

# EFFECT OF CHRONIC LEAD POISONING ON ERYTHROCYTE PROTOPORPHYRIN IN RATS

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## ABSTRACT

Research has shown that exposure to lead may have adverse effects at different blood lead concentrations. Lead inhibits at least two enzymes that are essential for the formation of heme, and because of the interaction of lead with these enzymes, no iron is inserted into protoporphyrin. Therefore the concentration of protoporphyrin increases in erythrocytes. The concentration of lead was measured by atomic absorption at 217.0 nm. The erythrocyte protoporphyrin was determined following extraction into 90% ethanol. The concentration of protoporphyrin in the blood of the lead exposed rat group was increased compared to that in the control group [12.46 +/- 0.57 (mean +/- S.D., n= 72) vs. 3.89 +/- 0.42, n=68,  $\mu\text{mol/L}$ ,  $p<0.05$ ]. The assay had excellent precision for both control and lead exposed groups (intra-assay and interassay coefficient of variation <5%). The method was linear from 0.0 to 12.46  $\mu\text{mol/L}$  of erythrocyte protoporphyrin. A good relationship was obtained between erythrocyte protoporphyrin value and blood lead concentration. The results suggest that the erythrocyte protoporphyrin value obtained by this method could be used as a screening test for lead poisoning.

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## INTRODUCTION

Several laboratory tests have been evaluated for occupational lead exposure. The determination of various metabolites such as coproporphyrin and delta-aminolevulinic acid has been widely used to detect occupational lead exposure.<sup>1,2</sup> Within the last few years, practical and effective tests for the diagnosis of lead poisoning have been developed. These include measurements of blood lead and erythrocyte protoporphyrin levels. In particular, measurement of erythrocyte protoporphyrin has become a useful test for increased lead absorption.<sup>3,4,5</sup> Lead inhibits at least two enzymes that are essential for the formation of heme, namely  $\delta$ -aminolevulinic dehydrogenase (ALA-D; E.C. 2.6.1.43) and ferrochelatase (EC 4.99.1.1.). Because of the interaction of lead with ferrochelatase in bone marrow, no iron is inserted into the substrate protoporphyrin IX, and the concentration of the latter is therefore increased in erythrocytes.<sup>6,7</sup> Lead has a strong affinity for bone and aorta, accumulating in

both tissues with age. In addition, lead poisoning has come to be recognized as a serious health hazard especially among ghetto children.<sup>8,9</sup> Clinical symptoms of lead poisoning appear only at toxic levels, and it is, therefore, important to be able to detect lead poisoning in the subclinical phase and prevent further contact with lead.<sup>10</sup> The concentration of erythrocyte protoporphyrin in the blood increases exponentially in lead poisoning.<sup>11</sup> Although lead intoxication perturbs many metabolic processes, the primary screening variables have been the concentration of Pb in blood and more recently the free erythrocyte protoporphyrin level in blood.<sup>12,13</sup> In case of iron deficiency and lead poisoning, zinc is incorporated into protoporphyrin IX instead of iron, and zinc protoporphyrin is produced instead of heme.<sup>14</sup> As stated in a recent document of the national committee for clinical laboratory standards, expression of the results of erythrocyte zinc protoporphyrin in different units is a main source of confusion which may hamper the wide use of this test. The possible interconversion of the ZPP values expressed

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as microgram/g Hb, microgram/dL blood and microgram/dL red cells was assessed.<sup>15</sup> A spectrofluorometric method has been designed for the determination of protoporphyrin IX.<sup>16-18</sup> The relationship between the concentration of 5-aminolevulinic acid in plasma and other biomarkers of lead exposure and its effect was investigated in lead-exposed children.<sup>19</sup> Protoporphyrin and zinc-protoporphyrin were measured in the erythrocytes of normal subjects and workers exposed to lead. Results showed a significantly higher level of zinc-protoporphyrin in the lead-exposed workers.<sup>20</sup> The purpose of this study was to determine the effect of chronic lead poisoning in rats. It is hoped that information from this study may be useful in detecting lead poisoning in the subclinical phase and preventing further contact with lead.

### MATERIAL AND METHODS

#### Reagents

Protoporphyrin, pyridine and ethanol were obtained from Sigma Chemical Company, St. Louis, Mo., USA. All other reagents and solvents were of analytical grade. Protoporphyrin standard was dissolved in pyridine for stock solution.

#### Subjects

Rats ( $n=72$ ) were fed with 5% lead acetate in the diet for 3 months. Control rats ( $n=68$ ) were fed with normal diet. Blood was collected, transferred to tubes, and preserved in an anticoagulant mixture of 0.03% heparin. All samples were protected from light before analysis and were analyzed the same day.

#### Extraction and assay method

50  $\mu\text{L}$  of whole blood was mixed with 250  $\mu\text{L}$  of deionised distilled water and hemolyzed completely. After 2 mL of 90% ethanol was added, the sample was shaken vigorously on a vortex mixer for two minutes and centrifuged at 3500g for 15 minutes. The supernatant solution was examined by using a Shimadzu RF-5000 spectrophotometer. The excitation wavelength was 457 nm and the fluorescence spectra was scanned from 400-600 nm. The standard solution of protoporphyrin was diluted 50 fold by adding ethanol. The mean of the triplicate analyses was reported as the concentration of lead in whole blood. It was analyzed by using an AAS-5FL atomic absorption spectrophotometer analyzer at 217.0 nm. The accuracy of the standards of whole blood was determined with addition of lead.

#### Calibration and quality control

Six levels (0.0 to 12.0  $\mu\text{mol/L}$ ) of calibrators were run in duplicate. The calibration curve was stable for one week. Controls comprising 3 levels (4, 8 and 12  $\mu\text{mol/L}$ ) of erythrocyte protoporphyrin were included in each run as quality

control.

#### Precision and sensitivity

Intra-assay coefficients of variation (CVs) were obtained by assaying the 3 controls (low, medium, and high) 10 times in the same run. Interassay CVs were obtained by assaying the controls in each run over 3 months. Also, interassay and intra-assay precision studies were conducted with rat plasma with 3 levels of erythrocyte protoporphyrin. The limit of detection was calculated as 3 SD above the mean response of the zero calibrator run 10 times.

#### Recovery and linearity

Mean recoveries of erythrocyte protoporphyrin were obtained by mixing various portions of controls, calibrators, and samples of different levels. Also, a high erythrocyte protoporphyrin sample was diluted manually by serial dilutions (1:2, 1:4, 1:8, 1:16) with the zero calibrator. Expected values were compared with obtained values.

#### Stability study

Stability of erythrocyte protoporphyrin was assessed by storing aliquots of fresh plasma from 6 rats at  $-70^{\circ}\text{C}$  up to 3 months. Thereafter, the samples were thawed and assayed for erythrocyte protoporphyrin. All measurements were done in duplicate.

#### Statistical analysis

Results are expressed as mean  $\pm$  SD of 10-12 experiments. The Student's t-test was applied to assess significant differences. Statistical significance was defined as a P value of less than 0.05.

### RESULTS

Chronic administration for 3 months by feeding 5% lead acetate in the diet caused a 4-fold increase of the erythrocyte protoporphyrin. The effect of chronic lead poisoning on erythrocyte protoporphyrin in the rat is shown (Table I). Figure 1 shows the fluorescence spectra given by the ethanol extraction method applied to the blood sample of a lead exposed rat. The fluorescence peaks were observed at 457 nm. The blood lead determinations were performed routinely on each sample by

Table I. Effect of chronic lead poisoning on erythrocyte protoporphyrin level in the rat.

	Control group	Lead-treated group
Erythrocyte Protoporphyrin ( $\mu\text{mol/L}$ )	3.89 $\pm$ 0.42 (n=68)	12.46 $\pm$ 0.57 (n=72)

Each value represents the mean  $\pm$  S.D. of 10-12 experiments.

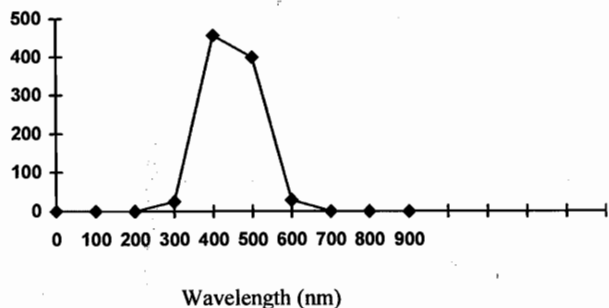


Fig. 1. Spectra of erythrocyte protoporphyrin by ethanol extraction method.

atomic absorption spectrometry. The concentration of lead in the blood of the lead exposed rat group is shown in Table II. The correlation between blood lead and level of erythrocyte protoporphyrin was determined by fluorescence method, and is presented in the scatter diagram of Figure 2. Intra-assay and interassay CVs are shown in Table III. Intra-assay CVs were between 0.55% and 1.4%. Inter-assay CVs were between 1.45% and 2.4%, from the lowest to the highest level. The mean erythrocyte protoporphyrin concentration of the zero calibrator was  $0.0025 \pm 0.001 \mu\text{mol/L}$ , and the low limit of sensitivity of the assay was  $0.12 \mu\text{mol/L}$ . The spectrofluorometric assay showed very good recovery ( $99.47\% \pm 1.45\%$ ). The linearity of the assay was confirmed by manual dilution of a high plasma sample ( $12.46 \mu\text{mol/L}$ ) as shown in Figure 3. Obtained values correlated well with expected values ( $r^2=0.99$ ).

## DISCUSSION

Clinical symptoms of lead poisoning appear only at toxic levels and it is, therefore, important to be able to detect lead poisoning in the subclinical phase and pre-

Table II. The concentrations of lead in the blood of the lead exposed rat group.

Time (day)	Blood lead ( $\mu\text{g/L}$ )
0	$0.012 \pm 0.001$
10	$0.031 \pm 0.001$
20	$0.054 \pm 0.001$
30	$0.063 \pm 0.003$
40	$0.072 \pm 0.002$
50	$0.083 \pm 0.002$
60	$0.092 \pm 0.003$
70	$0.104 \pm 0.002$
80	$0.126 \pm 0.003$

Each value represents the mean  $\pm$  S.D. of 10-12 experiments.

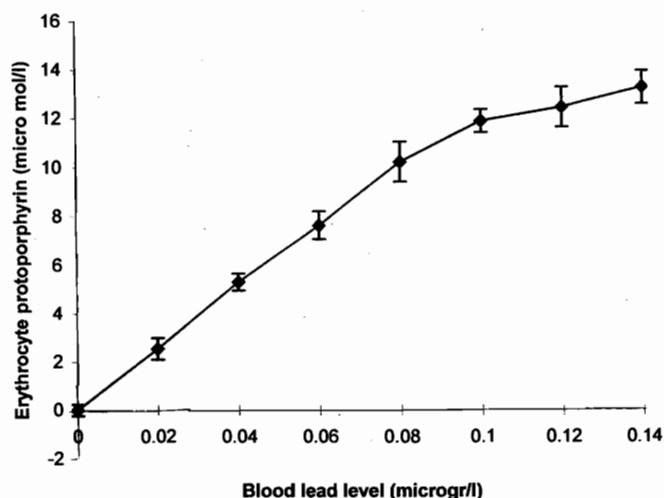


Fig. 2. Scatter diagram for erythrocyte protoporphyrin vs. blood lead level. Each points represents the mean  $\pm$  S.D. of 10-12 experiments.

vent further contact with lead. Within the last few years, practical and effective tests for the diagnosis of lead poisoning have been developed. These include measurements of blood lead and erythrocyte protoporphyrin levels. As shown in Figure 1 fluorescence peaks of erythrocyte protoporphyrin in ethanol were obtained at 457 nm. These results are consistent with those of previously published studies.<sup>1,8</sup> According to the results reported by several authors<sup>8,9,13,21</sup> the erythrocyte protoporphyrin value increases for a corresponding blood lead absorption in subjects with lead exposure lasting for at least three months and the linear relationship was derived between the logarithm of erythrocyte blood lead concentration. These results are very similar to the results obtained in the present study (Table I). As seen in Figure 2 there is a strong correlation between erythrocyte protoporphyrin and the concentration of lead in the blood. The results of this study confirms earlier ones that erythrocyte protoporphyrin and lead levels are significantly correlated.<sup>5,6,22</sup> The erythrocyte protoporphyrin assay had excellent precision at control and high levels (intra-assay and interassay coefficients of variation  $< 0.05$ ). The method was linear from 0.0 to  $12.0 \mu\text{mol/L}$  and displayed good correlation with blood lead levels. Erythrocyte protoporphyrin content was found to reflect whole body lead levels better than the circulating blood lead level. These findings lend support to the assumption that the erythrocyte protoporphyrin values obtained by this method could be used as a screening test for chronic lead absorption. The approach outlined in this study should be replicated in other exposed subjects, as well as in other occupational areas.

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**Table III.** Precision of the spectrofluorometric assay for measuring erythrocyte protoporphyrin.

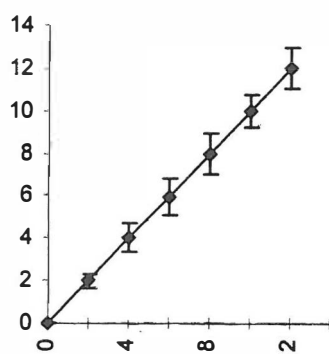
Sample	n	Mean (mmol/L)	SD	Coefficient of variation (%)
<i>Intra-assay</i>				
<b>Control</b>				
low	12	0.36	0.07	1.15
medium	12	7.51	0.12	0.91
high	12	12.45	0.15	0.57
<b>Sample</b>				
low	12	2.45	0.07	1.41
medium	12	7.53	0.01	0.11
high	12	12.44	0.05	0.15
<i>Interassay</i>				
<b>Control</b>				
low	12	3.71	0.14	2.11
medium	12	7.54	0.22	1.62
high	12	12.42	0.37	1.51
<b>Sample</b>				
low	12	3.75	0.16	2.12
medium	12	4.75	0.18	1.87

Each value represents the mean  $\pm$  S.D. of 10-12 experiments, each assayed in duplicate.

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Obtained value ( $\mu\text{mol/l}$ )



Expected value ( $\mu\text{mol/l}$ )

**Fig. 3.** Plot of the linearity presenting obtained vs. expected values. Equation of the relation: Obtained =  $(1.012 \pm 0.028) \times$  Expected  $- (0.866 \pm 0.712)$ ,  $r^2 = 0.99$ . Each value represents the mean  $\pm$  S.D. of 10-12 experiments.

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