THE EFFECTS OF SINGLE AND COMBINED CHEMOTHERAPY OF DL-Á-
DIFLUOROMETHYLORNITHINE AND DIMINAZENE ACETURATE IN EXPERIMENTAL
TRYPANOSOMA BRUCEI GAMIENSE INFECTION IN MICE

MBAYA*, A.W, ALIYU*, M.M and GAMBA†, B.

*Department of Veterinary Microbiology and Parasitology, †Department of Veterinary Medicine,
University of Maiduguri, P.M.B. 1069, Maiduguri, Borno state, Nigeria

*Correspondence: E-mail: awmbaya@yahoo.com, Tel: +234 8036011774

SUMMARY

The chemotherapeutic effects of DL-á-difluoromethylornithine (DFMO) and diminazene aceturate in Trypanosoma brucei gambiense infected mice were studied. All the infected mice developed parasitaemia 4 days post infection (P.I.). Weakness, increased respiration, rough hair coat, pallor of pinnae, snout and footpads were the major clinical signs observed in the acute phase of the disease. Terminally, there was progressive weight loss and hypsornia (somnolence) which occurred from day 22 P.I. Hepatomegaly, splenomegaly and atrophy of body fats were the major necropsy findings. All treatments commenced at the onset of parasitaemia by day 4 P.I. DFMO at 400mg/kg body weight was administered orally as a 4% concentration in Group B or in combination with diminazene aceturate at a single standard dosage of 3.5mg/kg body weight in Group C, caused a significant amelioration of all the clinical signs. There was a significant (P<0.05) decline in mean packed cell volume (PCV) but the decline in mean white blood cell counts (WBC) was not significant (P>0.05). The combined therapy of 4% DFMO and diminazene aceturate resulted in a faster attainment of PCV, WBC pre-infection values and the disappearance of the parasites from circulation than the other treatment regimes.

KEY WORDS: Chemotherapy, DL-á-difluoromethylornithine, Diminazene aceturate,
Trypanosoma brucei gambiense, Mice.

MATERIALS AND METHODS

Experimental animals
Thirty (30) adult (Balb/c) albino mice of both sexes weighing between 32-50g and obtained from the Department of Biochemistry, University of Maiduguri were used for the study. The mice were maintained on a standard diet (ECWA Feeds Ltd., Jos) and housed in clean plastic cages maintained at room temperature in the Laboratory of the Department of Veterinary Microbiology and Parasitology, University of Maiduguri. Clean water was provided ad libitum. They were allowed to acclimatize to their new environment for 14 days before the commencement of the experiment.

Source of trypanosomes and experimental drugs

Trypanosoma brucei gambiense strain (NITR/Abraka) was obtained from the Nigeria Institute for Trypanosomosis Research (NITR), Vom, Nigeria. The isolates were confirmed to be T.b. gambiense after they were subjected to the
negative Serum Incubation and Infectivity Test (SIT) (Kaguraka et al., 1988; Owen and Gillet, 1992). The parasites were passaged serially in donor rats. The experimental mice were infected with blood from the donor rats containing 1.5 x 10⁷/μl of T.b. gambiensense. Blood samples were diluted with phosphate-buffered glucose saline (pH 7.2). Twenty-five mice (25) were infected with the parasite intraperitoneally while the remaining five served as uninfected control. DL-α-difluoromethylornithine (DFMO) was obtained from Merrill Dow Research Institute Ohio, USA, in a white crystalline form, while diminazene aceturate (Berenil®) was obtained from Hoechst, Farbwerke, Germany.

**Experimental protocol**

The mice were randomly divided into 6 groups (A, B, C, D, E and F) of 5 mice each. Group A was treated with 2% solution of DFMO orally for 4 consecutive days starting from day 4 P.I. starting at the onset of parasitaemia. Group B was treated with 4% solution of DFMO for the same number of days starting at the onset of parasitaemia. Group C was treated with a combination of 4% solution of DFMO orally for 4 consecutive days and 3.5 mg/kg of diminazene aceturate as a single standard dose intraperitoneally from day 4 P.I. Group D was treated with 5.5 mg/kg of diminazene aceturate alone as a single standard dose intraperitoneally starting at the onset of parasitaemia from day 4 P.I. Groups E and F on the other hand served as infected and uninfected controls, respectively.

Estimation of parasitaemia, haematology and necropsy Parasitaemia was detected by the wet mount and buffy coat microscopy, thereafter it was estimated every 4 days by the rapid matching technique (Herbert and Lumsden, 1976). The packed cell volume (PCV) of the tail blood of the mice was determined by the microhaematocrit method, while the white blood cell counts (WBC) was determined by the Neubauer counting method every 4 days (Schalm et al., 1995). Dead mice and those routinely sacrificed at the end of the study were subjected to necropsy while the liver and spleen were carefully removed, washed and weighed using Metler’s electronic weighing balance (Hawksley, England).

**Statistical analysis**

The data obtained were analyzed using two-way analysis of variance (ANOVA) to detect significant differences between groups in tested parameters at 95% confidence limit (Maed and Curnow, 1983).

**RESULTS**

The clinical signs observed were weakness, rough hair coat, pallor of ears, snout and foot pads. These were, however, more pronounced during the first wave of parasitaemia for groups B, C, D and E, while group A experienced the same in the first wave and during relapsed parasitaemia. Hypersomnia (somnolence) was characteristically observed from day 22 P.I. These symptoms however, became less pronounced in Group C, treated with a combination of 4% DFMO and 3.5 mg/kg of diminazene aceturate. The pre-patent period for the infected groups ranged from 4 - 6 days.

The parasite scores of the mice treated with either DFMO or diminazene aceturate or its combinations is presented in Figure 1. The maximum survival period for the infected and untreated controls was 20 days, while those in the other groups did not manifest any death during the study period. A relapsed parasitaemia was however noticed by day 26 P.I. with a count of 5.5 x 10⁷/μl in the group treated with 2% DFMO. Detectable parasitaemia for all groups was 5.5 x 10⁷ / μl and it rose sharply to a peak of 25.5 x 10⁷/μl by day 10 P.I. but later declined sharply in all treatment groups. Parasitaemia reached a mean peak value of 50.0 x 10⁷/μl by day 12 and was maintained till day 16 P.I. and in the infected but untreated group it lead to the death of all mice.

Following infection, the PCV values showed
sharp decline in comparison to that of the uninfected controls, corresponding to the first wave of parasitaemia (Fig.2). However, those treated with either single or combined treatment experienced increase to pre-infection levels of their mean PCV values by day 28 P.I. Meanwhile, the infected but untreated control group which had a significant (P<0.05) drop in PCV value by day 18 P.I. with all the mice dead within the period. The decline in mean PCV between groups was most pronounced in the group treated with 2% DFMO due to relapsed parasitaemia encountered by day 20 P.I. The PCV of the healthy control remained relatively constant throughout the study period. All groups experienced minor fluctuations in WBC values which were not statistically (P>0.05) significant, except in the infected but untreated group, which experienced a significant (P<0.05) decline in mean WBC values. The WBC counts of the uninfected control, however, remained relatively constant throughout the period of the study (Fig.3). The weights of the liver and spleen of mice that died as a result of the infection or those routinely sacrificed were more in the infected but untreated control than in those treated. Among the treated groups, however, the mean weights of the liver and spleen of those treated with a combination of both drugs was much lighter, followed by those treated with 4% and 2% DFMO respectively (Table 1).

**Fig. 1:**

**Keys:**

DFMO = DL-4-difluoromethylornithine
Combin. = Combination of 4% DFMO and Berenil
Inf. & Untr. = Infected and Untreated control
Uninf. Cont. = Uninfected control
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FIGURE 2: Packed cell volume (%) of mice infected with *Trypanosoma brucei gambiense* and treated with either DFMO, Berenil® or their combination

**Keys:**
- DFMO = Dl-4-difluoromethylornithine
- Combin. = Combination of 4% DFMO and Berenil®
- Inf. & Untr. = Infected and Untreated control
- Uninf. Cont. = Uninfected control

FIGURE 3: White blood cell count (X10³/µl) of mice infected with *Trypanosoma brucei gambiense* and treated with either DFMO, Berenil® or their combination

**Keys:**
- DFMO = Dl-4-difluoromethylornithine
- Combin. = Combination of 4% DFMO and Berenil®
- Inf. & Untr. = Infected and Untreated control
- Uninf. Cont. = Uninfected control
TABLE 1: Mean (± S.D) liver and spleen weights (g/100g body weight) of mice infected with Trypanosoma brucei gambiense and treated singly with various concentrations of DL-α-difluoromethylornithine or in combination with diminazene aceturate with their controls.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Liver weight (g/100g body weight)</th>
<th>Spleen weight (g/100g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DFMO 2%</td>
<td>(n = 5) 6.4 ± 0.7a (3.2)*</td>
<td>4.0 ± 0.3b (2.2)*</td>
</tr>
<tr>
<td>B</td>
<td>DFMO 4%</td>
<td>(n = 5) 4.6 ± 0.3b (2.2)*</td>
<td>2.0 ± 0.3b (1.0)*</td>
</tr>
<tr>
<td>C</td>
<td>Combination</td>
<td>(n = 5) 3.0 ± 0.2c (1.0)*</td>
<td>1.2 ± 0.2c</td>
</tr>
<tr>
<td>D</td>
<td>Berenil®</td>
<td>(n = 5) 4.7 ± 0.3b (2.2)*</td>
<td>2.2 ± 0.3c (1.0)*</td>
</tr>
<tr>
<td>E</td>
<td>Infected control</td>
<td>(n = 5) 10.1 ± 0.8d (4.4)*</td>
<td>4.8 ± 0.3c (2.3)*</td>
</tr>
<tr>
<td>F</td>
<td>Uninfected control</td>
<td>(n = 5) 2.8 ± 0.3</td>
<td>1.2 ± 0.2c</td>
</tr>
</tbody>
</table>

n = number of rats in each group; * = number of times organ weights increased

\*a,b,c Values in columns with different superscripts differed significantly (P<0.05)

DFMO = difluoromethylornithine

DISCUSSION

Experimental infection of albino mice with virulent strain of Trypanosoma brucei gambiense led to anaemia demonstrated by low packed cell volume (PCV) and pallor of ears, snout and, which feet were the predominant symptoms encountered. Anaemia is a consistent feature of human trypanosomosis due to T. brucei gambiense (Damian et al., 1994; Radomski and Buguet, 1995; Hepburn et al., 1995), which is mainly haemolytic (Anosa, 1988). The virulent course of the T.b. gambiense strain in the mice differed from the typically chronic nature of the disease in man (Scott, 1970), but mimicked the course of an experimental T.b. brucei in animals (Losos and Ikede, 1972). The observation, however, confirmed the existence of a typical type II of T.b. gambiense with the resultant T.b. rhodiense-like syndrome in man (WHO, 1998; Abenga and Anosa, 2004). Secondly, the virulence of this strain might have been further enhanced by serial passages prior to sub-inoculation.

Serial passages have been shown to enhance virulence of the T. brucei sub-group (Mbaya et al., 2007). Such virulent course by the parasite leading to human sleeping sickness had been reported in Gboko, Nigeria (Emiribe, 1988) and has been described in an outbreak in Abraka, Nigeria (Enwezor and Ukah, 2000).

Treatment of the mice with 2% concentration of DFMO experienced relapse parasitaemia in contrast to those treated with 4% concentration. This shows that DFMO is dose - dependent as mice given the higher concentration of the compound singly and in combinations with Berenil® demonstrated greater and faster parasite clearance and subsequent reversal of clinical signs. Graded dose response has been reported as a consistent feature of DFMO in the treatment of coccidiosis of chickens (Jibike et al., 2002) and in animal trypanosomosis (Jibike et al., 1995). T.b. gambiense metabolizes glucose
to produce 4-hydroxy-4-methyl a-ketoglurate, which is inhibitory to the tricarboxylic acid cycle in the mitochondria and also destroys blood glucose due to aerobic glycolysis (Igbokwe, 1994). These might have been responsible for the profound body weakness encountered in the infected mice. The relapse parasitaemia encountered with 2% concentration of the compound is an indication of central nervous system involvement in the disease process. Trypanosomes sometimes evade the action of trypanocidal agents because the drug molecules are too large to cross the blood brain barrier in sufficient quantity to be curative (Jennings, 1991).

Hypersomnia in the mice is a cardinal sign encountered in human African trypanosomiasis. This occurrence in humans (Damian et al., 1994; Hepburn et al., 1995) and in primates experimentally infected with T.b. gambiense (Abenga and Anosa, 2004) has been attributed to the meningitis, which occurs during early infections.

The final stage in the pathogenesis is the breakdown of the choroidal plexus thereby compromising the blood brain barrier (Pantreath, 1995) with a subsequent movement of the parasites into localized areas of the brain. This was authenticated during the course of the infection when brain homogenates of a relapsed albino mouse injected intraperitoneally into a recipient albino rats produced parasitaemia after 4-8 days post-inoculation. This suggests that the brain in the later stages of the infection harboured the trypanosomes. Homogenates of the spleen, liver, kidney, heart and lymph nodes at this stage were however, non-infective. This is consistent with earlier reports in dogs infected with T. brucei (Chukwu et al., 1990).

The liver and spleen were enlarged in the positive control. This observation agrees with the findings of Anosa (1988). The degree of enlargement of these organs might have contributed to the more severe anaemia experienced by this group than their counterparts. Enlarged liver and spleen have been reported to contribute significantly to the level of the anaemia in African animal trypanosomosis (Anosa, 1988; Hepburn et al., 1995), through increased erythropagocytosis in the spleen and liver (Anosa, 1988) and also due to the formation and enlargement of germinal centres, proliferation of plasma cells and macrophages and accumulation of oedema fluid (Ikede, 1981). The combined therapy of 4% DFMO and diminazene aceturate was found to be more effective in the treatment of T. brucei gambiense with a faster attainment of pre-infection levels of haematological indices than all the other treatment regimes.

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