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Concentrations of isometamidium chloride (Samorin®) in sera of Zebu cattle which showed evidence of hepatotoxicity following frequent trypanocidal treatments

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The concentrations of isometamidium circulating in poorly nourished Zebu cattle which showed morbidity, mortality, and biochemical and histopathological evidence of hepatotoxicity, following frequent treatments with isometamidium chloride and diminazene aceturate were investigated using the isometamidium-ELISA. As few as two isometamidium treatments one month apart were associated with significant weight loss, and cattle treated with diminazene aceturate after three or four isometamidium treatments suffered a 50% mortality.

Although there were no obvious, marked elevations in isometamidium concentration which might have allowed the use of the ELISA as a predictor of a potential toxicity problem, concentrations did increase significantly with the number of monthly treatments administered, suggesting drug accumulation, and the increases were significantly higher in cattle to which diminazene had also been administered. In cattle treated with both trypanocides, weight loss and serum glutamate dehydrogenase levels were correlated with isometamidium concentrations. These observations, together with the histopathological findings, support the hypothesis that the morbidity and mortality observed were related to the repeated treatment with isometamidium in conjunction with diminazene aceturate, and that the pathogenesis involved a component of hepatic damage.

It is therefore recommended that cattle, particularly those under nutritional stress, are not subjected to repeated treatments with isometamidium at intervals as short as one month, and particularly not with concurrent administration of diminazene.

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INTRODUCTION

Control of African bovine trypanosomiasis continues to rely heavily on the use of a small number of therapeutic and prophylactic drugs. Of these, isometamidium chloride is the most widely used chemoprophylactic agent, and has been used successfully in many situations (Bourne & Scott, 1978; Leach & Roberts, 1981; Trail *et al.*, 1985). Recently, however, reports of unsatisfactory isometamidium prophylaxis have been attributed in part to the development of drug resistant trypanosomes (Kupper & Wolters, 1983; Pinder & Authié, 1984; Schönefeld *et al.*, 1987; Ainanshe *et al.*, 1992). To improve the understanding of resistance to isometamidium chemoprophylaxis, enzyme-linked immunosorbent assays (ELISA) have been developed for the detection and quantification of isometamidium in bovine serum (Eisler *et al.*, 1993, 1996) and have been used to elucidate the relationship between concentrations of the drug circulating in cattle, and susceptibility to tsetse challenge with pathogenic trypanosomes (Eisler *et al.*, 1994).

Increased dosage and frequency of administration of isometamidium has been used as a means of countering the problem of drug resistance (Fox *et al.*, 1991), but has under some circumstances been associated with a wasting syndrome causing high mortality (Dolan *et al.*, 1992). In the study reported here, the isometamidium-ELISA has been used to measure circulating concentrations of the drug in cattle in which an attempt was

made to recreate the syndrome under typical transhumant pastoralist conditions in a semi-arid area of Kenya, and to investigate a possible role for isometamidium in its pathogenesis.

MATERIALS AND METHODS

Cattle

Zebu steers ≈1½ years old were purchased from Maasai pastoralists at the foot of the Nguruman escarpment in South Western Kenya, and maintained on natural grazing in the same area for the duration of the experiment. The experimental site was as described by Stevenson *et al.* (1993). The area is semi-arid and had suffered a period of drought for two years prior to the start of the experiment, hence grazing was poor.

Trypanocidal drug treatments

Isometamidium chloride (Samorin®, Rhône Mérieux, Lyon, France) was administered as a 2% aqueous solution by deep intramuscular (i.m.) injection into the middle third of the neck at a dose level of 1.0 mg/kg body weight. Diminazene aceturate (Berenil® Hoechst AG, Frankfurt, Germany) was administered as a 7% aqueous solution by deep i.m. injection at a dose level of 7.0 mg/kg body weight.

Prior to the study, cattle had been maintained on an isometamidium prophylactic regimen. No isometamidium treatments had been administered within 5½ months of the start of the study, during which period trypanosome infections had been treated with diminazene aceturate. However, no diminazene treatment was administered within eight weeks of the start of the study.

Parasitological monitoring

Blood samples collected from an ear vein were examined weekly for trypanosome infections using the dark-ground/phase contrast buffy coat technique (Murray *et al.*, 1977), but with bright-field rather than dark-ground illumination. During the study, any animals found to be parasitaemic were immediately treated with diminazene aceturate.

Serum collection

Jugular venous blood was collected into plain Vacutainers (Becton Dickinson, Meylan, France), and the serum separated and stored at −20 °C until required for testing.

Experimental design

Forty cattle were allocated into five groups of eight animals. Group 2 comprised cattle in better body condition than Groups 1, 3, 4 and 5, and were maintained in an area of better grazing for the duration of the study (4 months). Groups 1, 2 and 3 received treatment with isometamidium chloride at the start of the experiment (23/8/91) and monthly thereafter for four consecutive months. In addition, Groups 1 and 2 received treatment with diminazene, three days after the third and fourth isometamidium treatments. Group 4 cattle were treated with diminazene at the same times as Groups 1 and 2, but were not treated with isometamidium. Group 5 cattle served as untreated controls. The drug administration to the different groups is summarised in Table 1.

Table 1. Experimental design: drug treatments and nutritional status

	Isometamidium*	Diminazene†	Nutritional status‡
Group 1	Yes	Yes	Poor
Group 2	Yes	Yes	Fair
Group 3	Yes	No	Poor
Group 4	No	Yes	Poor
Group 5	No	No	Poor

*Isometamidium chloride administered on D0, D30, D59 and D90, at a dose level of 1.0 mg/kg body weight by the intramuscular route.
†Diminazene aceturate administered on D62 and D93, at a dose level of 7.0 mg/kg body weight by the intramuscular route; diminazene was also administered to individual cattle (in any group) found to be infected with trypanosomes (see text).
‡Cattle in Group 2 were in better body condition at the start of the experiment, and maintained thereafter on better grazing than those in Groups 1, 3, 4 and 5.

Serum glutamate dehydrogenase (GLDH) levels were measured at the start of the trial, and both isometamidium concentrations and GLDH levels were measured approximately two weeks following the second, third and fourth isometamidium treatments. The precise dates were 16 days following the second isometamidium treatment, 14 days following the third isometamidium treatment, and 12 days following the fourth isometamidium treatment. Body weights were determined on these occasions, and on the days of isometamidium administration. Sera were not collected following the first treatment period for logistical reasons.

Six cattle, five comprising the weakest animal in each group, and another weak Group 1 animal were slaughtered on Day 102.

Isometamidium concentrations

Serum isometamidium concentrations were determined using a recently described competitive enzyme immunoassay (Eisler *et al.*, 1996).

Briefly, sera were prediluted 1/10 in an optimal dilution of isometamidium-horseradish peroxidase (HRP) conjugate in phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (Sigma; phosphate buffered saline-Tween (PBST). Ninety-six well microtitre plates (Immulon 4, Dynatech Laboratories, Billingshurst, UK) previously coated overnight with hyperimmune sheep anti-isometamidium serum were washed five times in PBST. Serum predilutions were transferred (100 µL per well) to duplicate wells of microtitre plates which were shaken for ten minutes at room temperature, and then incubated overnight at 4 °C, following which they were allowed to equilibrate to room temperature before five washes in PBST. Enzyme levels were

determined using 100 μL per well of a two-component horseradish peroxidase substrate containing 3,3,5,5 tetramethylbenzidine (Cambridge Veterinary Sciences, Littleport, UK) pre-warmed to 37 °C. After 10 min incubation at 37 °C with orbital shaking (Varishaker, Dynatech Laboratories, UK), the colour reaction was quenched by the addition of 100 μL per well of 2 M sulphuric acid. Absorbances were read at 450 nm using a multichannel photometer (Multiskan Plus Mk II, Labsystems Oy, Helsinki, Finland).

Standards prepared using pooled serum from isometamidium-naïve Kenyan Boran cattle and incurred quality control samples were included in duplicate on every microtitre plate. Samples were tested in duplicate, and provided the standards and quality controls had given the results expected, concentrations were derived by four-parameter logistic curve-fitting of calibration standards on the same ELISA plate.

Glutamate dehydrogenase concentrations

Serum glutamate dehydrogenase concentrations were determined as an index of hepatocellular damage (Mullen, 1976), using a test kit (GLDH Test Kit, Boehringer Mannheim GmbH Diagnostic, Mannheim, Germany).

Average daily body weight change

Cattle were weighed fortnightly using electronic weigh-bars (True Test, UK) accurate to 1 kg. Average daily body weight changes, expressed as percentages, were calculated in cattle in all five treatment groups over three periods of approximately one month following the first three isometamidium treatments, and over a 12 day period following the fourth isometamidium treatment, using the formula:

$$\frac{(\text{body weight at end of period} - \text{body weight at start of period}) \times 100}{(\text{body weight at start of experiment} \times \text{number of days in period})}.$$

The precise dates of the treatment periods used were: period 1, d.0–d.30; period 2, d.30–d.59; period 3, d.59–d.90; period 4, d.90–d.102. For four animals which died during the second half of period 3, a shortened period d.59–d.73 was used for the calculation of body weights.

Histopathological examination

Histopathological examination was carried out on all cattle that died, and those that were slaughtered. Tissues were preserved in neutral buffered formalin, and embedded, sectioned and stained using haematoxylin and eosin and Masson's trichrome, using standard histological techniques.

Statistical analysis

The experiment was divided into four periods (1–4), each following an isometamidium treatment of Groups 1, 2, and 3.

The treatment group structure was considered in two parts. Firstly, a factorial experimental design comprised Groups 1, 3, 4, and 5, with isometamidium treatments applied to Groups 1 and 3, diminazene treatments applied to Groups 1 and 4, and Group 5 as untreated controls). Secondly, the performance of two groups of animals (Groups 1 and 2), which were differentiated on the basis of starting body condition and quality of grazing, but which received identical drug treatments (isometamidium and diminazene), were compared.

The effects of isometamidium treatment, diminazene treatment and their interaction (using data from Groups 1, 3, 4 and 5), and the effects of body condition and nutrition (using data from Groups 1 and 2) on serum isometamidium concentrations were investigated by analysis of variance, using a matrix of orthogonal contrasts.

The significance of differences in isometamidium concentrations between individual treatment groups were tested using unpaired *t*-tests, and the significance of differences in concentrations within treatment groups over time were tested using paired *t*-tests, or where this was not possible because of the deaths of some animals, using unpaired *t*-tests.

The relationship between isometamidium concentration and average daily body weight change was analysed by linear regression for each of the three periods during which isometamidium concentrations were determined (periods 2, 3 and 4). Weight was modelled as the response variable using the main effects and interactions of treatment group and isometamidium concentration as explanatory variables. Animals not treated with isometamidium (Groups 4 and 5) were excluded from the analysis. Similarly, the main effects and interactions of isometamidium concentration, group, and treatment period on GLDH levels were fitted using linear models.

Statistical analyses were performed using Genstat 5 Release 2.2 (Numerical Algorithms Group Ltd, Oxford).

RESULTS

Trypanosome infections

Four animals were found to be infected on the first day of the study, one in Group 1 (*Trypanosoma vivax*), two in Group 2 (both *Trypanosoma brucei/Trypanosoma congolense* mixed infections), and one in Group 3 (*T. brucei*). Thereafter, only two trypanosome infections, both *T. vivax*, were detected, both on day 54 of the study and in Group 4 cattle. These infections were immediately treated with diminazene aceturate.

Body weight changes

Body weight changes have been reported elsewhere (Stevenson *et al.*, 1993). Average daily body weight changes are shown in Table 2. All animals began to lose weight after the first month of the experiment, when grazing was becoming scarce. The greatest weight losses were seen in Groups 1 and 3 during the second month of the experiment, and in Group 2 during the third month. During the fourth month of the experiment, when grazing had improved, increases in body weight were seen in all groups of cattle.

Treatment period†	1	2	3	4
Group 1	−0.0278 (0.1060)	−0.4933 (0.0609)	−0.5770 (0.2784)	0.3559 (0.4747)
Group 2	0.0266 (0.0628)	−0.1888 (0.0872)	−0.5941 (0.1977)	0.0691 (0.3122)
Group 3	−0.0504 (0.0738)	−0.5455 (0.1395)	−0.3837 (0.1615)	0.8570 (0.1528)
Group 4	−0.0351 (0.0772)	−0.3419 (0.1263)	−0.1611 (0.0748)	0.6688 (0.1332)
Group 5	−0.0202 (0.1107)	−0.3894 (0.0917)	−0.1268 (0.0535)	0.6094 (0.1533)

Table 2. Daily body weight changes* (%) of cattle following repeated intramuscular treatments with isometamidium chloride

*Mean (and standard deviation) daily body weight changes for †treatment periods 1–4 were expressed as percentages calculated using the formula:

$$\frac{(\text{body weight at end of period} - \text{body weight at start of period}) \times 100}{(\text{body weight at start of experiment} \times \text{number of days in period})}.$$

†Treatment periods: 1 = d.0–d.30; 2 = d.30–d.59; 3 = d.59–d.90; 4 = d.90–d.102.
For four animals that died during the second half of period 3, a shortened period d.59–d.73 was used for the calculation of body weights.

Isometamidium concentrations

Mean and standard deviation isometamidium concentrations in sera collected from each of the treated groups (Groups 1, 2 and 3) during the second, third and fourth treatment periods, determined using the ELISA are shown in Fig. 1. Isometamidium concentrations were not determined following the first drug administration as sera were not collected.

The serum isometamidium concentrations did not differ significantly ($P > 0.05$) between the three treated groups (Groups 1, 2 and 3) during either the second or third treatment periods (Fig. 1). However, during the third treatment period, the mean isometamidium concentration for each of these groups was greater than it had been during the previous treatment period, an increase significant (paired t-tests; $P < 0.05$) for Groups 2 and 3.

During the fourth treatment period, the mean isometamidium concentration in Group 2 (11.8 ng/mL) was significantly higher ($P < 0.05$) than that of Group 3 (5.6 ng/mL). The mean of Group 1 (8.5 ng/mL) fell in between, and was not significantly different ($P > 0.1$) from the means of either Groups 2 and 3.

In Group 2 cattle, the mean drug concentration in period 4 was significantly greater ($P < 0.05$) than that in period 3. This was in spite of the death during the fourth treatment period (days 95 and 99) of two Group 2 cattle (B6 and B61) which had the highest drug concentrations during the third treatment period.

In Groups 1 and 3, the mean isometamidium concentrations in period 4 were lower than those measured during the previous period. In Group 1 however, the mean concentration in the four cattle that survived period 4 rose from 5.8 ng/mL during period 3–8.5 ng/mL during period 4.

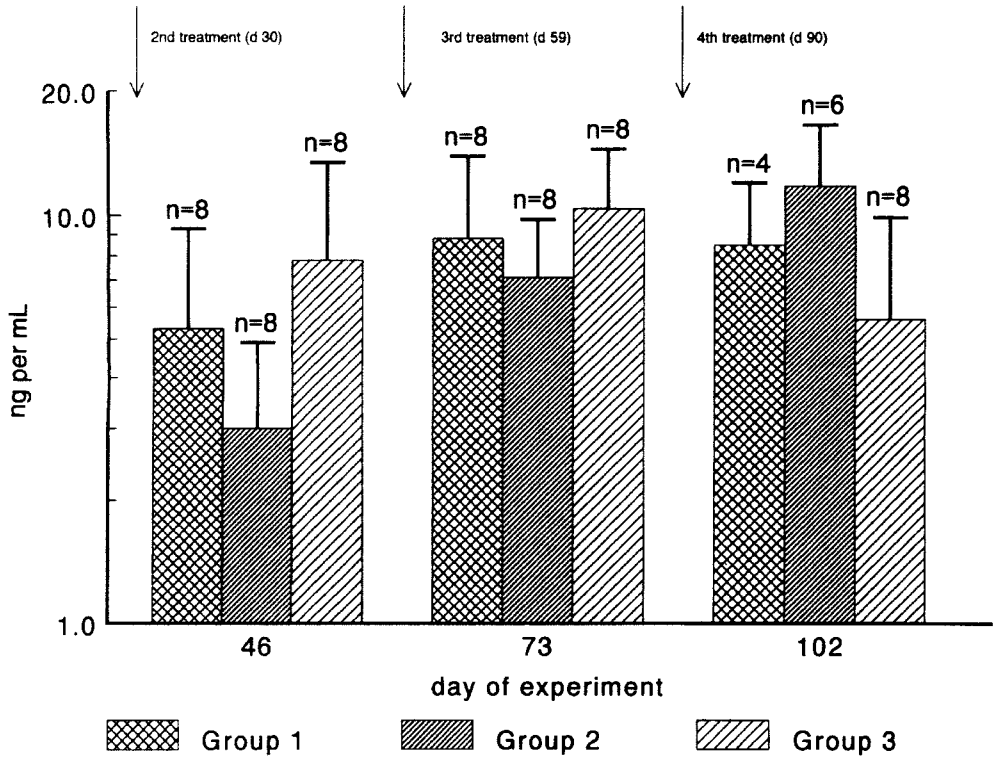

Fig. 1. The effect of diminazene and poor nutritional status on serum concentrations of isometamidium in Zebu cattle following repeated intramuscular treatments. Isometamidium was administered at a dose level of 1.0 mg/kg body weight on days 0, 30, 59 and 90 to all cattle in Groups 1, 2 and 3. Diminazene aceturate was administered to all cattle in Groups 1 and 2 at a dose level of 7.0 mg/kg body weight, 3 days after the third and fourth isometamidium treatments. Group 2 comprised cattle in better initial body condition and with access to better grazing than those in Groups 1 and 3. Decreases in numbers of animals in Groups 1 and 2 were due to deaths. Error bars: standard deviations.

Comparing Groups 1 and 3, diminazene had no effect ($P > 0.1$) on the concentrations of isometamidium during any of the treatment periods (periods 2, 3 and 4) for which it was determined.

Although Group 2 cattle were initially in better body condition and were better nourished than those in Group 1, the mean isometamidium concentrations did not differ significantly ($P > 0.1$) between these two groups in any treatment period. However, by the fourth period too few animals in Group 1 had survived ($n = 4$) for meaningful comparison.

Relationship of isometamidium concentration to body weight change

The relationship was significant ($P < 0.01$) only in Groups 1 and 2, and only during the third and fourth treatment periods, when body weight changes were best described by the model:

BW change $\%_{Group i}$ = $Group_i$ co-efficient × isometamidium concentration + constant

The model accounted for 54% and 68% of the overall variance in body weight changes during the third and fourth periods, respectively. The regression coefficients, standard errors and associated probabilities are shown in Table 3. Regression coefficients for Groups 1 and 2 were negative and significantly different ($P < 0.01$) to zero during both period 3 and 4, whereas Group 3 co-efficients were not significantly different to zero ($P > 0.5$) during either of these periods. Inspection of the standard errors (Table 3) shows that there was no significant difference between the regression co-efficients for Group 1 and for Group 2 in either treatment period 3 or in treatment period 4. Hence, during period 3, for which the regression constant was negative and significant ($P < 0.01$), a general weight loss was exacerbated in cattle treated with both drugs with increased isometamidum concentrations, regardless of their nutritional status. In period 4, the constant was positive and significant ($P < 0.01$), indicating that a general weight gain was reduced in cattle with increased isometamidium concentrations. Such effects could not be detected, however, in cattle treated with isometamidium alone. Finally, as expected, the regression constants were approximately commensurate with, and had the same sign as, the mean weight changes observed in Group 4 and 5 cattle which had not received isometamidium.

Table 3. Coefficients, standard errors and associated probabilities (of coefficients being zero) for the linear regression of isometamidium concentrations on body weight changes

Term	Regression coeffcient	Standard error	Probability
Treatment period 3			
Concentration.group 1	−0.043	0.0090	< 0.001
Concentration.group 2	−0.051	0.0125	< 0.001
Concentration.group 3	−0.016	0.00851	> 0.05
Constant	−0.217	0.0814	< 0.05
Treatment period 4			
Concentration.group 1	−0.060	0.0198	< 0.01
Concentration.group 2	−0.061	0.0129	< 0.001
Concentration.group 3	0.0041	0.0204	> 0.1
Constant	0.827	0.131	< 0.001

GLDH concentrations

The changes in GLDH concentrations have been previously described in brief (Stevenson *et al.*, 1993). At the start of the experiment, GLDH concentrations were less than 13 units per litre and thus within the normal range (Rushton, 1981). Marked increases were however, observed in some animals in Groups 1, 2, and 3 by day 73, and all animals that died had elevated concentrations of GLDH (18–88 u/L) in serum samples taken within two weeks prior to death. Mean and standard deviation GLDH concentrations for the five treatment groups are shown in Table 4.

Relationship between isometamidium and GLDH concentrations

The best fitting model accounted for 37% of overall variance, and included the terms:

$GLDH_{Group i, Period j}$ = $Group_i$ co-efficient × isometamidium concentration + $Period_j$ co-efficient × isometamidium concentration + constant

The regression constants, standard errors and associated probabilities are shown in Table 5. Overall there was a significant positive ($P < 0.01$) relationship between isometamidium concentration and GLDH level. The effects of isometamidium concentration became significant ($P < 0.05$), during the third and fourth treatment periods and were greatest during the fourth treatment period. The effect of isometamidium concentration on GLDH levels was significantly greater ($P < 0.01$) in Group 2, than that observed in either Group 1 or Group 3.

Table 4. Glutamate dehydrogenase concentrations (units per L)

Day of experiment	0*	46†	73‡	102§
Group 1	4.43 (1.73)	8.95 (5.74)	15.89 (10.16)	11.88 (9.49)
Group 2	3.44 (2.73)	7.94 (3.41)	22.08 (5.83)	38.38 (31.65)
Group 3	5.90 (1.51)	6.94 (5.50)	19.90 (9.29)	15.84 (6.94)
Group 4	5.43 (4.80)	5.38 (7.90)	6.31 (3.38)	6.06 (3.62)
Group 5	2.45 (1.72)	3.16 (1.97)	4.48 (2.73)	4.98 (1.56)

*Pre-treatment value.
†16 days following 2nd isometamidium treatment.
‡14 days following 3rd isometamidium treatment.
§12 days following 4th isometamidium treatment.

Table 5. Coefficients, standard errors and associated probabilities (of coefficients being zero) for the linear regression of isometamidium concentrations on GLDH levels

Term	Regression coefficient	Standard error	Probability
Concentration.period 2	−0.009	0.456	>0.5
Concentration.period 3	0.768	0.382	<0.05
Concentration.period 4	1.096	0.446	<0.05
Concentration.group 1	−0.172	0.385	>0.5
Concentration.group 2	1.287	0.422	<0.01
Concentration.group 3	0	NA	NA
Constant	8.61	2.67	<0.01

NA, not applicable.

Mortality

Nine animals died during the experiment. Four of the deaths occurred in Group 1 cattle within a period of four days (Days 80–83) during the second half of the third treatment period. Four further deaths occurred in Group 2 cattle, two during the first two weeks of the fourth treatment period (Days 95 and 99), and two 5–10 days later (Days 104 and 109). The remaining death occurred in Group 3 four weeks after the fourth isometamidium treatment (Day 119).

The relationship between isometamidium concentrations and mortality was investigated in isometamidium-treated cattle (Groups 1, 2, and 3). Isometamidium concentrations during the second, third, and fourth treatment periods in cattle that subsequently died were compared with concentrations in cattle that survived. In cattle treated with the isometamidium, concentrations were not significantly greater ($P > 0.05$) in those that subsequently died than in those that survived.

Histopathological findings

Pathological changes were found in the livers and kidneys of the nine cattle that died, and the six that were slaughtered. The major hepatic lesions in 13 isometamidium-treated cattle (Groups 1, 2 and 3) were congestion, intracytoplasmic vacuolation (fatty change) and loss of hepatocytes in the centrilobular areas. There were no centrilobular changes in livers of two cattle (Groups 4 and 5) that had not been treated with isometamidium. The renal lesions in isometamidium-treated cattle were an admixture of degeneration and regeneration, with intracytoplasmic vacuolation (fatty change) and hyperplasia of tubular epithelial cells, and congestion.

Areas of hydropic vacuolation of hepatocytes and renal tubular cells in isometamidium-treated cattle contained a fine reddish material best viewed on Masson's trichrome-stained sections. Other changes occurring in isometamidium-treated (Groups 1, 2 and 3) and untreated (Groups 3 and 4) cattle included increased cellularity in hepatic sinusoids and in renal glomeruli, due to polymorphonuclear leucocytes, and mild biliary hyperplasia and cholangiohepatitis.

DISCUSSION

The experiment described here was designed to investigate a syndrome suspected to be associated with frequent isometamidium and diminazene treatment of cattle under poor nutritional conditions (Stevenson *et al.*, 1990; Dolan *et al.*, 1992). As the experiment was apparently successful in this respect (Stevenson *et al.*, 1993), serum isometamidium concentrations were measured using a recently developed ELISA (Eisler *et al.*, 1994), to determine whether the different treatment regimens resulted in differences in drug concentrations, whether there was evidence of drug accumulation, and to investigate the relationship between drug concentrations and weight loss, liver damage, and survival.

The experiment was conducted intentionally in a location where, and during a period when, poor grazing resulted in weight loss in the absence of intensive trypanocidal treatment. The results therefore do not necessarily indicate what would happen in trypanocidal drug-treated cattle on a more adequate plane of nutrition and in positive energy balance.

Pharmacokinetic analyses of isometamidum in cattle conducted using the ELISA have shown the drug to have a large apparent volume of distribution associated with extensive tissue binding, both at the site of injection and elsewhere, and a very prolonged half life (Eisler, 1994). This might be expected to result in measurable drug accumulation following frequent i.m. treatments at a dose level of 1.0 mg/kg. However, two weeks after two, three or four injections of isometamidium chloride repeated at monthly intervals, circulating concentrations of the drug were not greatly elevated compared to those in laboratory cattle two weeks after only one injection at that dose level (Eisler *et al.*, 1994). Nevertheless, there was some evidence of accumulation. Following the third treatment, the mean concentrations in all isometamidium-treated cattle (Groups 1, 2 and 3) were higher than those following the second treatment (significant in Groups 2 and 3). In cattle treated with both isometamidium and diminazene (Groups 1 and 2), further increases in isometamidium concentrations (significant in Group 2) were observed in those that survived following the fourth isometamidium treatment until the date of sampling.

Isometamidium concentrations did not differ significantly between the various treatment groups, until four monthly injections of the drug had been administered. Hence, concentrations following two or three monthly injections were not influenced by initial body condition and nutritional status, nor by the additional administration of diminazene following the third of these doses. Following four monthly doses of isometamidium, however, drug concentrations in Group 2 cattle were significantly higher than those in Group 3. This suggests an effect of initial body condition and nutritional status, an effect of the administration of diminazene shortly after the third and fourth doses of isometamidium, or an interaction between both. Unfortunately, by this stage of the experiment, neither comparison of isometamidium concentrations in cattle on a single plane of nutrition, with and without diminazene treatment (Group 1 and Group 3), nor comparison of cattle treated with both

trypanocides but differing in terms of initial body condition and nutrition (Group 1 and Group 2) were possible. Decrease in group size caused by the deaths of four cattle in Group 1 and two cattle in Group 2 reduced the sensitivity of statistical tests, and there was further confounded by the non-random nature of the experimental drop-out: five of the six cattle that died had concentrations above average for their treatment groups in the two previous determinations. The effects of poor nutritional status or diminazene on isometamidium concentrations might be exerted through changes in the levels, or competition for plasma proteins. However, this seems unlikely since the binding of isometamidium to bovine albumin, the major plama protein, has been shown to be largely unsaturable, and hence probably weak and non-specific (Wilkes *et al.*, 1995). Furthermore, diminazene *per se* has been shown to have relatively little effect on the isometamidum ELISA (Eisler *et al.*, 1996). Other more indirect effects therefore, for example on hepatic function, would seem to be more likely causes of these interactions.

In cattle treated with isometamidium alone (Group 3), the mean serum concentration following four treatments decreased significantly compared with that following three treatments, and there was a similar decrease in GLDH levels over the same period. This was consistent with the explanation that improvement in nutritional status during this period permitted recovery of hepatic function, and increased elimination of the drug burden.

Although there was no evidence that better initial body condition or better grazing had any effect on isometamidium concentrations (Group 2 compared with Group 1), there was evidence of an inverse relationship between isometamidium concentrations and body weight gain following multiple treatments with both isometamidium and diminazene (Groups 1 and 2, during the third and fourth treatment periods). This relationship did not differ significantly between Groups 1 and 2, implying that it was not influenced by their differences in initial body condition and nutritional status.

A direct relationship between isometamidium concentrations and GLDH levels was observed in cattle (Groups 1, 2 and 3) during the third and fourth treatment periods, irrespective of whether or not diminazene was administered or not. This relationship was most pronounced in Group 2 cattle, but this apparent effect of initial body condition and nutritional status may have in part been due to non-random drop out in Group 1, in which deaths occurred in period 4 in cattle with high GLDH levels in period 3.

The major histopathological lesions included fatty change and loss of hepatocytes in the centrilobular areas of the liver, findings consistent with hepatotoxicity. This could have been due either to increased vulnerability of the centrilobular zone to circulating toxic material, owing to its distance from the portal supply (relative anoxia), or to a direct toxic effect on this area. Similar changes were reported previously in toxicity studies in monkeys (Philips *et al.*, 1967). The reddish material (hydropic change) within hepatocytes and renal epithelial cells may have been accumulations of isometamidium or its metabolite. Pigment deposits have been observed in hepatic and renal tissues in acute toxicity studies of isometamidium in both dogs and monkeys (Philips *et al.*, 1967), and previous work has suggested that these tissues may be predilection sites for storage of isometamidium (Philips *et al.*, 1967; Kinabo & Bogan, 1988). Other histopathological changes including increased cellularity in the hepatic sinusoids and the renal glomeruli may have been a reflection of increased circulating white blood cells perhaps during terminal septicaemia in severely debilitated animals.

Intravenous injections of cattle and other species with isometamidium may be associated with signs of acute systemic toxicity (Schillinger *et al.*, 1985; Sutherland *et al.*, 1991). More long-term adverse effects of phenanthridinium drugs, including isometamidium were reviewed by Williamson (1970). Dimidium was associated with delayed toxicity, resembling photosensitization, with periportal fatty infiltration of the liver. Climate, nutritional status and dietary photodynamic substances may also have been significant factors. Homidium, pyrithidium, and metamidium were also considered capable of producing liver lesions, but only at doses considerably higher than the therapeutic level.

Overall, the findings of the present study support the hypothesis that the morbidity and mortality observed was related to the frequent administration of repeated doses of isometamidium in conjunction with diminazene aceturate, and that the pathogenesis of the syndrome involved a significant component of hepatic damage. On that basis, and in concurrence with Stevenson *et al.* (1993) it is recommended that cattle, particularly those under nutritional stress, are not subjected to repeated treatments with isometamidium at intervals as frequent as one month, and particularly not with concurrent administration of diminazene.

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