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Effects of BHA Administration in the Rat on Red Blood Cell Hemolysis and Sulfhydryl Liberation

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The physical and structural properties of synthetic antioxidants are important considerations to determine their hemolytic effects. They may influence red blood cells and their cellular components by one of the following methods: (i) free radical scavenging (Wattenberg and Lam 1983), (ii) inhibition of lipid peroxidation (Stohs et al. 1984), (iii) enzymatic induction linked to P-450 group of enzymes (Nakagawa et al. 1979).

2,(3)-tert-Butyl-4-hydroxyanisole (BHA) is one of the hindered phenolic antioxidants widely used as preservatives (Stuckey 1968). The relative significance of its solubility, absorption/adsorption and mobility across cell membranes in determining their cytotoxic, precarcinogenic or carcinogenic effects has been suggested to be correlated to their reactive metabolites and the metabolic pathway it follows in vivo. BHA has been suggested to have a protective effect in carcinogenesis induced by several chemical compounds (Wattenberg 1978). However the mechanism(s) by which BHA act(s) is(are) unclear. It includes enhancement of activities of several detoxification enzymes (Hassan et al. 1985).

The cytotoxicity of such substances can be determined by estimating the damage incurred on plasma and lysosomal membranes (Allison 1971). One of the simple methods of analysis of disruptive effects of substances on membranes, is based on the rupture of the erythrocyte membranes, i.e. rate of in vitro hemolysis (Jaurand et al. 1980).

Since rat is the most frequently used animal in lab studies, we have chosen to study the in vivo effects of BHA on rat RBCs. The red blood cell membrane is perhaps the most complex natural membrane, prone to deformability in vitro by (i) changes in osmotic pressure, (ii) by injury as in the case of chrysotile

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fibres (Pele and Calvert 1983), (iii) change in surface charge (Light and Wei 1977), and (iv) metabolic and enzymatic changes in the erythrocytic membrane and within the red cell.

In this study we have attempted to assess the rate of hemolysis with BHA, in an in vivo set up to elucidate the mechanism of action of hemolysis and the possible biochemical steps involved in the erythrocytic membrane disruptions. The hydrophobic BHA undergoes a series of enzymatically induced metabolic changes, converting to more electrophilic metabolites which are very active, before they are excreted. This is effected by the Mixed function oxygenases and Glutathione is particularly significant in determining the potency of such compounds in vivo (Sparnins and Wattenberg 1981). Glutathione is one of the several nonprotein sulfhydryl (-SH) compounds, which bind to electrophiles (metabolites of BHA, in this case). We have also seen the -SH release in hemolysates with time, to help elucidate the mechanism of toxicity of the antioxidant BHA.

MATERIALS AND METHODS

Male albino, Wistar-derived strain rats maintained and bred in Animal House, JNU were used for experimentation. They were housed four per cage (polypropylene) with rice husk padding and divided into four groups as follows : Controls received only vehicle (1 ml veronal) through i.p. injection and the other three groups received 1 ml of 0.1% BHA (5 mg/kg bw) suspended in veronal buffer for different times i.e. 7, 15 and 21 days through i.p. injection; 35 mg, 75 mg and 105 mg BHA per kg bw respectively was administered over the course of experiment. Through out the duration of experimentation the animals had free access to standard animal feed (Hindustan Lever Ltd., Bombay), except 12-16 hours before sacrifice the animals were starved. Water was available ad libitum. Animals were sacrificed by cervical dislocation and blood was collected by cardiac puncture.

BHA, DTNB and Potassium Chloride was purchased from Sigma Chemical Co., USA and all other chemicals used were obtained locally of the highest available purity analytical grade. Heparin (5000 iu/5 ml vials) was preferred to Citrate-saline for blood collection. Blood was collected in heparinised 5 ml capacity disposable syringes.

The blood was washed three times in normal saline and suspension of RBCs were made in saline to a concentration of 1% RBCs. Aliquots of 1 ml veronal buffer with 1 ml of 1% RBC for various time intervals ranging from 0,1,2,3,4,5,6,7,8,9,10,15,30, 45 and 60 minutes were incubated in a water bath shaker at 37°C. The positive controls constituted of 1 ml of 0.2% Triton X-100 to mark 100 percent hemolysis in place of veronal (Jaurand et al. 1980). The reaction was terminated with 0.5 ml of 2.5% gluteraldehyde solution made in cacodylate buffer. These solutions were centrifuged at 1000-1500 rpm in a table top Remi-centrifuge to allow broken membranes and unbroken cells settle at bottom and absorbance of supernatant was taken at 540 nm in a double-beam Beckman spectrophotometer using 3 ml quartz cuvettes of 1 cm pathlength to assess the percentage of hemolysis. The average O.D. of three replicates served to determine percent hemolysis.

1 ml of the hemolysate from each tube was precipitated with 2 ml of 5% TCA and centrifuged. The precipitate was dissolved in 3 ml of 1 N NaOH and the pH of this solution adjusted to 8.0 with 3 ml of phosphate buffer.

Each sample was divided into 2 test tubes of 3 ml each. To one added 20 µl of DTNB reagent made in phosphate buffer pH 7.0. After 25 minutes, the absorbance was measured in Beckman double beam spectrophotometer at 412 nm. The quantity of sulfhydryls (-SH) in blood was calculated using the formula :

$$Co = \frac{A}{E} D$$

Co - original concentration
A - absorbance
E - Molar Extinction Coefficient (13600/M/cm)
D - dilution factor

The -SH levels in blood is expressed as mmoles -SH per litre of blood.

RESULTS AND DISCUSSION

The percentage hemolysis at various time intervals for each of 0, 7, 15 and 21 days are presented in Figure 1.

The sulfhydryl levels in the hemolysates are presented in Figure 2.

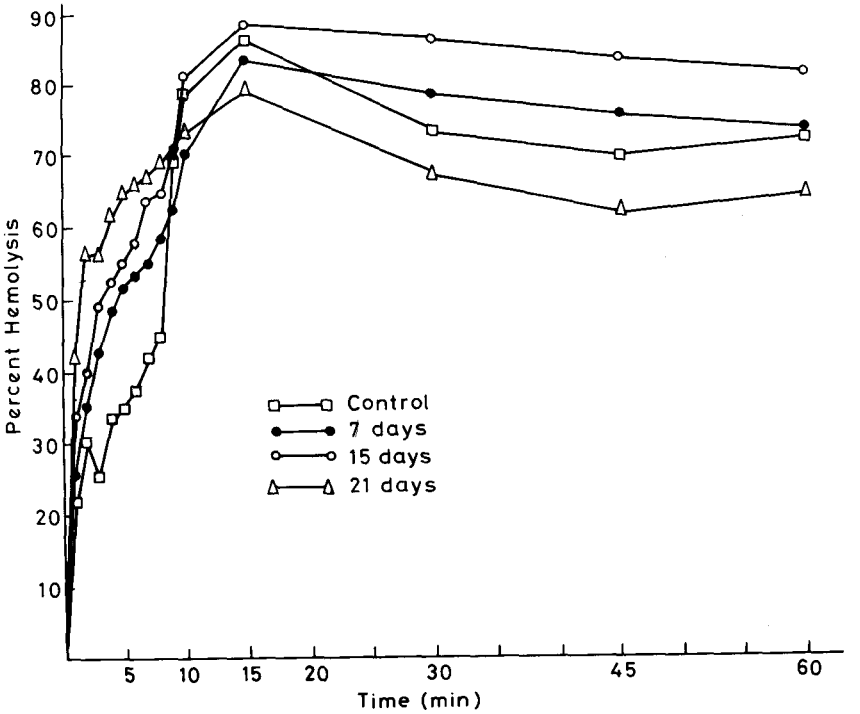

Figure 1. In vitro Dose-effect curve of percent hemolysis in 0.1% BHA treated rats. Each group of study comprised three rats. Three groups received 1 ml of 0.1% BHA each day through i.p. injection for 7, 15 and 21 days. Control animals received only vehicle (Veronal).

Statistical analysis for the correlation of time series shows a direct positive correlation of % hemolysis with respect to -SH liberation in hemolysates. The 'r' values for 0, 7, 15 and 21 days are 0.993, 0.996, 0.990 and 0.870 respectively. A regression analysis of % hemolysis and -SH showed linear correlation in each of 0, 7, 15 and 21 days given by the following slopes and intercepts respectively :

	Slope	Intercept
0 days	0.103	0.544
7 days	0.097	0.886
15 days	0.099	0.662
21 days	0.081	1.540

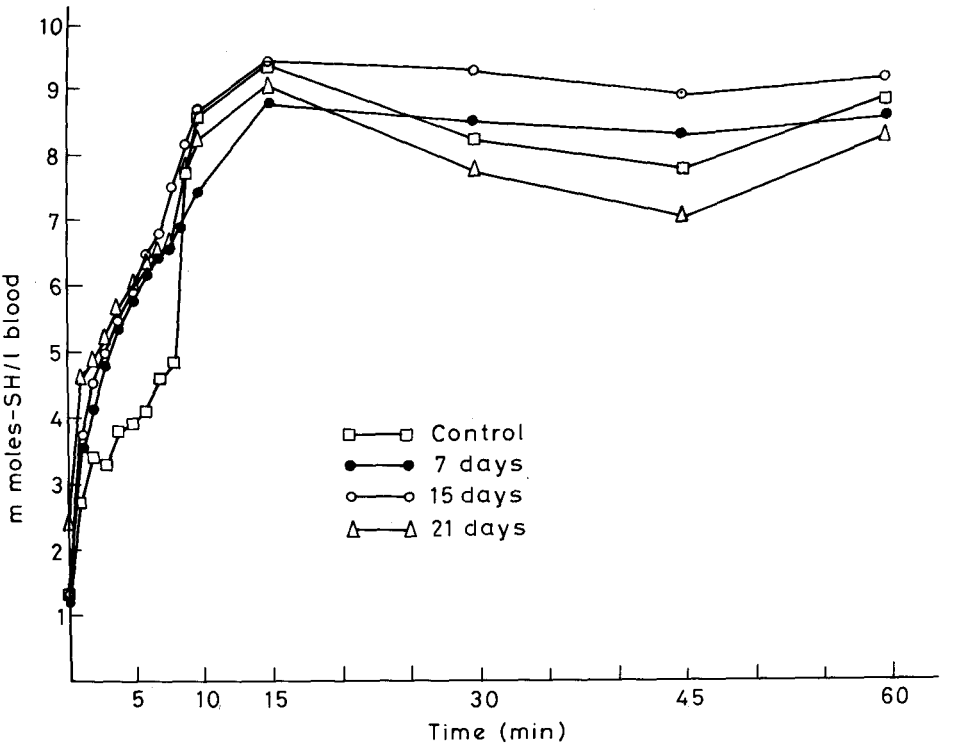

Figure 2. Sulfhydryl liberation in hemolysates of BHA treated rats. Hemolysates were derived from same rats as explained in figure 1.

Tissue distribution and pharmacokinetics of ^{14}C-labelled 3-BHA showed its appearance in the plasma within half an hour reaching a peak between 6-18 hrs. The half life of absorption in the plasma was 9.16 hrs. Its distribution was found to be 581.0 nmol eq/gm hr. Label was detected upto 72 hrs after which no detectable label was seen at 240 hrs after a single dose study by Ansari and Hendrix (1985).

Our in vivo studies on the hemolytic effects of BHA have shown a sharp increase in percent hemolysis within 10 minutes of incubation, tested after 16-18 hrs of the last administration of various duration regimens.

The 10 minute auto correlation between 7 day treated and 21 day treated rats was found to be of little

significance, inspite of apparent difference in slope of the curves. 50% hemolysis occured in 8.216 minutes in control rats, in 4.296 minutes in 7 day treated rats, in 3.25 minutes in 15 day treated rats and 1.549 minutes in 21 day treated rats. This shows a positive disruptive effect which is dose-time dependent in causing damage to RBCs. Blood levels of BHA and its metabolites in such dose dependent responses could lead to better interpretation of hemolytic studies.

Such in vivo dosing of BHA has been shown to enhance Glutathione-S-transferase activities and non-protein sulfhydryl levels (Benson et al. 1979). The very positive correlation between % hemolysis and sulfhydryls in hemolysates may perhaps be due to the natural mechanism within the RBCs to combat the damaging effects of some active metabolite(s) of BHA that has attacked the membrane.

The accumulation of such metabolite(s) in the plasma over multiple dosage regimens like ours elucidate the importance of an in vitro technique of assay in an in vivo continuous exposures. Such studies offer a quick testing bioassay which can be conveniently studied in man especially during on-going occupational exposures to membrane-damaging substances which reach the blood stream.

Erythrocyte deformability, due to the presence of antioxidants from the diet to the bloodstream is a quantifiable entity, both in the form of filterability (Levander et al. 1980) and hemolysis in vitro (Jayalakshmi and Sharma 1986). In vitamin E deficient rats, incubations with various pro-oxidants markedly decreased filterability in vitro whereas feeding a synthetic antioxidant protected against it (Levander et al. 1977) and is highly correlated to the extent of lipid peroxidation.

The deformability of erythrocyte membrane depends directly on the state of the membrane proteins, particularly spectrin (Cantor and Schimmel 1980) and more so by selective membrane expansion due to incubation of red cells with certain cationic drugs that intercalate preferentially in the inner half of the membrane (Deuticke 1968).

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