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IN VIVO AND *IN VITRO* EFFECTS OF ARTEMISININ GROUP OF DRUGS ON TRYPANOSOMOSIS IN MICE

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ABSTRACT

This study was done to study the effect of artemisinin groups of drugs on mice experimentally infected with the protozoan *Trypanosoma brucei* and on *Trypanosoma brucei* invitro. Commercial artesunate and artemether were used with diminazene aceturate serving as control. It was discovered that artemether administration resulted into seven days of aparasitaemia of *trypanosomosis in vivo* and reduced motility of the trypanosomes *in vitro*. There was synergistic effect in the action of artesunate and diminazene aceturate. *In vitro* analysis gave a similar result in that the trypanosome were found to be sensitive to artemether with an MIC of 0.6µl, artesunate with berenil combination and the artemether treatment group 2.5µM. The implication of these results is discussed and advice is given on the potential adaptation of artesunate for treatment of trypanosomosis and planting of *Artemisia annua* tree in the country.

Key words: Artemether, artesunate, diminazene aceturate, *in vitro* and *in vivo,* trypanosomosis.

INTRODUCTION

Human African Trypanososmosis (HAT), or sleeping sickness, is a disease caused by infection with the protozoan *Trypanosma brucei gambiense* or *Tryapanosoma brucei rhodesciense*, two morphologically identical subspecies of *trypanosome brucei*. The two forms of the disease are transmitted by tsetse flies of the genus *Glossina* (order *Diptera*) and restricted to sub Saharan Africa. (Trouiler *et al.,* 2000).

HAT is a protozoan disease that affects 36 countries in Sub- Saharan Africa. The most recent World Health Organisation prevalence estimates are 50,000- 70,000 cases worldwide, based on a total number of 17,500 new HAT cases per year (WHO, 2006).

The therapeutics currently used in human and cattle African trypanosomosis show a number of lethal or sublethal side effects (Kuzoe, 1993). Moreover the increasing appearance of chemoresistant parasite strains (Mbwambo *et al.,* 1998) necessitates research for new active chemical series (Farrell *et. al.,* 1984, Kinnamon *et al.,* 1989).

Human and livestock are exposed to the risk of infection with trypanosomes in an area of approximately 10x10^{6}km^2 in Sub Saharan

Africa infested with tsetse flies (Leach and Robert, 1981).

Currently chemotherapy and chemoprophylaxis are the main methods of trypanosomosis control. However the increasing appearance of pathogenic trypanosomes that are resistant to the limited number of commercial trypanocides calls for the development of new drugs (Rottcher *et al.,* 1985).

HAT is the prototype of a neglected disease, affecting the poorest people of the poorest continent (Trouiler *et al.,* 2002). The development of new diagnostic tests and drugs has been severely affected by this neglect. (Francois *et al.,* 2005).

Artemisinin, a peroxide-containing sesquiterpene lactone isolated from the herb *Artemisia annua,* has been found to possess potent antimalaria activity and low toxicity both in animals and humans (China cooperative Research group on Quinghaosu and its derivatives as antimalarias, 1982).

Artemisinin was originally developed in 1972 in China (Chinese Institute of material medicine) from the plant *Artemisia annua* (sweetworm wood), a sesquiterpene lactone (empirical formula $C_{15}H_{22}O_5$). Artemisinin is the active ingredient in qinghao, a Chinese herb tea that have been used for 150 years to treat malaria and haemorrhoids. It grows in the wild in China and has been found to grow in other parts of the world too, though the species may vary a bit. Locally it is prepared as an infusion of the dried leaves. (China cooperative Research group on Qinghaosu and its derivatives as antimalarias, 1982).

Because of their low solubility in either water or oil and the short plasma half-life of artemisinin, artesunate and artemether have been studied, in particular, sodium artesunate (Benakis *et al.,* 1997).

All artemisinin derivatives are metabolized to an active metabolite: dihydroartemisinin, whose half-life is 2 hours compared with the 45 minutes half life of artesunate (Bich, *et al.,* 1996).

Artesunate has now been widely used for years with great success for the treatment of malaria, and its combination with other drugs has been strongly promoted by the world health organization (WHO) in sub-Saharan Africa because of high efficacy and excellent tolerability. (McIntosh and Olliaro, 1998).

In general, the endoperoxides have several advantages over existing antimalarial drugs, firstly there is little or no cross resistance with other antimalarial drugs. Secondly, the endoperoxides clear the peripheral blood of malaria parasites more rapidly than other available drugs do. Finally resistance to the endoperoxides has not yet developed despite widespread clinical use. (White, 1994).

This study is necessary because of increased need for alternative therapy in the treatment of both human and animal trypanosome. It becomes imperative to develop a drug that is cheap and easily available and administered with short course of administration and to which resistance has not occurred. Therefore, this study is aimed at evaluating the activity of different artemisinin group of drugs against *Trypanosoma brucei* both *in vivo* and *in vitro* with a view to comparing the effectiveness of artemisinin group of drugs using *in vivo* and *in vitro* assay methods.

MATERIALS AND METHODS

Source of parasite

Rats that have been infected with *Trypanosoma brucei* (Lafia strain) were obtained from the Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ibadan. Blood from the infected rats was used to infect mice after five days at a parasitaemia level of about $3X10^3$ parasites/ml of blood.

Determination of parasitaemia

Parasitaemia was monitored using blood obtained from the tail, presterilized with methylated spirit for both rats and mice. The number of parasites was determined microscopically using the rapid matching method of Herbert and Lumsden (1976); microscopic counting of parasites in blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2).

In vivo test

Forty mice weighing between 25-35g obtained from the Experimental Animal Unit of the Department of Veterinary Physiology and Pharmacology, University of Ibadan were kept in wooden boxes, stabilized for two weeks and fed with commercial growers ration gotten from a local feed mill in Abeokuta and water was provided *ad libitum*. The mice were separated into 5 groups (A, B, C, D and E) each having 8 mice and were stabilized for about 2 weeks before they were infected with Trypanosomes. Each of the mice was given $1 X 10^3$ parasites intraperitoneally in 0.2 ml blood/PBS solution.

They were left for 5days to develop parasitaemia.

After 7 days when parasitaemia was established, the mice in were treated. The following drugs were used; artesunate, 50mg Artesunat® a commercial artesunate in tablets made by Mekophar chemical Pharmaceutical Joint stock Company, Artemether, Gvither® (artemether injection 80mg/1ml ampoule) a commercial brand of artemether marketed by Greenlife pharmaceuticals limited No 2, Banklane Ilupeju, Lagos Nigeria was used. Diminazene aceturate *Diminal® (445mg diminazene diaceurate*+ 555 mg *phenazone/g*, by Eagle Chemical Company LTD, Ikeja , Nigeria.

- Group A was treated with diminazene aceturate at a dose of 3.5mg/kg given intramuscularly,
- Group B was given artesunate at a dose of 100mg on the first day and 50mg for the next five days orally using an oral intubator;
- Group C was treated with artesunate per os and diminazene aceturate intramuscularly at 50mg/kg on the first day and 25mg/kg for the next four days and 1.75mg/kg respectively,
- Group D with artemether at a dose of 3.2 mg/kg on the first day and 1.6 mg/kg for the next four days intramuscularly and group E were infected but not treated to serve as control.

In vitro test

Blood sample was taken from infected rat with a parasitaemia of about $3X10^3$ trypanosome/ml (Herbert and Lumsden 1976) into an ethylene diamine tetra acetic acid (EDTA) containing bottle, 5µl blood was pipetted into each of the 96 well of microtitre plate, 10µl phosphate buffer saline (PBS) was added to aid the survival of the trypanosomes.

Trypanocidal activity was tested for in duplicates in 96 well micro titer plates (Flow laboratories Inc., McLean, Virginia 22101, USA).

Fifteen microlitre of blood containing about 20-25 parasites per field obtained as described above was mixed with 5 µl of the tested drugs.

Artesunate was thoroughly mixed in distilled water before usage. Artesunate was mixed with diminazene aceturate at half their conventional dose for the combination therapy before pouring into the microtitre plate. Reduction in/stoppage of motility under the microscope of trypanosomes in wet mount was taken as effective treatment for the infection.

To ensure that the effect monitored was that of the drug tested alone, a set of control was included which contained the parasite suspended in PBS only.

For standard control, test was also performed with the same concentrations of *Diminal® (445mg diminazene diaceurate*+ 555 mg *phenazone/g*, Eagle Chemical Company LTD, Ikeja, Nigeria).

The microtitre plate was incubated for five minutes at 37ºC in an incubator. After incubation few drops of the mixture in each well were examined under the microscope to check for motility and to determine the degree of the motility. Motility was assessed for two hours after which there was no movement. Whereas the trypanosome survived for 4-5 hours in the control well in which no drug was added.

RESULT

Clinical Signs: The infected mice were weak, anorexic and emaciated with the skin coat appearing rough and starry. There was also paleness of the mucous membrane of the eyes in groups A-D.

Parasitaemia: The parasites were detected in the peripheral blood 5 days post infection (dpi). The initial appearance of the trypanosomes in the blood was followed by a rapid increase in the degree of parasitaemia in the mice. The parasitaemia rapidly increased during the acute phase of the infection and fluctuated during the remaining course of infection (Fig. 1)

At three days post treatment there was reduction in activity of the trypanosomes in three groups namely: diminazene aceturate group, artemether group, artesunate and diminazene group.

Five days after treatment there was complete clearance of parasite from three groups: diminazene aceturate group, artemether group, artesunate and diminazene group.

Seven days post treatment reduction was noticed in the other two groups (artesunate and control groups) but the control group started dying afterwards.

After 7days, complete clearance was established in three groups viz: diminazene aceturate group, artemether group, artesunate and diminazene group.

Clinical signs such as dullness and paleness of mucous membrane disappeared in the infected and treated groups.

The *in vitro* trypanocidal action of artemissinin group is as presented in Table 2.

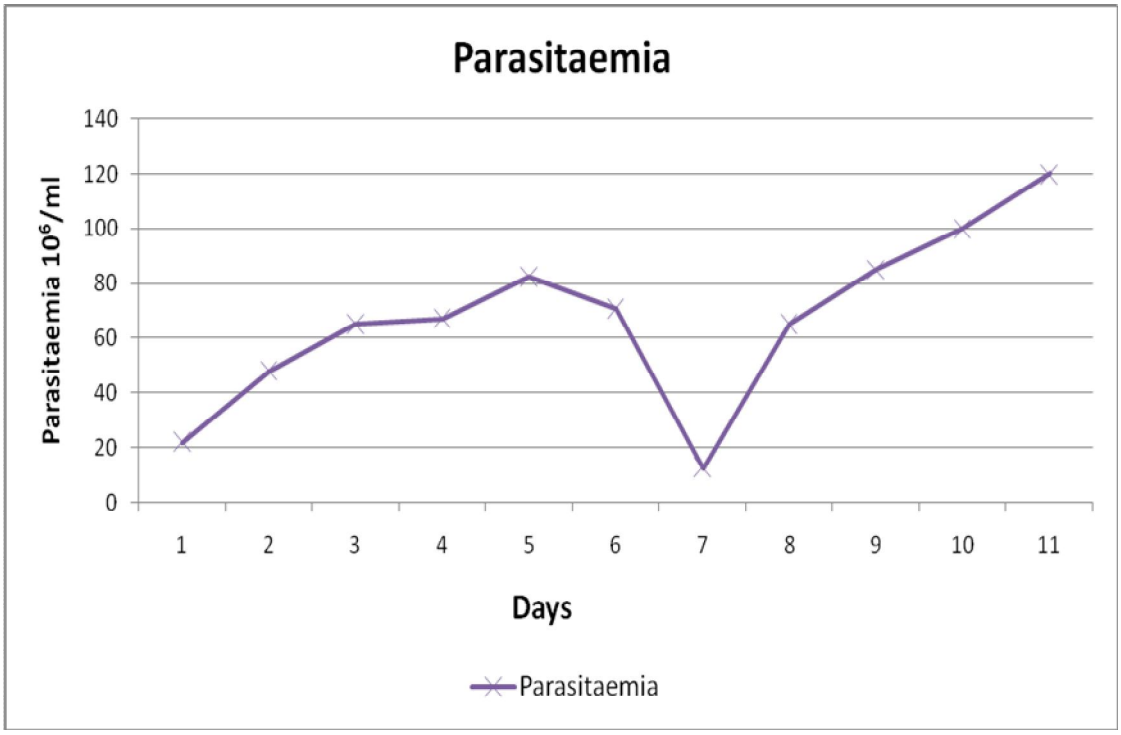

Figure 1: Graph showing the degree of parasitaemia in infected mice

Table 1: In vivo analysis of artemisinin group on trypanosomosis in mice

TREATMENT GROUPS	PARASITAEMIA DIMINAZENE	NUMBER OF MICE TREATED	DIED	RECOVERED	SACRIFICED
ACETURATE	7.8	8	0	8	8
ARTESUNATE	7.8	8	1	0	7
ARTEMETHER	7.8	8	0	8	8
ARTESUNATE & DIMINAZENE ACETURATE	7.8	8	2	6	6
CONTROL (UNTREATED)	7.8	8	4	0	4

Table 2: In vitro Analysis of Artesmisinin Group on Trypanosomosis

DRUG & CONCENTRATION MINUTES	SURVIVAL OF TRYPANOSOMES IN						
	0	30	60	120	180	240	300
DIMINAZENE ACETURATE	+++	+++	++	-ve	-ve	-ve	-ve
ARTESUNATE	+++	+++	+++	++	-ve	-ve	-ve
ARTEMETHER	+++	+++	++				
ARTESUNATE & DIMINAZENE ACETURATE	+++	+++	++	-ve	-ve	-ve	-ve
CONTROL (UNTREATED)	+++	+++	+++	+++	+++	-ve	-ve

LEGEND:
+++: HIGH PARASITAEMIA
++: MODERATE PARASITAEMIA
+: LOW PARASITAEMIA
-VE: NO PARASITAEMIA

DISCUSSION

A total of forty mice were used in the *in vivo* analysis from which it was discovered that artemether given at the conventional dose of 3.2mg stat was found to be effective in the treatment of *Trypanosoma brucei* infection.

In vitro effectiveness of artemether is in line with the discovery of Yuliya *et al.* (2007). The effectiveness observed with artesunate and diminazene aceturate combination in this study could be due to a synergistic effect earlier shown by Biobaku *et al.* (2008).

Artesunate not been effective in the in vivo analysis might be due to oral absorption prevention by the dry feed with which the mice were fed.

The effectiveness of diminazene aceturate still support the fact that it is still an effective trypanocide and still possess high potency when used correctly.

The group treated with artemether at 3.2mg/kg stat and 1.6mg/kg/day intramuscularly for another four days showed reduction in activity of the trypanosomes and complete clearance of parasite at three and five days after the commencement of treatment. All the mice became active again and were all sacrificed. This was the same thing that was observed in the diminazene aceturate group which is the current available treatment for Trypanosomosis. Thus, it can be concluded that intramuscular artemether given at above stated dose is effective against trypanosomes and the potency is comparable to that of diminazene aceturate.

The group treated with artesunate at 100mg stat and 50mg daily for five days with an oral intubator did not show reduction in activity or complete clearance at three and five days

after the commencement of treatment. Reduction in the activity of the trypanosomes in this group was noticed at seven day after the commencement of treatment in this group. This was also noticed in the control group. It can be concluded that artesunate given at 50mg/kg dose orally is not effective against Trypanosomoses. The fact that one mouse in the artesunate treated group died as against four in the control group could mean that artesunate has some potency against trypanosomes. The route of administration is another thing to consider as artesunate was the only agent given orally knowing fully well that bioavailability of most drugs is higher when given parentarally than orally. The dry feed could have also reduced the absorption of artesunate.

Further study using either higher dose orally or a parenteral route needed to be done to validate this. It has also being said that the absorption of artemether is fat dependent which might also be responsible for its effect.

The group treated with artesunate and diminazene aceturate in half doses of the individual drug also showed reduction in activity of the trypanosome and complete clearance at three and five days after the commencement of treatment. This could mean that diminazene aceturate given in half dose is as potent as the current standard dose when supplemented with drugs that have antitrypanosomal property. It could also mean that artesunate have a synergestic effect with the diminazene aceturate. These would need further study. The fact that two mice in this group as against none in the diminazene aceturate treated group were aparasitaemic for 7days could mean that the combined drugs in half doses are not as effective as diminazene aceturate alone given in full dose.

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