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Full Length Research Paper

The effects of pyrimethamine sulfadoxine and berenil on glucose-6-phosphate dehydrogenase activity in *Trypanosoma brucei brucei* and *Trypanosoma brucei congolese* infected rats

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The effects of pyrimethamine sulfadoxine (PS) and Berenil on the glucose 6-phosphate dehydrogenase (G6PD) activity in the blood, brain, heart, kidney, liver and skeletal muscle tissues of *Trypanosoma brucei bruccei* (Tbb) and *Trypanosoma brucei congolense* (Tbc) infected Wistar rats were investigated. Forty-eight rats with a mean weight of 140.0 g were divided into 2 equal groups and separately treated with PS and Berenil. Each group was further subdivided into uninfected, infected with Tbb and infected with Tbc subgroups. The drugs were administered after parasitemia was confirmed via microscopic examination. G6PD activity, total organ protein and haemoglobin levels were determined spectrophtometrically in the hemolysate and homogenates of brain, heart, kidney, liver and skeletal muscle tissues. The results were statistically analysed by using the two-tailed student's t-test. The results of the two types of trypanosomal infections showed that they were comparable and showing similar patterns. The hemolysate indicated the highest activity of G6PD followed by the homogenates of the brain, liver, heart, kidney and muscle in that order. PS- treated animals did not demonstrate a significantly different G6PD activity when compared with those treated with Berenil ($p > 0.05$) and the values between uninfected and infected animals (both Tbb and Tbc) did not differ significantly ($p > 0.05$). This suggests that the mechanism by which PS and Berenil effect anti-trypanosomal actions may not be via the regulation of the pentose phosphate pathway.

Key words: Trypanosome, infection, glucose-6-phosphate dehydrogenase, homogenate, haemolysate.

INTRODUCTION

Recently, a need has arisen to explore the rationale in the choice of drugs for the treatment of trypanosome infections in livestock and humans. This is based on observations which have been made that sometimes, the major drugs (Homidium, Isometamidium and Diminazene) used in the treatment of livestock are either in short supply or difficult to administer. The WHO (1998) reported that 3 - 5% of those treated in the late stage of the illness, die from the treatment itself. Other problems encountered include oral absorption, acute toxicities, side effects, high cost and uncertainty of action and the emergence of trypanosome resistance (Wang, 1995). One of the several candidate pathways involved in the mechanism of action of PS and Berenil in the treatment of trypanosomiasis is the glucose metabolism pathways (Opperdoes and Michels, 2001). The glycolytic pathway enzymes have been exhaustively investigated but the pentose phosphate pathway has only recently been explored (Opperdoes and Michels, 2001). The regulatory enzyme of the pathway, glucose 6-phosphate dehydrogenase (G6PD) has recently been purified, localized and characterized in *T. brucei bruei* (Tbb) (Heise and Opperdoes, 1999) and its molecular characteristics with that of the 6-phosphogluconate dehydrogenase (6PGDH)

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has also been reported (Duffieux et al., 2000).

We have earlier discussed the prospects of pyrimethaminesulfadoxine (PS) in the chemotherapy of trypanosomiasis in a laboratory animal model where we noted, the reduction in parasitemia and the reversal of pathologic imbalances caused by trypanosomiasis in rat serum alanine transferase, serum aspartate transferase and serum alkaline phosphatase activities (Coleshowers and Okochi, 2001; Coleshowers and Etoh, 2004). In this study, we determined whether the mechanism by which PS and Berenil elicit anti-trypanosomal actions may involve the regulation of G6PD activities. We are reporting our fin-dings on the effects of PS and Berenil on G6PD activity in the blood, brain, heart, kidney, liver and skeletal muscle of rats infected with *T. brucei brucei* (Tbb) and *T. brucei congolense* (Tbc) respectively.

MATERIALS AND METHODS

All chemicals used were of analytical grade obtained from Sigma Chemical Company (USA).

Animal care

Forty-eight male Wistar rats (aged 7 weeks) with a mean weight of 140g obtained from the Laboratory Animal Production Unit of the Nigeria Institute of Medical Research (NIMR), Yaba, Lagos were used in the study. All animals used were treated in accordance with the principles of laboratory animal care as indicated in the Guide for the Care and use of Laboratory Animals of the National Institute of Health (1978). Animals were placed in a temperature controlled room (25 ± 2°C) in which a 12 h light: 12 h dark cycle was maintained for one week to condition the animals before the start of experiment. A standard rat chow and tap water were provided *ad libitum*.

The rats were divided into two main groups and treated with, a-25 mg/kg body weight pyrimethamine-sulfadoxine (PS) and b-7 mg/kg body weight diminazene diaceturate (Berenil) respectively. Each group was further sub-divided into three subgroups: uninfected rats, infected with *T. brucei brucei* (Tbb) and infected with *T. brucei congolense* (Tbc). The drugs were administered to each group of rats orally for PS and intramuscularly for Berenil, after parasitemia was confirmed microscopically in the parasite-inoculated rats.

Parasite inoculation and parasitemia

T. brucei brucei (Tbb) and *T. brucei congolense* (Tbc) were obtained from the Department of Parasitology of the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos, Nigeria. They were maintained in the laboratory by serial passage of blood of infected rats to uninfected. Inoculums, calculated to be approximately 8300 parasites/ml of blood was obtained from the tail vein of infected rats, collected in normal saline and passaged to experimental animals. Standard procedure of calculating parasitemia, using methanol and Giemsa stain on one cell-thick film, as parasites/1000 blood cell per field under X 40 magnification light microscope was followed.

Blood sample collection and preparation of haemolysate

All animals were sacrificed under chloroform anaesthesia after 5 days and 3 - 5 ml blood were collected into heparinzed tubes at 4°C for immediate preparation of a 1:20 haemolysate. This was prepared by collecting 0.1 ml of washed red blood cells suspended in normal saline, with 0.9 ml of hemolysing solution (0.05 ml β-mercaptoethanol, 10 ml – 10% sodium EDTA and distilled water) and shaken vigorously. The tubes were then placed in ice for 5 min and the samples were centrifuged at 13,000 rpm for 10 min. The haemolysate was aspirated and used to determine hemoglobin concentration from hemoglobin standard curve and G6PD activity.

Preparation of organ/tissue homogenates

The brain, heart, kidney, liver and skeletal muscle of chloroform anaesthesized rats were quickly removed and thoroughly washed with isotonic saline. Individual tissues were homogenized in 10 volumes of ice cold (0 - 4°C) medium containing 50 mM Tris–HCl, pH 7.4 and 300 mM sucrose, using a homogenizing machine. The homogenates were centrifuged at 6,500 rpm for 10 min to remove nuclei and debris. The supernatant was immediately used for G6PD activity measurement at 37°C. Total protein levels were determined according to Lowry et al. (1951).

G6PD assay of haemolysates and homogenates

G6PD assays were done by using the method of Beutler (1975). This assay measures the rate of reduction of NADP+ to NADPH when the hemolysate or homogenate is incubated with glucose 6-phosphate (G6P). The assay mixture consisted of 6 mM Glucose 6-phosphate (0.1 ml), 2 mM $NADP^+$ (0.1 ml), $MgCl_2$ (15 mM), Tris buffer pH 8.0 (0.68 ml) and either haemolysate or homogenate (0.02 ml). The assay for haemolysate and homogenates was monitored with a background wavelength of 550 nm and a real wavelength of 340 nm over a 5 min time course on the spectrophotometer at 25°C. A full-scale reading of 1.0 absorbance unit was maintained for the spectrophotometer (Beckmann 7400). G6PD activity at 37°C was calculated in hemolysates and homogenates from the equation

$$E = 1000A/Hb$$

where A = number of enzyme units per millilitre and expressed as $A = \Delta Abs \text{ at } 340/6.22 \times V_C/V_A$ and where 6.22 is absorbance of a 1 ml solution of NADP(H).
V_A = Volume of hemolysate or homogenate in the system
V_C = Volume of cuvette (1.0 ml)
Hb = Hemoglobin concentration in g/100 ml

G6PD activities (E) in homogenates were evaluated against total protein without the 1000 factor in the numerator, that is, E = A/total protein (mg/ml). The conversion factor from 25 to 37C is given as

$$\frac{\text{G6PD activity at 25°C}}{\text{G6PD activity at 37°C}} = 0.559 + 0.034$$

Statistical analysis

The data were analysed by using the two-tailed student's t-test and the level of significance was set at $P < 0.05$.

RESULTS

Table 1 shows G6PD activity and haemoglobin levels in

Table 1. G6PD activity in haemolysates of Tbb and Tbc infected rats and uninfected rats.

Groups		Hb (g/100 ml)	∆Abs/min @ 340 nm	G6PD@25°C IU/gHb	G6PD@37°C IU/gHb	t-test
Uninfected	a	1.47	0.0063	3.594	6.429	-
	b	1.43	0.0061	3.577	6.399	-
Infected with Tbb	a	1.46	0.0062	3.561	6.370	P>0.05
	b	1.49	0.0065	3.658	6.544	P>0.05
Infected with Tbc	a	1.45	0.0063	3.644	6.519	P>0.05
	b	1.48	0.0065	3.683	6.589	P>0.05

haemolysates of Tbb and Tbc infected rats and uninfected rats. Superscript “a” represents the PS group while subscript “b” represents the Berenil group. Student’s t-test was the comparison of respective uninfected group with infected groups. Further comparison between Tbb and Tbc groups did not reveal significant differences in pattern of G6PD activity however, parasitemia of Tbb was observed after 48 h while Tbc was only observed after 72 h. The values displayed are the mean values of the 8 animals in each group. Standard deviations were not displayed for clarity but considered in the statistics.

The result reveals that the status of G6PD activities in both uninfected and infected rats is not significantly differrent. Infected but untreated animals (data not shown) however indicated increased G6PD activities.

Table 2 shows inter-organ G6PD activities of homogenates. Superscript “a” represents PS-treated rats while subscript “b” represents Berenil-treated rats. Values displayed are the mean of 8 rats, while SD was not shown for clarity but used in t-tests. Results reveal that the brain has the highest G6PD activity followed by the liver, heart, kidney and skeletal muscle in that order. Infected but un-treated animals showed a general increase in G6PD activities in all homogenates, though marginally (data not shown).

DISCUSSION

The differences in the ways trypanosome species interact with their hosts have frustrated efforts at designing new effective drugs (Barret et al., 2003). The current strategy of research is multi-faceted, the majorly involving combination chemotherapy regimens (Kelser et al., 2001) and rational drug designs (Douglas, 1994).

We explored the regulatory enzyme of the pentose phosphate pathway, G6PD which assists in the generation of the NADPH -hydrogen donor in biosynthetic processes and as defense against oxidative attacks by infected hosts (Duffieux et al., 2000; Singh and Pathak, 2007), as a potential target.

We have earlier reported the prospect of employing pyrimethamine-sulfadoxine in trypanosomiasis chemotherapy (Coleshowers and Etoh, 2004). Pyrimethaminea diaminopyrimidine and dihydrofolate reductase inhibitor has been very successful as a causal prophylactic and suppressant against malaria attacks (Chio and Queener, 1993). Sulfadoxine inhibits folate production by competitively inhibiting the incorporation of p-aminoben-zoic acid (PABA) into dihydropteric acid, the immediate precursor of folic acid (Rollo, 1964). Its toxicity causes haemolytic anemia, especially in cases of G6PD deficiency (Rollo, 1964). However, in combination with pyrimethamine-sulfadoxine used to be one of the last lines of anti-malaria therapy in Africa (Plowe et al., 1996). It has however being succeeded by Artesunate due to observed resistance to the combination (Alloueche et al., 2004; Cissé et al., 2006).

A general elevation of G6PD activity and other enzyme activities have been observed (Coleshowers and Etoh, 2004) in both Tbb and Tbc infected animals. This suggests that the infection is responsible for such elevation. However, interactions between PS and G6PD were not noticeable as evident in Tables 1 and 2 among uninfected animals, both for PS and Berenil. This finding suggests that there may not be any interaction between the mechanisms by which PS or Berenil elicit anti-trypanosomal action and the regulation of the pentose phosphate pathway.

The patterns revealed by both Tbb and Tbc infections are similar for both hemolysates and organ homogenates (Tables 1 and 2). Apparently high values of G6PD activity observed in hemolysates do not directly suggest a higher activity than that observed in organ homogenates when taking into account that the measurements in both experiments are different. However, these results reveal the ease of determining the G6PD activity in animal hemolysate than organs homogenates.

Considering that major metabolic processes take place in the liver, the total protein values are quite consistent with such process. However, the higher G6PD activities in brain homogenates when compared to the liver in Table 2, confirm increased glucose metabolism in the brain. Among the organs, the activities of G6PD relatively

Table 2. Inter-organ G6PD activities of homogenates.

Homogenate	Groups		Total protein mg/ml	∆Abs/min @ 340 nm	G6PD@25°C IU/mg/ml	G6PD@37°C IU/mg/ml	t-test
Brain	Uninfected	a	0.87	0.0513	0.494	0.884	-
		b	0.89	0.0507	0.478	0.855	-
	Infected with Tbb	a	0.86	0.0511	0.498	0.891	P > 0.05
		b	0.88	0.0512	0.488	0.873	P > 0.05
	Infected with Tbc	a	0.85	0.0455	0.479	0.857	P > 0.05
		b	0.87	0.0461	0.496	0.887	P > 0.05
Heart	Uninfected	a	0.85	0.0455	0.449	0.803	-
		b	0.87	0.0461	0.455	0.813	-
	Infected with Tbb	a	0.86	0.0459	0.448	0.873	P > 0.05
		b	0.86	0.0456	0.445	0.796	P > 0.05
	Infected with Tbc	a	0.85	0.0460	0.454	0.812	P > 0.05
		b	0.87	0.0462	0.445	0.796	P > 0.05
Kidney	Uninfected	a	0.89	0.0466	0.439	0.785	-
		b	0.86	0.0459	0.448	0.801	-
	Infected with Tbb	a	0.87	0.0463	0.446	0.798	P > 0.05
		b	0.88	0.0465	0.443	0.792	P > 0.05
	Infected with Tbc	a	0.88	0.0471	0.449	0.803	P > 0.05
		b	0.89	0.0453	0.427	0.764	P > 0.05
Liver	Uninfected	a	0.87	0.0505	0.487	0.871	-
		b	0.89	0.0453	0.461	0.824	-
	Infected with Tbb	a	0.92	0.0503	0.458	0.819	P > 0.05
		b	0.89	0.0489	0.461	0.825	P > 0.05
	Infected with Tbc	a	0.89	0.0498	0.469	0.839	P > 0.05
		b	0.90	0.0501	0.467	0.835	P > 0.05
Skeletal muscle	Uninfected	a	0.81	0.0411	0.426	0.762	-
		b	0.83	0.0419	0.423	0.757	-
	Infected with Tbb	a	0.82	0.0417	0.426	0.762	P > 0.05
		b	0.81	0.0416	0.426	0.771	P > 0.05
	Infected with Tbc	a	0.82	0.0418	0.427	0.764	P > 0.05
		b	0.82	0.0414	0.423	0.757	P > 0.05

decrease in the following order: brain > liver > heart > kidney > muscle. The relatively higher G6PD activities of the heart over the kidney suggest that, more glucose is mobilized in the heart to perform its function than in the kidney. The lowest G6PD activity as observed in the skeletal muscle suggests that, despite its large mass, the

pentose phosphate pathway may not be a major pathway of glucose utilization unlike the glycolytic pathway.

These results suggest that, the mechanism by which PS elicit anti-trypanosome actions may not involve the regulation of the pentose phosphate pathway though it was expected that the presence of sulfadoxine in the combination therapy should have shown an effect on the G6PD activity.

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