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**Erythrocyte Membrane Deformation and Antihemolytic Effect of Antituberculosis Drugs in Rats**

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**Abstract:** The commonly used antituberculosis (antiTB) drugs; pyrazinamide (PZA), isoniazid (INH), ethambutol (ETB) and rifampicin (RMP) were administered to albino rats for the purpose of investigating the toxic consequences of combination therapies. The drugs were evaluated by simulating the normal clinical dosage and a 24 week gavage studies of standard therapy with antituberculosis drugs in albino rats without disease were carried out. The doses employed were: Isoniazid, 5 mg kg$^{-1}$; rifampicin, 10 mg kg$^{-1}$; ethambutol, 20 mg kg$^{-1}$ and pyrazinamide, 25 mg kg$^{-1}$. The effect of clinically equivalent antiTB regimen and established oxidant drug, aminopyrine, on membrane lipid peroxidation and osmotic fragility in rat red cells was examined. The regimen showed evidence of protection against hemolysis both in vitro and in vivo although there are evidences of distorted erythrocyte membranes which appeared to be approaching a star-shaped configuration and an enhanced antihemolytic effect in hypotonic saline solution. Since star-shaped erythrocytes have been shown to be devoid of ATP, this may have consequences on the physiological function of the red blood cells.

**Key words:** Antituberculosis, antihemolytic, RBC membrane deformation

**INTRODUCTION**

A wide variety of hematological manifestations can be observed in patients with tuberculosis and these have a chronic inflammatory nature (Mehmet et al., 2002). Studies have demonstrated that there is a high level of oxidative stress during early stages of tuberculosis (TB). Mice infected with *Mycobacterium tuberculosis* have also been shown to exhibit erythrocytic oxidative stress (Guleria et al., 2002); these are manifested as significant enhancement of erythrocytic catalase and glutathione peroxidase activities along with elevated levels of erythrocytic total thiols and plasma lipid peroxidation as compared to normal animals. The infected animals were also reported to exhibit significantly decreased activity of superoxide dismutase and levels of glutathione in erythrocytes (Guleria et al., 2002).

In a study carried out by Wiid et al. (2004) to evaluate the Total Antioxidant Status (TAS) in healthy and *M. tuberculosis*-infected persons, active TB patients showed a significantly lower TAS compared to community controls. This low values were however found to be reversed by antituberculosis (antiTB) therapy (Wiid et al., 2004) and the levels of TAS was found to be highly correlated with plasma micronutrients such as vitamin A and zinc, but vitamin E remained unaffected.

Antituberculosis drugs are administered for a period ranging from 6 months to 9 months before effective control of the disease is achieved. This long duration means that the red blood cells are exposed to the drugs for extensive periods and there has been reports in the literature implicating rifampicin and para aminosalicylic acid in hemolytic anemias (Bakhshi et al., 2004). Hemolytic anemia cases due to tuberculosis have also been previously reported in the English literature (Bakhshi et al., 2004; Gupta and Bathia, 2005) and these have been corrected by full treatment with antituberculosis drugs. While the data presented is not adequate for evaluating the possible effect of antiTB drugs on erythrocyte membranes, antiTB therapy may have stabilized the membranes and reversed TB-induced hemolytic anemia; possibly by reducing the phagocytic activity and subsequent hemolysis in the TB patients studied. In contrast, hemolytic anemia produced by antituberculosis drugs, rifampicin, streptomycin and para-aminosalicylic acid have been reported in the literature (Khurana and Singh, 2006; Panos et al., 2006) and this was regarded as a major complication of TB therapy.

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Mehmet et al. (2002) reported the disappearance of autoimmune hemolytic anemia in a Coombs' positive patient following antiTB therapy; although this is a very rare type of hemolytic anemia in TB sufferers, it however gives weight to the fact that antituberculosis drugs may be capable of preventing erythrocyte hemolysis. There are also reports in adult literature that hemolysis associated with tuberculosis has responded to antitubercular therapy alone (Kuo et al., 2001).

Combination therapy with antiTB drugs is a common practice in treatment of TB and AIDS patients. Although toxic effects of most individual therapies are known, the toxicity potential of combination therapy has not been established (Rao et al., 1998).

The conflicting reports on the consequences of antiTB therapy in relation to the RBC integrity needs to be resolved. We have attempted to elucidate this using standard approaches by screening for hemolysis, MDA-reactivity and erythrocyte microscopy in rodents maintained on clinically employed combination regimen of antiTB drugs.

**MATERIALS AND METHODS**

**Animals and treatment:** Rats weighing between 150 to 200 g were obtained from the animal house of the College of Medicine of the University of Lagos, Nigeria, between January-December 2004. Animals were given free access to rat pellets and water and were housed in group cages in a constant temperature room (30°C) with a 12 h light-dark cycle.

Groups of at least six rats, one group serving as control, were started on the four-drug regimen including isoniazid (INH) 5 mg kg⁻¹, rifampicin (RFP) 10 mg kg⁻¹, ethambutol (ETB) 20 mg kg⁻¹ and pyrazinamide (PZA) 25 mg kg⁻¹ for two months. At the end of the second month of antituberculosis exposure, ethambutol and pyrazinamide were withdrawn from the regimen and exposure was continued with isoniazid and rifampicin for the remaining four months. In another set of experiments, groups of rats were given standard hepatotoxic for a 24 h period with or without hepatoprotective agents. Oral route was used for the administration of all drugs using the oral cannula, while blood samples were obtained by cardiac puncture.

At time intervals over six months after dosing with the combination antiTB drugs, animals were fasted for 12 h, weighed, sacrificed by cervical dislocation and blood samples were collected for determination of blood count.

**Lipid peroxidation:** RBC membrane lipid peroxidation in blood samples collected from the control and treated rats was assessed by the thiobarbituric acid (TBA)-reactivity of malondialdehyde (MDA), an end product of lipid peroxidation (Stocks and Dormandy, 1971) as reported by Jain (1989). Absorbance at 600 nm was also subtracted from absorbance at 532 nm before calculating the TBA reactivity of RBC. MDA values in nmol mL⁻¹ RBC were determined using the extinction coefficient of the MDA-TBA complex at 532 nm (1.56 × 10⁵ per cm per molar solution).

**In vitro RBC hemolytic study:** Heparinized blood was collected from rats, centrifuged and washed three times with phosphate-buffered saline (PBS made up of 8.1 g of NaCl, 2.302 g of Na₂HPO₄, 0.194 g of NaH₂PO₄, pH 7.4). Ten percent RBC suspension was prepared from this and incubated in medium containing INH, RFP and ETH. The concentrations of antituberculosis drugs used were equivalent to that obtained at steady state, given doses of INH, RFP and ETH as in the in vivo study. Known erythrocyte membrane oxidant, aminopyrine and established hepatotoxin, bromobenzene were also incubated with erythrocytes at established concentrations-aminopyrine (1.665 × 10⁻⁶ g mL⁻¹) and bromobenzene (0.094 × 10⁻⁶ g mL⁻¹). Aliquots were obtained periodically for determination of hemolysis and glutathione content over a period of 3 h. All incubations contained 10 µL of pen strept mL⁻¹ of cell suspension to vitiate any microbial growth. Pen-strept contained 300 mg of penicillin and 500 mg of streptomycin in 10 mL of distilled water (Jain, 1989).

**Measurement of red blood cell hemolysis in antiTB exposed rats:** Red blood cell hemolysis in rats maintained on antiTB regimen was determined by the method of Young et al. (1981). Cell lysis was measured at 37°C by mixing 0.05 mL of blood with 5 mL (approximately 1%) of both isotonic saline (0.9% NaCl) and hypotonic saline (0.5% NaCl) for 1 h. The suspension was centrifuged and the extinction of the supernatant was measured at 540 nm. This value was related to the extinction obtained when complete lyses was achieved by osmotic shock or addition of Triton X-100 and the result was used as an indication of the degree of cell lyses.

**Red blood cell microscopy:** Blood obtained from treated rats was prepared for microscopic examination by using the hemocytometer method. The supporting ridge of the chamber was moistened and the coverslip applied by pressing firmly so that Newton’s rings appear. The cells in suspension were mixed and a drop was transferred to the hemocytometer chamber. The appearance of the red
blood cells were observed and a photomicrograph taken for further analysis. The 40x objective was used in the micrography.

**Drugs and chemicals:** Thiobarbituric acid (TBA) was obtained from Adrich Chemical Company, Milwaukee, Wisconsin, USA. All other chemicals and drugs were purchased from Sigma Chemical Company St Louis, Mo 63178, USA.

**Statistical analysis:** Statistical analysis was performed using GraphPad Instat, GraphPad software, version 2.04a. Numerical values for parameters were recorded as mean±SEM. Comparisons between groups were calculated using the unpaired t-test.

**RESULTS**

**Treatment duration with antituberculosis drugs on hemolysis and MDA reactivity of rat erythrocytes:** Lipid peroxidation as measured by MDA reactivity was decreased significantly by 8 weeks (p<0.05) of antituberculosis regimen. This followed a sharp drop in week 1 and a subsequent increase in week 2 (Table 1). A reversal towards normal values was observed from week 18 and by week 24, the MDA-reactivity had significantly overshot the control values (p<0.001). Relative hemolysis of RBC in isotonic solution showed a consistent decrease up to week 18 followed by an increase in week 24. In hypotonic solution, the erythrocytes followed a similar trend but were much more susceptible to hemolysis than isotonic solution. The lowest relative hemolysis for isotonic and hypotonic solutions were 6.1 and 15.2%, respectively taking the control values as 100%. The pattern of hemolytic effect in 0.1, 0.55 and 0.9% saline solutions are shown in Fig. 1.

**Effects of hepatotoxins on hemolysis of rat erythrocytes:** Bromobenzene (0.9M kg⁻¹), carbon tetrachloride (0.3 mL kg⁻¹) and acetaminophen (600 mg kg⁻¹) resulted in decreased hemolysis of erythrocytes in isotonic solution. Mean percentage decrease compared to control erythrocytes were found to be insignificant. However, bromobenzene and acetaminophen also resulted in decrease hemolysis in hypotonic solution of 0.55% saline. The decrease by bromobenzene was slightly different from control, those of acetaminophen was significantly lower than control (p<0.05). Carbon tetrachloride showed a slight insignificant increase in hemolysis compared to control erythrocyte (Fig. 2). The

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### Table 1: Effects of treatment duration with antituberculosis drugs on hemolysis and MDA reactivity of rat erythrocytes

<table>
<thead>
<tr>
<th>Duration (Weeks)</th>
<th>MDA nmol mL⁻¹ RBC</th>
<th>Hypotonic solution (0.55% NaCl)</th>
<th>Isootic solution (0.9%NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.05±0.18</td>
<td>100±4.5±36.9</td>
<td>100±5.3±34.1</td>
</tr>
<tr>
<td>1</td>
<td>0.61±0.05</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>0.95±0.13</td>
<td>79.1±47.2</td>
<td>90±440.7</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>71±22.0</td>
<td>60±435.4</td>
</tr>
<tr>
<td>*8</td>
<td>0.46±0.01***</td>
<td>64±24.3</td>
<td>37±5.6**</td>
</tr>
<tr>
<td>18</td>
<td>0.71±0.07</td>
<td>15±2.0</td>
<td>6.1±2.8</td>
</tr>
<tr>
<td>24</td>
<td>2.01±0.13****</td>
<td>41±22.7</td>
<td>67±5.5</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM (n = 6). Asterisks indicate significant differences between the control group without drugs and the group treated with drugs: NA values not available; *Withdrawal of pyrazinamide and ethambutol from the regimen: **p<0.05, ***p<0.01, ****p<0.001 (Student’s t-test).
Hemolytic effect of \textit{in vitro} incubation with antituberculosis drugs and selected hepatotoxins

Washed normal RBC was resistant to hemolysis until 40 min after incubation in normal saline Fig. 3. Percentage hemolysis increased between 40 and 60 min and decreased by 120 min. Beyond 120 min, there was a sharp increase in percentage hemolysis. Total percentage hemolysis at the end of the incubation period were 192.7% for control, 81% for isoniazid, 3.4% for rifampicin, 58% for ethambutol, 120% for aminopyrine and 82% for bromobenzene (Fig. 3). All agents showed effect on hemolysis upon initial exposure with isoniazid, rifampicin, ethambutol aminopyrine and bromobenzene; recording 12.06, 4.41, 2.94, 2.94 and 1.47% hemolysis, respectively for isoniazid, rifampicin, ethambutol, aminopyrine and bromobenzene. The concentration of the antituberculosis drugs corresponds to their equivalent steady-state plasma concentration while for the hepatotoxins, the concentrations chosen were 1.665×10^{-5} g mL^{-1} for aminopyrine and 0.094×10^{-3} g mL^{-1} for bromobenzene.

Red blood cell morphology: Figure 4 shows the appearance of red blood cells in rats exposed to antituberculosis treatment for two weeks. The shapes of the cells are showing signs of distortion and loss of the normal biconcave character. The membranes are becoming star-shaped and wrinkled.

**DISCUSSION**

Exposure to clinical doses of combination antiTB drugs resulted in a dual effect on erythrocyte hemolysis. A gradual and consistent decrease in hemolysis was apparent up to 18 weeks of daily exposure and the lowest hemolysis was at this point while a reversal of this trend was noticeable by 24 weeks of drug treatment. Lipid peroxidation as measured by MDA reactivity was decreased significantly by 18 weeks of exposure to antiTB regimen; following the same trend as was observed in hemolysis. If lipid peroxidation is reduced, it follows logically that the oxidant effect and subsequent hemolysis is prevented by the drugs which our study confirmed. The relationship between the percentage decreased hemolysis and lipid peroxidation as given by the MDA value was observed to be directly proportional throughout the duration of the study. The same can be seen at 24 weeks exposure when increasing hemolysis was followed by a high value of MDA which significantly exceeded the normal value (p<0.001). Lipid peroxidation has been implicated in membrane damage during oxidative stress that may lead to cell lysis (Chance et al., 1979; Bidlack and Tapped, 1973) and it has also been shown that the initial reactive species of oxygen generated during such stress involves superoxide dismutase (Fridovich, 1978).
The reversal of hemolytic effect after week 18 did not correspond to the withdrawal of pyrazinamide and ethambutol from the regimen. The time lag does not suggest that the two drugs have any significant contribution to the observed antihemolytic effect.

This study further revealed that hemolysis of RBC in isotonic solution was gradually reduced to 61% by week 18, interestingly though, a high protection was also conferred on the RBCs exposed to highly hypotonic (0.1%) saline solution. This reduction in hemolysis may confirm the suspicion that the drugs actually prevented oxidative stress or that the effects of the drugs may confer a protective effect on erythrocyte membrane. In 0.1% saline solution however, the erythrocytes were slightly more susceptible to hemolysis up to week 4. There are several reasons that have been suggested for this type of observation, susceptibility to hypotonic solution is said to be probably due to leakages at areas of membrane instability and weakness (Tufano et al., 1984) as a result of loss of membrane lipids, which may be related to stearic alterations of spectrin (Loyer et al., 1979); plasticity of the erythrocyte membranes due to reduced ATP (Tufano et al., 1984); alteration of membrane protein due to intracelluar oxidation with formation of cross-links between sulphhydric radicals because of reorganization of spectrin as a result of dephosphorylation (Liu and Palek, 1979); or aggregation of non-hemoglobin proteins on the inner membrane surface of erythrocytes (Allen et al., 1983) which reduces its flexibility. The combination therapy may have affected any of the above or a combination of two or more of the above mechanisms. The shape of the erythrocytes as shown in Fig. 4 may be responsible for protection against hemolysis and it has been reported that star shape in erythrocyte morphology indicates ATP deficit in erythrocytes (Hochmuth and Mohandas, 1972) and our study corroborated this by revealing considerable star-shaped erythrocytes. Whatever effect the drugs might have on the membranes of RBC, it definitely affected their inherent elasticity.

Of the three known hepatotoxins tested, only bromobenzene and acetaminophen showed a decrease in hemolysis with acetaminophen showing the greater effect. Carbon tetrachloride on the other hand resulted in an increase in hemolysis particularly in 0.55% saline (Fig. 2). The differences in the effects of these hepatotoxins on erythrocyte membranes may be due to differences in metabolite generation and possibly different molecular interaction with the membrane.

Short term exposure of erythrocytes to the steady state concentrations of antiTB drugs and hepatotoxins revealed the relative effects on hemolysis; all the drugs tested showed a similar pattern of effects on hemolysis with a near complete protection within 40 min of exposure followed by a slight susceptibility between 40 to 60 min (Fig. 3). By 180 min of exposure, all the agents showed relative protection against hemolysis compared to control values. Present study showed that rifampicin provided the greatest protective effects amongst the antiTB drugs since it shows almost complete prevention of hemolysis (Fig. 3). This corroborates an earlier study by Oguz et al. (1989), who reported that they did not observe any hemolytic adverse effect in a patient they were treating for tuberculosis with rifampicin and streptomycin as well as the report by Mehnert et al. (2002) who observed the disappearance of autoimmune hemolytic anemia in a Coombs’ positive patient following antiTB therapy. The in vivo result confirmed the in vitro observation that the entire antiTB drugs tested protect the erythrocytes against hemolysis. Earlier observation of hemolytic effect seen in rifampicin treatment had been attributed to irregular rifampicin therapy (Oguz et al., 1989).

The precise mechanism by which antiTB regimen brings about the decrease in hemolysis is not clearly understood but this study did show that the regimen was able to bring about a significant reduction in lipid peroxidation and since increased lipid peroxidation has been implicated in hemolytic effect of substances, this may be the single most important beneficial effect of the drug combination on RBC integrity. Peroxidation of membrane lipids has been suggested to be capable of resulting in inactivation of enzymes and cross-linking of membrane lipids and proteins and ultimately in cell death (Chio and Tappel, 1969; Bidlack and Tappel, 1973; Pflegerott et al., 1982; Jaim, 1989). It has also been reported that lipid peroxidation and cell damage could result from a failure to catabolize effectively hydroperoxides (Dabson et al., 1981; Chance et al., 1979).

Osmotic fragility, autohemolysis and acidified glycerol lysis are screening tests currently in use for erythrocyte membranopathy in a routine hematology Laboratory (King et al., 2000). There is erythrocyte membranopathy in the animals used in this study and the major visual defects appear to be spherocyte formation and star-shaped erythrocytes. This apparently is contributory to the effects observed.

There are so many mechanisms suggested for the manifestation of lipid peroxidation and as such, the apparent protection against lipid peroxidation and subsequent anti-hemolysis by antiTB drugs may be due to several reasons: glucose-induced membrane lipid peroxidation and osmotic fragility have been shown to be blocked in RBC by pretreatment with fluoride, an inhibitor of glucose metabolism; vitamin E, an antioxidant; para-
chloromercurobenzoate and metyrapone, inhibitors of the cytochrome P-450 system, or with dimethylfuran, diphenylamine and thiourea, scavengers of oxygen radicals (Jain, 1989). Though measurement of glucose levels was not taken in this study, the effect attributed to the antiTB regimen may have been through interference with glucose metabolism or any of the above-mentioned mechanisms since the agents are known to be heterogeneous in their mechanisms of pharmacological action. Investigation of possible effects of the regimen on any of the above parameters will help in explaining this phenomenon.

In conclusion therefore, antiTB treatment, used as combination therapy in TB infection, has shown the capability to reduce hemolysis of red blood cells and rifampicin showed the greatest effect in vitro and may be responsible for the extension of the protective effect even beyond the withdrawal of pyrazamide and ethambutol from the initial combination by the fourth week. The interference with the mechanisms that lead to lipid peroxidation may be the singular most important reason for this effect and the outcome of this study corroborates the earlier observations by Siribaddane and Wijesundera (1997) as well as Mehmet et al. (2002) who reported the disappearance of hemolytic anemia due to tuberculosis and Coomb's positive autoimmune disorders respectively. The findings of this research has revealed the protective effect that combination antiTB regimen confers on the RBC membrane. In addition to clearing the system of the tubercle bacilli therefore, this regimen also protects the patient from lethal hemolytic consequences of TB infection.

REFERENCES


