MATERIAL AND METHODS

Animals and treatment

Male Wistar rats weighing 180-200 g were fed rat chow (Purina, São Paulo, Brasil) *ad libitum*. HCB dissolved in corn oil by sonication, was administered by gavage (25, 50 and 100 mg/kg body wt.) 5 days a week for 4 weeks.

Control animals received isovolumetric amount of corn oil (Mazola®). Animals were euthanized 24 hours after the last dose by cervical dislocation.

5-aminolevulinic acid (ALA) determination by HPLC

Chemicals

5-Aminolevulinic acid hydrochloride (ALA) and *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) were purchased from Sigma (St. Louis, MO, USA), formaldehyde, acetylacetone, methanol, 2-mercaptoethanol and trichloroacetic acid were bought from Merck (Darmstadt, Germany). The water used was bidistilled and subsequently deionized.

Chromatographic conditions

The HPLC system consisted of a Model 510 Waters pump coupled with a 1046 A Hewlett Packard fluorescence detector and a SP4600 Data Jet Thermo Separation integrator. The analytical conditions were as follow: mobile phase, methanol-water (500:500, V:V); analytical column, Supelcosil LC-18 Supecol (150 x 4.6 mm I.D., 5 mm particle diameter); flow-rate, 0.5 mL/min; and injection volume, 20mL. The excitation and emission wavelengths were set at 370 and 460 nm, respectively.

Standard solution

Stock standard solution of ALA was prepared by dissolving 5 mg of 5-aminolevunilic acid in 50 mL of deionized water and storing at 4 °C. This solution was diluted to give appropriate concentrations of ALA.

Sample Collection

Liver (1g) non-perfused was homogenized (1:3 w:v) in 10 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] buffer pH 7.0 with a Teflon pestle in a tight-fitting glass vessel and centrifuged for 5 min at 1000 x g at 4 °C. The pellet was discarded and the supernatant was used for ALA determination.

ALA determination

A modification of the method described by Okayama *et al*. (1990) was employed, replacing 2-iodoacetamide by 2-mercaptoethanol to prevent thiol formation.

Liver homogenates supernatant (250 mL), as according described above, was added with 0.06 M 2-mercaptoethanol (15 mL) and, after two minutes, trichloroacetic acid 35% (60 mL). The mixture was centrifuged at 1600 x g for 10 min, and the supernatant (25 mL) was mixed with acetylacetone (100 mL), acetic acid (625 mL) and formaldehyde solution 10% (250 mL). The mixture was mixed in vortex, heated at 100 °C in a multiblock heater for 10 min, cooled in an ice-cold water bath to stop the derivatization reaction in a glass to the dark conditions, and filtered through a 0.22 mm filter. The filtrate (20 mL) was injected into the HPLC system.

Liver lipid peroxidation parameters

Sample preparation

Animals were euthanized by cervical dislocation.

After opening the peritoneal cavity was done the excision of a sample for the ALA determination, the liver was perfused with cold 0.9% NaCl by portal vein, it was removed, weighed and homogenized (1:3 w/v) in 140 mM NaCl 40 mM Phosphate buffer pH 7.4 with a Teflon pestle in a tight-fitting glass vessel and centrifuged for 20 min at 1000 x g at 4 °C. The supernatant was used for lipid peroxidation evaluation as thiobarbituric acid reactants (TBARs) and chemiluminescence (CL).

Thiobarbituric acid reactants (TBARs) measurement

The supernatant of liver homogenates was incubated at 37 °C in a shaker water bath, diluted (1:3 v/v) in 0.1 M potassium phosphate buffer pH 7.0. After a 2 hour incubation, samples were precipitated with 5% TCA and TBARs determined in the supernatant according to Junqueira *et al*. (1986).

Chemiluminescence (CL) emission

The supernatant of liver homogenates was diluted (1:8 v/v) in 140 mM NaCl 40 mM phosphate buffer pH 7.4 and incubated for 30 min at 37 °C in a shaker bath. Chemiluminescence of the incubated liver homogenates (3 mL) was measured in a Packard 1700R liquid scintillation analyzer using the single photon monitor mode at 25 °C. The values were expressed as cpm per gram of tissue (Boveris *et al*., 1983).

Protein determination

Protein concentration was estimated employing the biuret reagents (Layne, 1957).

Statistics

Mean values were compared by ANOVA followed by Tukey's test. Differences were considered statistically significant when *p* values were less than 0.05.

RESULTS

ALA analysis

Validation of method

Typical chromatograms of the ALA derivative (200 mg/ L ALA standard solution), a liver sample to which ALA has been added to prepare 200 mg/ L of ALA in the homogenate and liver sample from rats treated with 100 mg of HCB/ kg body wt. are show in Figure 1.

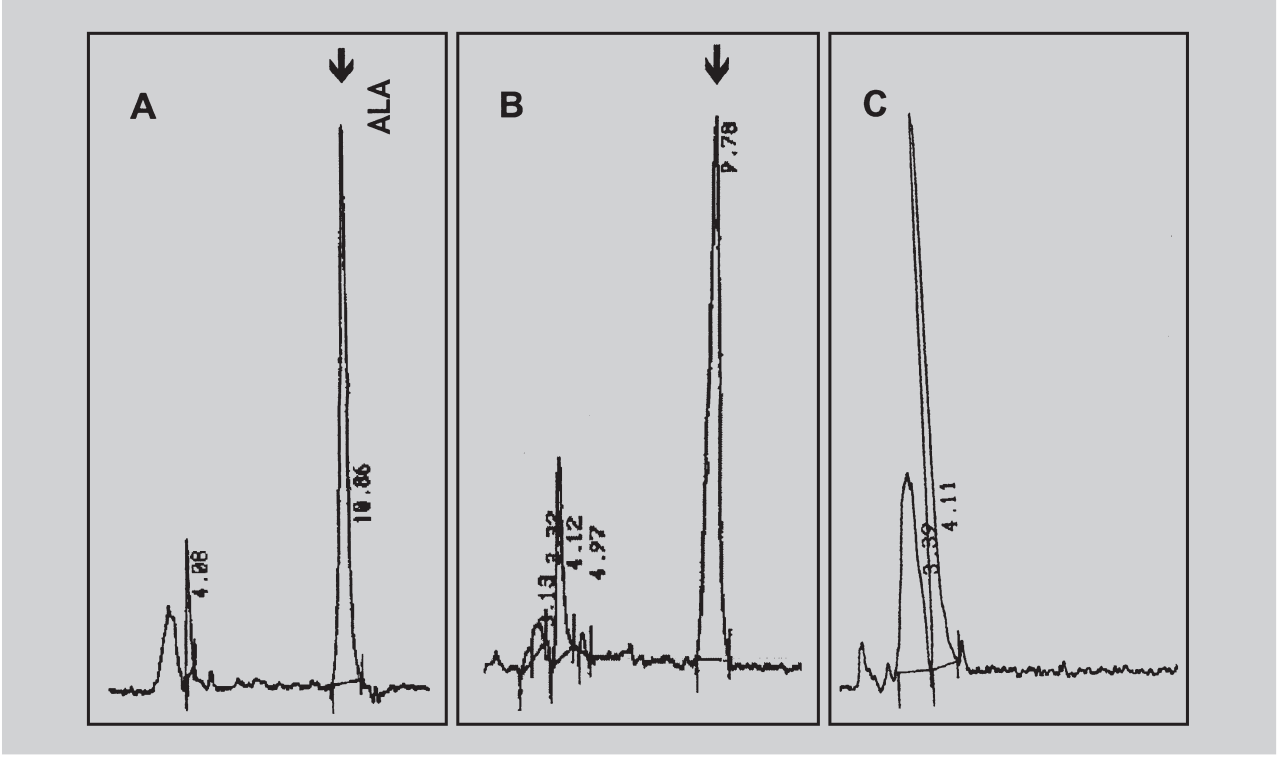

FIGURE 1 - HPLC chromatogram of ALA with fluorescence detector. (A) Chromatogram of 5-aminolevulinic acid (ALA) in standard solution (200 mg/ L, left); (B) liver homogenate + ALA (prepared at 200 mg/ L, middle); (C) liver sample from rats treated with HCB (100 mg/ kg body wt, right). Arrow = peak of ALA derivative. Retention of time: 15 min (ALA). Chromatographic conditions in the text.

TABLE I - Lipid peroxidation parameters liver of non-treated and HCB-treated rats

	TBARS (nmol/mg of protein/120 min)
Control rats	1.49 ± 0.27 (8)
25 mg of HCB/kg	2.85 ± 0.57 (8) *
50 mg of HCB/kg	2.55 ± 0.61 (6) *
100 mg of HCB/kg	2.85 ± 0.59 (8) *
	Chemiluminescence (counts/min.10^6/mg of protein
Control rats	1.5 ± 0.7 (6)
25 mg of HCB/kg	2.8 ± 1.2 (6) *
50 mg of HCB/kg	3.5 ± 1.6 (6) *
100 mg of HCB/kg	3.7 ± 1.6 (6) *

Values represent the means ± SD. The number in parenthesis represents the number of animals per group
(*) Significantly different from control rats ($p< 0.05$)

The concentration of ALA in the standard solution (12.5-200 mg/L) linearly correlated to the fluorescence intensity given by peak areas (r = 0.997). The calibration curve for standard ALA can be represented by the equation y = -9921+1070.5x, and that of the ALA liver homogenate spiked with authentic ALA, by the equation y = -12591.2 + 1223x (r = 0.998).

The reproducibility of ALA quantification was tested in the liver samples with addition of ALA standard solution. The variation coefficients ranged from 2.4 to 8.9% for interday assay and 7% and from 17.5% for intraday assay. The recovery of ALA added to liver homogenate (12.5, 50 and 200 mg/L) was 100%. The detection limit was 6.25 mg/L or 0.74 µmol/20 mL of ALA.

ALA concentration in liver homogenate of both control and HCB-treated (25, 50 and 100 mg/kg body wt) were under the detection limit of the method (2.27 mg of ALA/g liver).

Lipid peroxidation parameters in the liver of control and HCB-treated rats

All animals treated with HCB showed increased levels of TBARs and CL, as compared to control animals (Table I).

DISCUSSION

In order to investigate the role of ALA in HCB-induced liver oxidative stress, we validated the method proposed by Okayama *et al*. (1990) for ALA determination in the hepatic tissue of rats. The detection limit obtained was 0.74 µmol/ injection, or 2.27 µg/g of liver, and the high recovery percentage (100%) shows that ALA was not lost during the extraction procedure.

The validated method was subsequently employed to determine ALA concentration in hepatic tissue of non-treated and HCB-treated animals (25, 50 and 100 mg of HCB/ kg b.w.). The results indicated levels of ALA lower than 2.27 mg/g of liver, below the detection limit of the method. According to McGillion *et al*. (1974), quantification of ALA in hepatic tissue of control animals by isotopic methods resulted in 0.06 mg/g of liver. This value is much below our detection limit of ALA and could explain why ALA was not detected in the controls. The fast elimination kinetics of ALA could be another determinant. According to McGillion *et al.* (1974), ALA could not be found in hepatic tissue of rats after 24 hours of an intraperitoneal injection. Considering that the animals were sacrificed after 24 hours of exposure to HCB, we decided to further assess this possibility. Rats were exposed to a high concentration of HCB (1 g/ kg b.w.) for 3 days, enough time to induce hepatic lipid peroxidation, as shown by Almeida and Barros (1994). Even though animals were sacrificed after 30 minutes of the last the administration of HCB, ALA levels in the liver remained below the detection limit (results not shown).

Almeida *et al.* (1997) demonstrated that superoxide anion production by the endoplasmic reticulum is increased in rats treated with daily doses of 25 mg of HCB/ kg b.w. for 30 consecutive days. These increased levels of superoxide anion could lead to the production of hydrogen peroxide by Fenton reaction. According to De Matteis *et al.* (1988), H_2O_2 can be responsible for the oxidation of uroporphyrinogen, with the production of a sufficiently stable oxidative derivative, which in turn could act as inhibitor of URO-D.

Although we did not demonstrate an increase of ALA in the liver of HCB-treated animals, our results

indicate an oxidative stress condition in the liver of these animals (Table 1). Wainstok De Calmanovici *et al.* (1984) demonstrated a decrease in the activity of hepatic URO-D after 4 weeks in rats treated with 1 g of HCB/kg b.w., however, ALA levels were found increased only after 6 weeks of treatment. The results reinforce the hypothesis that oxidative stress actually precedes ALA accumulation, suggesting that, at an earlier time of exposure, other factors, such as induction of cytochrome P450 (CYP450) (Smith *et al*., 1990; Franklin *et al*., 1997, Almeida *et al*, 1997), followed by increase in superoxide anion production (Almeida *et al*, 1997), may contribute to the observed oxidative stress condition in the liver. Nevertheless, one cannot exclude the possibility of ALA participation as an inducer of hepatic oxidative stress at a later phase of HCB exposure.

RESUMO

Ácido delta-aminolevulinico e estresse oxidativo hepático na fase inicial da intoxicação experimental por hexaclorobenzeno

Este trabalho avaliou os níveis de ácido delta-aminolevulínico (ALA) em fígado de ratos expostos a diferentes doses de hexaclorobenzeno (HCB) (25, 50 e 100 mg/kg de peso corpóreo) durante 4 semanas e correlacionou com os parâmetros de peroxidação lipídica. Os níveis de ALA foram determinados por cromatografia líquida de alta eficiência após derivatização com acetilacetona e formaldeído, seguida de detecção de fluorescência. A metodologia foi cuidadosamente validada, apesar disso, os níveis hepáticos de ALA em todos os animais tratados ou não foram abaixo do limite de detecção do método (2,27 mg de ALA/ g de fígado). Por outro lado, para a peroxidação lipídica, avaliada como produção de reagentes ao ácido tiobarbitúrico e quimiluminescência, os resultados foram significativamente elevados em todos os animais tratados em comparação com os do grupo controle ($p < 0,05$). Estes resultados sugerem que o estresse oxidativo hepático observado nos animais após tratamento com HCB não deve necessariamente estar associado com o aumento das concentrações de ALA no fígado. Uma outra possibilidade seria o aumento nos níveis de ALA no fígado, mesmo que abaixo do limite de detecção do método, sendo suficiente para induzir estresse oxidativo hepático.

UNITERMOS: Hexaclorobenzeno. Estresse oxidativo. Ácido delta-aminolevulínico

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