EFFICACY OF REPEAT AND COMBINATION TREATMENT WITH DIMINAZENE ACETURATE AND/OR ISOMETAMIDIUM CHLORIDE AGAINST DRUG RESISTANT *TRYPANOSOMA CONGOLENSE*

R.S. Silayo and Fortunata V. Silayo *Department of Veterinary Microbiology & Parasitology, Sokoine University of Agriculture, P.O. Box 3019, CHUO KIKUU, MOROGORO*

SUMMARY

Mice infected with *Trypanosoma congolense* "ADRI" previously shown to be resistant to diminazene aceturate (70 mg/kg) and isometamidium chloride (20 mg/kg), were treated at four days post – infection with either drug at doses of 21 mg/kg or 42 mg/kg and 10 mg/kg or 20 mg/kg intraperitoneally, respectively, and treatment repeated at 48 hours interval with the same drug or the alternative drug. Similarly, infected mice were treated with single doses of diminazene aceturate (56 – 75 mg/kg) and isometamidium chloride (10 – 22 mg/kg). For each repeat and combination treatment there was a corresponding treatment of uninfected mice to check on suspected toxicity of short – interval trypanocidal drug treatment. Single dose treatment with diminazene aceturate (56 – 70 mg/kg) resulted in only two out of 31 mice (6.5%) being cured while treatment with isometamidium chloride (10 – 22 mg/kg) at a single dose resulted in 10 out of 38 animals (26%) being cured. In contrast, eight out of 10 animals (80%) which received two doses of diminazene aceturate (21 or 42 mg/kg) were cured while all five mice (100%) which received two doses of isometamidium chloride 10 mg/kg were cured. In combination drug treatment involving diminazene aceturate 42 mg/kg or 21 mg/kg and isometamidium chloride 20 mg/kg or 10 mg/kg, 23 out of 24 animals (96%)were cured. The efficacy of diminazene aceturate and isometamidium chloride given in two repeat doses compared to single dose treatment were significantly greater ($P < 0.001$). Similarly the greater efficacy of combination treatment as compared to single dose treatment was highly significant ($P < 0.001$). There was no evidence of toxicity following repeat or combination treatment. Single doses of diminazene aceturate greater than 70 mg/kg were highly toxic to mice. At a dose of 75 mg/kg, three out of five mice died within two hours of treatment. These results demonstrated that a two dose treatment at 48 hours interval or drug combination is highly efficacious against trypanosome infections resistant to therapeutic single doses of diminazene aceturate and isometamidium chloride.

INTRODUCTION

Development of trypanosome drug resistance is among the most serious problems associated with the use of trypanocidal drugs for the control of African animal trypanosomiasis, undoubtedly one of the most important diseases hindering livestock development in Sub-Saharan Africa. At least five drugs have been in wide scale field application but only two, namely diminazene aceturate and isometamidium chloride remain reliably efficacious (Williamson, 1976; Anon, 1979; Holmes and Scott, 1982). Even with these two drugs there are increasing field reports of development of drug resistance in both West and East African strains of *T. congolense* and *T. vivax*. (Jones –Davies, 1967, 1968; Mwambu and Mayende 1971;

Njau, *et al*; 1983; Pinder and Authie, 1984; Mbwambo, *et al*, 1988).

Despite the magnitude of the animal trypanosomiasis problem in Africa, prospects for availability of a new trypanocidal drug for field use in the near future are meager because of increasing drug development costs in face of diminishing drug purchasing powers of the affected countries (Williamson, 1976; Anon, 1979; Holmes and Scott, 1982; Williamson, March and Scott – Finnigan, 1982). Therefore there has been a need to study new drug application regimes utilizing presently available drugs to combat development of drug resistance.

The presently used single dose therapy in treatment of African animal trypanosomiasis is suspected to partly cause reduced trypanocidal efficacy. Recent studies in goats (Silayo, Aliu, Mamman, Peregrine and Moloo, in press) have shown that efficacy of diminazene aceturate treatment of animals infected with a drug resistant *T. congolense* was improved by two-dose treatment at 8 and 24 hour intervals, compared to single dose treatment. Other studies in mice infected with a different stock of drug resistant *T. congolense* revealed that with a treatment interval of four days, repeat treatment with isometamidium chloride and spaced diminazene-isometamidium treatment was highly efficacious while repeat treatment with diminazene aceturate was not (Silayo and Lekaki, 1989). Both studies indicated that the treatment interval was critical in determining the efficacy achieved by the repeat or combined drug treatment, and it was suggested that this could be correlated with the duration of plasma therapeutic drug concentrations and the time it takes to reduce parasitaemia to subpatent levels following the first treatment.

Based on previous observations that trypanosomes, even of known diminazene aceturate resistant *T. congolense* tend to become subpatent within 48 hours of treatment, we decided to examine the relative efficacy of two-doses of either diminazene aceturate or isometamidium chloride given at an interval of 48 hours and that of combining these drugs.

MATERIALS AND METHODS

Animals

Random – bred, female, Swiss-white mice 6 to 10 weeks old weighing 20 – 40g were used for trypanosome maintenance and drug sensitivity tests. They were individually identified with picric acid markings and kept in plastic cages with wood shavings as bedding. Mice meal (NMC, Dar es Salaam) and water were provided *ad libitum*.

Trypanosomes

T. congolense "ADRI" used in the present studies was an uncloned stock obtained from Animal Disease Research Institute (ADRI) Temeke, Dar es Salaam and has been described elsewhere as *T. congolense* Kibaha (Mbwambo *et al*, 1988). It has been shown to be highly resistant to diminazene aceturate, the minimum curative dose (MCD_{80}) of which was greater than 70 mg/kg in mice (Silayo and Marandu, 1989). The drug resistance was stable over prolonged serial passage (Silayo and Marandu 1989; Silayo and Lekaki, 1989).

Mouse Inoculation and Monitoring of Parasitaemia

For drug sensitivity tests, mice were inoculated intraperitoneally with

approximately 2 x 10^5 trypanosomes in mouse blood diluted with phosphate buffered saline glucose (pH 8.0).
The mice were randomly allocated from a large cage into treatment groups. Parasitaemia was monitored by wet film microscopic examination of tail blood and at least 20 high power fields (x 400) were examined before declaring a sample negative. Mice were examined daily from two days pre-treatment to seven days post-treatment and then twice a week up to day 70 after first treatment. Mice which became aparasitaemic following treatment and remained so for 70 days after first treatment were declared cured.

Drugs

Diminazene aceturate (BERENIL®, Hoechst Ag Frankfurt, Germany) and isometamidium chloride (SAMORIN®, RMB Dagenham, England) were used. Solutions of each drug were prepared freshly using sterile distilled water at room temperature. Dilutions prepared were such that a mouse weighing 20 g would receive 0.20 ml of drug solution.

Experimental design

The experiment was carried out in two parts: Part one consisted of single dose treatments to establish whether *T. congolense* "ADRI" was indeed still resistant to diminazene aceturate and isometamidium chloride, and part two comprised repeat and combination treatment. Drug solutions were administered intraperitoneally into mice in groups of five. Single dose diminazene aceturate treatments were 56 mg/kg, 60 -70 mg/kg (at 2 mg/kg intervals) and 75 mg/kg. Isometamidium chloride single dose treatments were 10 -22 mg/kg (at 2 mg/kg intervals). Repeat and combination treatments chosen were B - B, BB - BB, B - S, BB - S, S - S, S - B, S - BB whereby BB and B represent diminazene aceturate 42 mg/kg and 21 mg/kg, respectively, and SS, S represent isometamidium chloride 20 mg/kg and 10 mg/kg, respectively. Initial treatment was carried out at day 4 post-infection and the second treatment in the case of repeat or combination treatments was carried out 48 hours later, following previous observation that parasitaemia was generally subpatent 48 hours after treatment with diminazene aceturate. For each repeat or combination treatment of infected mice, a corresponding treatment of uninfected mice was carried out to check on suspected toxicity of short-interval trypanocidal drug treatment. Untreated controls were included both for infected and uninfected mice.

Statistical analysis

Chi-square test was used to test the significance of cure rate differences between single dose, repeat and combination drug treatments. The two by two contingency tables were constructed using cumulative data from all single dose treatments (≥ 56 mg/kg for diminazene and ≥ 10mg/kg for isometamidium chloride), all repeat treatments for each drug and all the combination drug treatments.

RESULTS

The sensitivities of *T. congolense* "ADRI" to single doses of diminazene aceturate at doses of 56 - 75 mg/kg and isometamidium chloride at doses of 10 - 22 mg/kg are shown in Tables 1 and 2.

In the diminazene aceturate treated animals, parasitaemia was cleared in all mice by day 2 post-treatment (Table 1). Only two mice, one in each of the groups treated at doses of 68 and 70 mg/kg were cured. All mice in

Table 1: **Sensitivity of *T. congolense* "ADRI" (number of parasitaemic mice/total treated) to treatment with different doses of diminazene aceturate administered at day 4 post-infection in mice.**

	Dose (mg/kg) Number of parasitaemic mice/total treated								
Days after treatment	0.00*	56.0	60.0	62.0	64.0	66.0	68.0	70.0	75.0
0	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
1	5/5	1/5	1/5	3/5	4/5	5/5	4/5	2/5	2/2
2	5/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	–
3	5/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	–
7	5/5	3/5	1/5	2/5	3/4	2/5	0/5	1/4	–
14	5/5	4/5	4/4	5/5	4/4	5/5	3/4	3/4	–
70	5/5	5/5	4/4	5/5	4/4	5/5	3/4	3/4	–

* Untreated control

Table 2: **Sensitivity of *T. congolense* "ADRI" (number of parasitaemic mice/total treated) to treatment with different doses of isometamidium chloride administered at day 4 post-infection in mice.**

	Drug dose (mg/kg) Number of parasitaemic mice/total treated							
Days post Treatment	0.00*	10.0	12.0	14.0	16.0	18.0	20.0	22.0
0	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
1	5/5	3/5	4/5	5/5	5/5	5/5	3/5	4/5
2	5/5	2/5	0/5	5/5	0/5	1/5	1/5	2/5
3	5/5	2/5	1/5	0/5	0/5	1/5	1/5	2/5
7	5/5	5/5	5/5	0/5	2/5	3/5	0/5	1/5
14	5/5	5/5	5/5	3/5	3/5	3/5	2/5	1/5
70	5/5	5/5	5/5	4/4	4/5	3/4	2/5	1/5

* Untreated control

the rest of the single dose treatment groups had relapse parasitaemia. In the group treated with diminazene aceturate at a dose of 75 mg/kg, three mice out of five died within two hours of drug treatment, necessitating the removal of the group from the experiment.

In some mice treated with single doses of isometamidium chloride, disappearance of parasitaemia was delayed until 3 – 4 days after treatment. All mice treated at a dose

Table 3: Sensitivity of *T. congolense* "ADRI" (number of parasitaemic mice/ total treated) to treatment with two doses of either diminazene aceturate or isometamidium chloride and their combination at days 4 and 6 post-infection in mice.

	Drug treatment Number of parasitaemic mice/total treated									
	0.00*	B-B	BB-BB	S-S	SS	B-S	S-B	BB-S	S-BB	SS-BB
0	5/5	5/5	3/5	5/5	3/5	5/5	5/5	5/5	5/5	4/5
1	5/5	4/5	1/5	5/5	4/5	5/5	4/5	5/5	5/5	5/5
2	5/5	0/5	0/5	2/5	1/5	0/5	0/5	0/5	1/5	0/5
3	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
14	5/5	0/5	0/5	0/5	1/5	1/5	0/4	0/5	0/5	0/5
21	5/5	1/5	0/5	0/5	4/5	1/5	0/4	0/5	0/5	0/5
70	5/5	1/5	1/5	0/5	4/5	1/5	0/4	0/5	0/5	0/5

* Untreated Control
BB = Diminazene aceturate 42mg/kg B = Diminazene aceturate 21mg/kg
SS = Isometamidium chloride 20mg/kg S = Isometamidium chloride10mg/kg
DPT = Days post-treatment

Table 4: Survival of uninfected mice (number surviving/initial total) following short-interval two-doses of either diminazene aceturate or isometamidium chloride and either combination.

	Drug treatment Number of mice surviving/initial total									
DPT	0.00*	B-B	BB-BB	S-S	SS	B-S	S-B	BB-S	S-BB	SS-BB
0	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5
1	5/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5
2	5/5	5/5	4/5	5/5	5/5	5/5	5/5	4/5	5/5	0/5
3	5/5	5/5	3/5	5/5	5/5	5/5	5/5	3/5	4/5	0/5
14	5/5	5/5	3/5	5/5	5/5	5/5	5/5	3/5	4/5	0/5
70	5/5	5/5	3/5	5/5	5/5	5/5	5/5	3/5	4/5	0/5

* Untreated control
BB = Diminazene aceturate 42mg/kg B = Diminazene aceturate 21mg/kg
SS = Isometamidium chloride 20mg/kg S = Isometamidium chloride 10mg/kg
DPT = Days post-treatment

Table 5: Chi-square test on efficacy of single dose, versus repeat dose treatment

Drug	Regime (dose)	Cured[4]	Not cured	Total	Significance
Diminazene aceturate	a) Single[1]	2	29	31	
	(b) Repeat[2]	8	2	10	P <0.001
	Total	10	31	41	
Isome-tamidium chloride	(c) Single[3]	10	28	38	
	(d) Repeat	5	0	5	P < 0.001
	Total	15	28	43	

1. Cumulative single doses 56 – 70mg/kg (from Table 1)
2. Cumulative repeat doses B and BB (21 and 42 mg/kg) from Table 3
3. Cumulative single doses 10 – 22mg/kg (from Table 2 and 3)
4. Cure = Aparasitaemia for at least 70 days after first treatment

of 10 or 12 mg/kg developed relapse parasitaemia after treatment. At doses of 16, 18, 20 and 22 mg/kg, cure rates were 20, 25, 60 and 80%, respectively. Based on the above results, curative doses CD_{50} and CD_{80} for *T. congolense* "ADRI" in the experiment were 19 mg/kg and 22 mg/kg diminazene aceturate, respectively, using probit analysis (Finney 1971).

The sensitivity of *T. congolense* "ADRI" to two doses and combination treatment with diminazene aceturate and isometamidium chloride is shown in Table 3. Only one mouse in each of the groups treated with two doses of diminazene aceturate (21 or 42 mg/kg) and the group treated with combination of diminazene aceturate (21 mg/kg) followed by isometamidium chloride (10 mg/kg) had relapse parasitaemia. All mice in the rest of the combination and repeat treatment groups were cured.

Treatment at a dose of 20 mg/kg of isometamidium resulted in four out of five mice relapsing.

Table 4 indicates the survival of

uninfected mice following two-dose and combined trypanocidal drug treatment. All uninfected treated mice were in good health throughout except for those in groups BB – BB, BB – S in each of which two out of five mice died and S – BB in which one out of five mice died. Post-mortem examination revealed that deaths were due to trauma inflicted by fighting.

Table 6: Chi-square test on efficacy of single dose versus combination treatment with diminazene aceturate and isometamidium chloride.

Regime (dose)	Cured[4]	Not cured	Total	Significance
(a) Single B[1]	2	29	31	
(e) Combination	23	1	24	P < 0.001
Total	25	30	55	
(c) Single S[3]	10	28	38	P < 0.001
(d) Combination	23	1	24	
Total	33	29	62	
(b) Repeat B[2]	8	2	10	
(e) Combination	23	1	24	P = NS
Total	31	3	34	
(d) Repeat S	5	0	5	
(e) Combination	23	1	24	P = NS
Total	28	1	29	

1. Cumulative single doses 56 – 70 mg/kg (from Table 1)
2. Cumulative repeat doses B and BB (21 and 42 mg/kg) from Table 3
3. Cumulative single doses 10 – 22mg/kg from Tables 2 and 3
4. Cure = Aparasitaemia for at least 70 days after first treatment

NS Not Significant. B = diminazene aceturate
S Isometamidium chloride

Tables 5 & 6 summaries the results in two by two contingency tables for cumulative single dose, repeat dose and combination drug treatment. The difference between cure rates following two-dose and combination drug treatment compared to single dose treatment were significant ($P < 0.001$), while that between two-dose and combination drug treatment was not significant.

DISCUSSION

The results presented here have shown the superior efficacy of 48-hour repeat dose and combination drug treatment compared to single dose treatment in curing drug resistant *T. congolense* infections. It is probable that the time interval is critical in determining efficacy achieved. Previous studies in mice had indicated that when the treatment interval was four days, cure was effected by combination drug treatment and repeat isometamidium chloride treatment but not repeat diminazene aceturate treatments (Silayo and Lekaki, 1989). In goats infected with *T. congolense* IL 3274 (resistant to diminazene aceturate) repeat diminazene aceturate treatment at 8 and 24-hour intervals resulted in 30% cure rate compared to nil for single dose treatments (Silayo *et al.*, in preparation). It is not clear why cure rates obtained following 48 - hour repeat dose and combination drug treatment were significantly higher than single dose treatment cure rates even though the single dose was equal to or larger than the total of the repeat doses. It may be that plasma therapeutic concentrations were maintained for at least 48 hours after the first dose and that the second dose resulted in therapeutic concentrations being maintained for a minimum of four days required for elimination of drug resistant populations. It is also probable that success could be attributed to the fact that the second dose was administered at a time when parasite numbers were minimal, thus increasing chances of eliminating drug resistant individuals with the aid of the immune system. There are studies which have indicated that the efficacy of trypanocidal drug treatment is influenced by the parasite load at the time of treatment at least *in vitro* and in mice (Hawking, 1963; Walker and Opiyo, 1973), although other studies in rabbits and in goats do not support such views (Gilbert, 1983; Peregrine *et al.*, 1988).

On combination drug treatment, it is likely that drug synergism can partly explain the success, as has been suggested by Williamson (1976) for diminazene aceturate and isometamidium chloride which are considered to have different modes of action, since they are absorbed to a different extent by the trypanosome and bind differently to DNA.

It is clear that further studies are needed, firstly, to confirm the applicability of the murine studies to cattle, sheep, goats, equines, pigs, and dogs, and to explain the success. Confirmation is likely to have far reaching impact on trypanocidal application regimes to minimize development of drug resistance. Presently, undue weight is given to labour and drug costs resulting in single dose treatment being practiced. Such repeat dose treatment even in drug susceptible infections would likely apply also especially where the host species such as the dog is sensitive to toxic effects of the full single dose. In the present studies, two doses of diminazene aceturate, 21 mg/kg totalling 42 mg/kg were shown to effect 80% cure compared to 25% cure effected by diminazene aceturate at a single dose of 70 mg/kg.

Mortality suspected to be associated with repeat and combination drug treatment (Silayo and Lekaki, 1989) has not been confirmed although five out of 40 mice that received a repeat dose or the alternative drug died. These were all from groups treated with the higher dose of diminazene aceturate repeated after 48 hours or in combination with isometamidium chloride. There were however, no corresponding mortalities in similarly treated uninfected mice. Although gross post-mortem examination did indicate that the deaths were associated with fighting, there is a need for more studies involving serum enzyme profiles to confirm that the short interval trypanocidal drug treatment is not more toxic than single dose treatment. Such studies would best be carried out in large animals especially in cattle in which field reports have associated close interval dimininazene/ isometamidium treatment with mortalities (Anon, 1967). Also,this would help support or refute manufacturer's recommendation that isometamidium chloride should not be given within a month of application of other trypanocidal drugs.

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REFERENCES

Anon (1967) Department of Veterinary Services Rhodesia. Field Instruction 6/67, April 1967, Harare, Zimbabwe.

Anon (1979). "The African Trypanosomiases" – Report of a joint WHO Expert Committee and FAO expert consultation, Rome 8–12 November,1976. FAO Animal Production and Health Paper No. 14, FAO, Rome.

Finney, D.J. (1971). "Probit analysis" 2nd Edition Cambridge at University Press.Cambridge, England.

Gilbert, R.J. (1983). Studies in rabbits on the disposition and trypanocidal activity of the anti-trypanosomal drug diminazene ceturate. Brit. J. Pharmac. 80: 133–139

Hawking,F. (1963). Chemotherapy of trypanosomiasis. In: "Experimental chemotherapy" by Schnitzer, R.J. and Hawking F (Eds) Academic Press, New York & London pp. 129 – 156.

Holmes, P.H. and Scott, J.M. (1982). Chemotherapy against animal trypanosomiasis In:"Perspectives in Trypanosomiasis Research" J.R. Baker (Ed), Research Studies Press, Chichester & New York, pp. 59 – 69.

Jones – Davies, W.S. (1967). A berenil –resistant strain of *T. vivax* in cattle. Vet. Rec. 18:567–568.

Jones – Davies, W.S. (1968). Berenil resistance in naturally occurring *T. congolense*. Bull. Epiz. Dis. Afr. 16:213–216.

Mbwambo, H.A.; Mella, P.N.P. and Lekaki, K.A. (1988). Berenil (diminazene aceturate) – resistant *Trypanosoma congolense* in cattle under natural tsetse challenge at Kibaha Tanzania. Acta Trop. (Base) 45:239–244.

Mwambu, and Mayende , J.S. (1971). Occurrence of berenil resistant strain of *T. vivax*. Trans. Roy. Soc. Trop. Med. Hyg. 65:254–255.

Njau, B.C.; Mkonyi, P.M. and Kundy, D.J. (1983). Berenil resistant *Trypanosoma congolense* isolated from naturally infected goats in Tanga region Tanzania. In: ISCTRC 17th Meeting 1981, Arusha, Tanzania. OAU/STRC Publ. No. 112 pp. 289 –298.

Peregrine, A.S., Ogunyemi, O., Whitelaw, D.D., Holmes, P.H., Moloo, S.K., Hirumi, H., Urquhart, G.M. and Murray, M. (1988). Factors influencing the duration of isometamidium chloride (samorin) prophylaxis against experimental challenge with metacyclic forms of *Trypanosoma congolense*. Vet. Parasitol. 28:53–64.

Pinder, M. and Authie , E. (1984). Appearance of isometamidium resistant *Trypanosoma congolense* in west Africa. Acta Trop. (Basel) 41:247–253.

Silayo, R.S. and Lekaki, K.A. (1989). Trypanocidal drug combinations for treatment of drug resistant *Trypanosoma congolense* infection. In: Proceedings of 7th Tanzania Veterinary Association Scientific Conference 5–7th December 1989, Arusha Tanzania. pp. 79 – 84.

Silayo, R.S. and Marandu, W.P. (1989). The stability of drug resistance and relative apathogenicity of a strain of *Trypanosoma congolense* maintained in mice. In: Proceedings of ISCTRC 20th Meeting Mombasa Kenya April 1989 OAU/STRC, 382 –388pp.

Walker, P.J. and Opiyo, E.A. (1973). The effect of the parasite burden on the curative dose in experimental trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg. 67:281–282.

Williamson, J. (1976). Chemotherapy of African Trypanosomiasis. Trop. Dis. Bull. 73:531–542.

William.; March, J.C.; and Scott–Finnigan, T.J. (1982).Drug synergy in experimental African trypanosomiasis. Tropenmed. Parasit. 33:76–82.