Estimation of Cytochrome B_5 Reductase Activity in Red Blood Cells of *Rattus Norvegicus* after the Treatment of Trichloroacetic Acid

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Summary

Cytochrome b_5 reductase is a red cell enzyme and is responsible for the reduction of methemoglobin back to hemoglobin. Its activity is increased during malarial infections and ascorbate deficiency. Certain antithyroid and neuroleptic drugs have been reported to affect the expression of cytochrome b_5 reductase activity. In the present study we have attempted to study the activity of cytochrome b_5 reductase activity in red blood cells of rats, after the trichloroaceticacid (TCA) treatment. TCA was given at 0.2% concentration in drinking water *ad libitum* for 60 days continuously, and the activity was measured after 20, 40 and 60 days. A significant increase in the activity of cytochrome b_5 reductase was observed after the TCA treatment as compared to control groups. The results suggest that TCA affects the activity of cytochrome b_5 reductase activity in red blood cells of rats.

Keywords: Cytochrome b_5-reductase, trichloroacetic acid, red blood cells.

Introduction

Trichloroacetic acid is a skin and eye irritant, and affects the kidneys and liver of experimental animals. It was reported as a liver carcinogen in a single study in mice (1). It has also a number of industrial applications, including use as a synthetic intermediate, in medication and reagent for albumin detection (2). It was negative in most mutagenicity studies in bacterial strains (3,4). It was positive for micronucleus to C57BL mice (5) and for chromosomal aberrations in Swiss albino mice (6). It induced DNA strand breaks in Sprague-Dawley rats and $B6C3F_1$ mice (7). TCA increases 8-hydroxy-2-deoxyguanosine (a marker for oxidative stress) in B6C3 F_1 mice (8) Cytochrome b_5 and its reductase can directly detoxity certain bioactive compounds (9). Its activity can be measured ex vivo using the reduction of ferrihemoglobin in erythrocytes (10). In the present study we have studies the effect of trichloroacetic acid on the cytochrome b_5 reductase activity in red blood cells of *Rattus norvegicus*.

Materials and methods

Chemicals: EDTA (Qualigens, India); Sodium Citrate (Qualigens, India); Potassium ferricyanide (Qualigens, India); Human hemoglobin (Sigma); NADH (SRL, India); Trichloroacetic acid (SRL, India); sodium chloride (SRL); Carbon tetrachloride (SRL, India).

Animals: Rats (Rattus norvegicus) 4 months of age with an average weighing 150-200 g were procured from Lucknow, U.P., India and were housed in 5 groups, each group containing 5 rats. The animals were housed at 23±2°C in a daily light/dark cycle. All animals were fed on barley, wheat ad libitum in polypropylene cages, and received human care according to the criteria outlined in the 'Guide for the care and use of Laboratory Animals' prepared by the National Academy of Science and published by National Institute of Health (10). The departmental ethical Committee granted the permission for the experiment.

Dose Selection: Trichloroacetic acid was given at 0.2% (~0.012M) concentration in drinking water ad libitum for 60 days continuously (1). At this concentration, it was reported as a carcinogen in vivo (1).

Experimental groups: The rats were divided into 5 groups (5 in each group). Group I as a control and the remaining groups (II-V) were the treated groups receiving 0.2% of TCA orally daily for the 60 days in drinking water. The blood samples were collected after 20, 40 and 60 days of the treatment and the activity of cytochrome b_5 reductase was measured.

Preparation of red cell lysate: Red cell lysate was prepared according to the protocol suggested by Verma et al., (11). One ml of blood was collected in 0.1 ml of 10% EDTA and centrifuged at 3000 rpm for 15 min. The plasma was removed and the RBCs were washed three times with normal saline. To the packed RBCs 3 times normal saline was added and mix thoroughly with pasture pipette and then centrifuged and the supernatant was removed. After the last wash to the packed red cells equal amount of distilled water and half the amount of carbon tetrachloride was added. The tubes vortexed vigorously for 5 mins. and then centrifuged at 3000 rpm for 25 mins. The upper layer was collected as red cell lysate.

Measurement of hemoglobin concentration [Hb] in red cell lysate:

The concentration of hemoglobin [Hb] in each prepared red cell lysate was calculated by the Drabkin reagent. About 20 µl of lysate was added to 5 ml of drabkin reagent. After 5 min the absorbance was noted at 540 nm and the concentration was expressed in g/dl. The hemoglobin standard was taken as 12 g/dL. The concentration of the hemoglobin was calculated as follows:

$$\text{Hemoglobin [Hb] (g/dL)} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of standard tube}} \text{ X Hemoglobin concentration of standard } (12\, g/dL)$$

Ferrihemoglobin reduction as a marker of cytochrome b5 reductase activity in erythrocytes: Methemoglobin (Hb^{3+}) is a natural subtrate for cytochrome b_5 reductase. However, the reduction of methenoglobin (Hb^{3+}) to hemoglobin (Hb^{2+}) is too slow to determine the in vitro activity of the enzyme in clinical samples. The rate of NADH-dependent reduction of a methemoglobin – ferrocyanide complex can be used as a marker for cytochrome b_5 reductase activity. An increase in optical density due to the reduction of this complex is monitored spectrophotometrically at 575 nm (9). To a 1.5 ml amber microcentrifuge tube, the following reagents (Prewarmed to 37°C) were added in the order as 19 µl of 0.27M EDTA (pH 7.0), 98 µl of 50 mM sodium citrate buffer (pH 4.7), 294 µl of 0.5 mM potassium ferri cyanide, 196 µl of

1.224% human hemoglobin and 323 μl distilled water. The two reaction mixtures were prepared for each tested sample. The tubes were vortexed for 1 min, and 20 μl of diluted (1:20) red cell lysate was added. After incubating for 10 min at 37°C, 50 μl water (for control reading) and 50 μl 2 mM NADH (for experimental reading) was added and optical density (OD) was taken at 575 nm at 1 min intervals for 12 min. The first two min reading was discarded because the reaction rate is non-linear.

ΔOD (i.e. OD_{12} min – OD_2 min) for control and NADH-treated samples were calculate and the enzyme activity was calculated by using following formula:

$$\mu\,\text{mol ferri Hb reduced/min/gHb} = \frac{\Delta\,\text{OD sample} - \Delta\text{OD control}}{[\text{Hb}]} \text{ X } 238$$

[Hb] is the concentration (g/dL) in the RBC lysate. The factor 238 is a constant calculated from the optical density difference between ferrohemoglobin and ferrehemoglobin (9).

Statistical analysis: Student 't' test was performed using statistica Soft Inc.

Results and discussion

The results of the present study reveal that the treatment of TCA increased the expression of cytochrome b_5 reductase activity (Table 1). The concentrations of reduced methemoglobin-ferrocyanide are given in Table 1. After 20 days of treatment the values in Group II, III, IV and V were 3.58±0.128, 3.44±0.143, 3.66±0.156 and 3.46±0.132 respectively and were significant as compared to control (2.38±0.066) (group I) (Table 1). After 40 days of treatment the values in Groups II, III, IV and V were 4.46±0.107, 4.68±0.086, 4.66±0.169, 4.62±0.135 respectively, significant as compare to control group I (2.50±0.089). After 60 days of treatment the values in Groups II, III, IV and V were 5.28±0.037, 5.42±0.135, 5.52±0.139 and 5.30±0.045 respectively and significant to control group I (2.64±0.120) (Table 1). Methemoglobin is the derivative of hemoglobin in which the iron of the heme group is oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. The primary reaction that reduces methemoglobin back to hemoglobin is catalyzed by the reduced form of nicotinamide-adenine dinucleotide (NADH) – cytochrome b_5 reductase (b_5R) (12). However, the reduction of methemoglobin (Hb^{3+}) to hemoglobin (Hb^{2+}) is too slow to determine the *in vitro* activity of the enzyme in clinical samples. Alternatively the rate of NADH-dependent reduction of a methemoglobin-ferrocyanide complex can be used as a marker for cytochrome b_5 reductase activity (9). The carcinogenicity of TCA in mice have been reported by succession of hyperplastic and neoplastic lesion in the liver, ranging from apparently pre-neoplastic changes to fully malignant lessions (13). TCA has been reported to increase the activity of AST and ALT in plasma of rats mainly due to the leakage of these enzymes from liver cytosol into the blood stream (14). Dehydrogenases are important and critical enzymes in biological systems as they are responsible for detoxification, metabolism and biosynthesis of energetic macromolecules for different essential functions (15). TCA induced peroxisome proliferation in mouse liver and it is the important marker of carcinogenesis (13).

Table 1: Mean values of μ mol ferri Hb reduced/min/g Hb after the treatment of trichloroacetic acid

Duration	Control	Treated Groups			
	Group I	Group II	Group III	Group IV	Group V
After 20 days	2.38±0.066	3.58±0.128*	3.44±0.143*	3.66±0.156*	3.46±0.132*
After 40 days	2.50±0.089	4.46±0.107*	4.68±0.086*	4.66±0.169*	4.62±0.135*
After 60 days	2.64±0.120	5.28±0.37*	5.42±0.135*	5.52±0.139*	5.30±0.045*

*P < 0.05 (Significant with respect to control).

Our present study was aimed to study the effect of TCA on cytochrome b_5 reductase activity. The results shows that the treatment of TCA increased the activity of cytochrome b_5 reductase. The reported normal values for NADH cytochrome b_5 reductase activity in adults human are 3.4±0.5 μmol ferri Hb reduced/min/g Hb (16). However some authors have obtained 4.6±1.0 μmol Ferri Hb reduced/min/g Hb. Although factors that affect the expression of cytochrome b_5 and its reductase have not been well studied, but ascorbate deficiency, malarial infection and antithyroid and neuroleptic drugs have been reported to affect the expression and / or activity of this pathway (9). Like other markers such as SOD, MDA, ALT, AST, GST for oxidative stress and toxicity, our study shows that cytochrome b_5 reductase can also be used as a marker for the stress/ toxicity studies. The treatment of 0.2% TCA for 52 days has been report as a carcinogenic. The values presented in our study are for 20, 30 and 60 days treatments. The increased activity of cytochrome b_5 reductase can also be taken as a marker for possible carcinogenesis.

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