No Evidence for Synergy Between Nitroxynil and Triclabendazole Against Juvenile, 4-week-old, Triclabendazole-resistant Fasciola hepatica in Sheep

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ABSTRACT

Triclabendazole is currently the only single flukicide with licensed efficacy claims against all parasitic stages of Fasciola hepatica, including young immature flukes; it has been widely used in sheep and cattle for more than 30 years. Triclabendazole resistance was first confirmed in 1995 in Australia and since then has been reported in many other countries. Previous studies have shown synergy between triclabendazole and other flukicides against triclabendazole-resistant (TCBZ-R) liver fluke isolates. A study was conducted in sheep to investigate whether synergy exists between nitroxynil and triclabendazole, when administered at their recommended doses, in the treatment of early, immature (4-week-old) TCBZ-R *F. hepatica* infections. Lambs (68) were allocated by live weight to six groups and each animal was dosed with either 250 triclabendazole-susceptible (TCBZ-S) metacercariae (Groups 1 and 3) or 250 TCBZ-R metacercariae (Groups 2, 4, 5 and 6). Treatments were administered 28 days after infection. Groups 3 and 4 were dosed with triclabendazole (10 mg/kg); Group 5 with nitroxynil (10 mg/kg), Group 6 with triclabendazole (10 mg/kg) plus nitroxynil (10 mg/kg); Groups 1 and 2 were not treated with a flukicide. The livers of two lambs from each group were examined post-mortem 48 hours after treatment; the remaining lambs were submitted for necropsy eight weeks post-treatment (12

weeks post-infection) to determine efficacy. Amongst groups infected with TCBZ-R metacercariae, there were no significant differences ($P<0.001$) in the numbers of fluke recovered 12 weeks post-infection, irrespective of treatment. The mean number of flukes recovered at 12 weeks in Groups 2, 4, 5 and 6 was 53, 63, 43 and 51, respectively. There was no evidence from this study that, at their recommended dosages, triclabendazole alone, nitroxynil alone or a combined treatment with triclabendazole plus nitroxynil were effective in eliminating infections with 4-week-old TCBZ-R *F. hepatica* in sheep.

INTRODUCTION

In the absence of effective vaccines and because of practical limitations in management options to control the snail intermediate hosts, the control of *F. hepatica* infection and disease in livestock relies heavily on the use of flukicidal anthelmintics (Fairweather and Boray, 1999a; Parr and Gray, 2000),

Of the currently available flukicides, triclabendazole (TCBZ) has the broadest activity against different stages of TCBZ-susceptible (TCBZ-S) isolates of F. hepatica, with efficacy observed from two-day-old immature flukes through to mature adults (Boray et al., 1983). This profile allows for strategic treatments to be applied at extended intervals, because of the elimination of egg-shedding for ~12 weeks or more, after treatment (Parr and Gray, 2000). However, what makes TCBZ critically important is in the treatment of acute fasciolosis in sheep, which is caused by the hepatic peregrinations of immature fluke (Boray, 1967; Fairweather and Boray, 1999a). Most of the other flukicides have activity against either adult (≥10-12 week old) fluke only, e.g. oxyclozanide, albendazole, or adult and late immature (>6 to 8-week-old) fluke, e.g. rafoxanide, closantel and nitroxynil (Fairweather and Boray, 1999b).

TCBZ-R in F. hepatica was first documented in Australia (Overend and Bowen, 1995) and later in Europe and South America (Mitchell et al., 1998; Moll et al., 2000; Olaechea et al., 2011). The extent to which TCBZ-R contributes to treatment failures has been the subject of some debate (Fairweather, 2011b; Sargison and Scott, 2011a), but the economic consequences of poor treatment responses are manifest (Sargison and Scott, 2011b). Treatment of acute fasciolosis in sheep in areas where TCBZ is no longer effective is problematic and a potential cause of serious welfare problems.

Whilst other flukicides, e.g. closantel, nitroxynil and oxyclozanide, have been shown to be effective against TCBZ-R isolates of adult *F. hepatica* (Coles and Stafford, 2001; McKinstry et al., 2009; Mooney et al., 2009), this does not address the issue of treatment of acute fasciolosis. An alternative approach is to use anthelmintic combinations, which, in an additive, complementary or synergistic way, enhance efficacy against certain parasite stages and/or against resistant isolates (Geary et al., 2012).

Studies in sheep have shown synergy with several flukicide combinations showing enhanced activity against mature and immature *F. hepatica* infections and against TCBZ-S and TCBZ-R isolates, as compared to either component alone (Boray, 1994; Fairweather and Boray, 1999a, b). Laboratory studies have also demonstrated synergy in efficacy against adult fluke, for example with combinations of triclabendazole and clorsulon (Meaney et al., 2006, 2007).

Nitroxynil is a halogenated phenol with high activity against adult *F. hepatica* and good, but somewhat inconsistent, efficacy (>70%) against immature flukes aged 6-8 weeks (Fairweather and Boray, 1999a). Nitroxynil is licensed in several countries for the treatment of immature and adult fluke in both cattle and sheep and its efficacy profile has been determined in various studies, (Colegrave, 1968; Lucas, 1967; Rapic et al., 1988; Reid et al., 1970; Richards et al., 1990) for example. It has also been shown to be effective against TCBZ-R adult liver fluke (Coles and Stafford, 2001; McKinstry et al., 2009; Mooney et al., 2009).

An in vitro study showed that a com-

Table 1. *Details of Treatment Groups*

Group Number	No. of sheep in group	Infection by a single oral gavage of 250 metacercariae	Treatment at 28 days post-infection
1	10	TCBZ-S F. hepatica	None
2	10	TCBZ-R F. hepatica	None
3	12	TCBZ-S F. hepatica	10mg/kg triclabendazole
4	12	TCBZ-R F. hepatica	10mg/kg triclabendazole
5	12	TCBZ-R F. hepatica	10mg/kg nitroxynil
6	12	TCBZ-R F. hepatica	10mg/kg triclabendazole + 10mg/kg nitroxynil

bination of approximately half the recommended dose rates of triclabendazole and nitroxynil was effective against TCBZ-R adult *F. hepatica* (McKinstry, 2008; McKinstry et al., 2005), consequently it was decided to investigate this further in sheep.

This paper describes in full a study that was designed to investigate whether such synergy between nitroxynil and triclabendazole can also be demonstrated in lambs, when administered at their licensed dose rates, in the treatment of immature (4-week-old) TCBZ-R *F. hepatica* infections (McCoy et al., 2005). The defined end-point for determining efficacy was the mean number of adult fluke recovered eight weeks after treatment.

MATERIALS AND METHODS

Sixty eight lambs of the same breed type from an early lambing (January) flock were sourced for the study; each lamb was identified by a unique ear-tag number and double tagged. The lambs had been reared indoors and had no prior exposure to infection with F. hepatica. All lambs were fed the same ration which comprised haylage *ad libitum*, supplemented with a proprietary concentrate mix. Water was available ad lib from troughs. All lambs were housed for the duration of the study and kept in similar pens.

Lambs were allocated to the trial according to sex and live weight, which was determined 7 days prior to the start of the infection phase of the trial. Within sex, lambs were ranked by weight and blocked into sixes; within each block, individuals were randomly allocated to one of the six treatment groups, giving a group size of ten in control groups (Groups 1 and 2) and 12 in treatment groups (Groups 3, 4, 5 and 6).

Two hundred and fifty (250) metacercariae of either TCBZ-S (Cullompton) or TCBZ-R resistant (Sligo) isolates (Fairweather, 2011a) were administered by oral gavage to each lamb, according to its treatment group.

The treatments, given singly or in combination, were 5% triclabendazole (Fasinex®, Novartis Animal Health UK Ltd.), administered at 10 mg/ kg live weight by oral drench and 34% nitroxynil (Trodax®, Merial Animal Health Ltd.) administered at 10 mg/ kg live weight by subcutaneous injection. The composition of the treatment groups is shown in Table 1. Treatments were administered 28 days after initial infection; the appropriate dose was calculated individually from known body weight and delivered by calibrated syringes to the nearest 0.1 ml.

All groups of lambs were examined daily for any general signs of ill health or fasciolosis. The lambs were weighed on the day of allocation (Day –7), on the day of treatment (Day 28) and at fortnightly intervals until the end of the study at Day 86. Faecal samples (50g) were collected from fresh dung from all trial lambs 7 days prior to artificial infection and examined for the presence of fluke eggs. Subsequently,

Table 2. *Post-mortem Fluke recovery, dimensions and fluke egg count on Day 86*

Treatment group	Mean Fluke count ±Standard Deviation	Mean Fluke length (mm)	Mean EPG
1. TCBZ-S,			
no treatment	117.4±22.3	27.2	16.6
2. TCBZ-R,			
No treatment	53.2±14.8	19.2	9.4
3. TCBZ-S, TCBZ treatment	0.2±0.4*	20.0	0
4. TCBZ-R, TCBZ treatment	62.5±17.7	18.6	17.9
5. TCBZ-R, nitroxynil treatment	43.4±9.9	18.9	3.7
6. TCBZ-R, nitroxynil + TCBZ treatment	50.6±19.5	19.8	7.0

samples were taken per rectum on the day of infection (Day 0) and Days 14, 28, 42 and 49 and twice-weekly thereafter until the end of the study. The techniques used were Standard Operating Procedures (SOP) for zinc sulphate flotation, based on the Manual of Veterinary Parasitological Laboratory Techniques, MAFF (MAFF, 1986) and expressed as eggs per gram (EPG).

Post-mortem examinations were conducted on twelve lambs (two per group) on Day 30 in order to determine that the experimental infection had established. Subsequently the remainder of the lambs were necropsied at the end of the study, 12 weeks after infection (Day 86). The livers were removed immediately after euthanasia and subjected to detailed dissection, washing and screening, using standard procedures (Flanagan et al., 2011; MAFF, 1986) to isolate, measure and count all liver fluke present in the liver tissue or bile ducts.

Kruskall Wallis (Siegel and Castellan Jr, 1998) non-parametric tests were used to determine if any differences in the mean number of flukes recovered from each group at the end of the experiment were significant ($P<0.05$). Paired comparisons were carried out on the mean rank of each group to determine differences. Because of the absence of any evidence for synergy against TCBZ-R isolates on Day 86, reflected in the lack of any significant differences fluke burdens, descriptive statistics alone were used for the other parameters measured.

RESULTS

None of the lambs showed any clinical signs of *F. hepatica* infection and there were no significant differences in live weight amongst the groups over the duration of study. Infections in the untreated groups first became patent in week 9 for lambs infected with the TCBZ-R isolate (Group 2) and in week 11 for those infected with the susceptible isolate (Group 1). In group 3, in which lambs infected with the TCBZ-S isolate were treated with TCBZ on Day 28, all the samples were negative with the exception of those from 2 lambs in week 11, which had very low (<2 EPG) counts. At the end of the study, 12 weeks post-infection, the mean fluke-egg counts in all the groups bar Group 3 (TCBZ-S treated with TCBZ) had positive egg counts, the values ranging from 3.7 to 17.9 eggs per gram (EPG) (Table 2).

Livers were examined from two animals per group (one male, one female) on Day 30 after infection. The mean numbers of immature fluke recovered ranged from 71 to 103 in lambs infected with TCBZ-S metacercariae, indicating 34.2% establishment. Corresponding numbers for the TCBZ-R isolate were 20 to 45 immature fluke, representing an establishment rate of 13.85%.

By 12 weeks post-infection (Day 86) there was a significant difference ($P<0.001$) in the number of adult fluke recovered in Group 3 compared to the untreated control infected with the TCBZ-S isolate; treatment with TCBZ resulted in a >99% reduction in TCBZ-S fluke burdens. There were no significant differences between the numbers of flukes recovered from any of the groups (2, 4, 5 and 6) infected with the TCBZ-R isolate (Table 2).

DISCUSSION

The establishment rates (13.35 to 34.2%) of the experimental infections were somewhat less than those previously described with infective doses of 200 metacercariae (44.37%) (Boray, 1967), particularly in the resistant isolate (13.35%). In agreement with the work of Boray, the level of infection (250 metacercariae/lamb) used in this study did not result in clinical signs of fasciolosis, nor in a reduction in growth rate in the lambs.

There were some indications of differences in the biology of the two isolates insofar as the mean length of the flukes at 12 weeks was 27.2mm in the TCBZ-S specimens from untreated lambs and 19.2mm in the TCBZ-R control group. Furthermore, in the TCBZ-S control groups the RBC, PCV and Hb values (data not shown) were all lower than those from the TBCZ-R groups, suggesting greater pathogenicity and a trend towards anaemia.

The lack of synergy between nitroxynil and TCBZ in this study contrasts with previous findings in an in vitro experiment in which severe histological damage to the tegument and internal organelles was observed when both 4-week old and adult liver fluke were exposed to nominally half strength concentrations of nitroxynil and TCBZ simultaneously (McKinstry, 2008). The damage was more disruptive following exposure to the two drugs in combination than with either compound alone.

Although synergy between nitroxynil and TCBZ was not demonstrable in the current in vivo study, nitroxynil has been shown to synergise with another flukicide, clorsulon, in the treatment of immature fluke, against which neither compound individually is effective. Administration of this combination to cattle reduced counts of 2-week -old TCBZ-S *F. hepatica* by 95% and 4-week-old liver fluke by 99% (Hutchinson et al., 2009). Subsequently, a broad-spectrum, injectable, anthelmintic product was licensed, which contains a combination of nitroxynil, (dosage 10.2 mg/kg live weight) and clorsulon (2 mg/kg), in addition to ivermectin (0.2 mg/kg) (Nitromec®, Virbac, Australia Pty.). The inclusion of clorsulon in this combination may be pivotal as previous studies have shown that clorsulon is particularly effective when used in synergistic combinations with other flukicides (Boray, 1994; Fairweather and Boray, 1999b).

In conclusion, whilst the present study failed to demonstrate synergy between nitroxynil and triclabendazole against 4-week-old TCBZ-R liver fluke in sheep, there is evidence from other research that flukicide combinations can play important roles in the treatment of immature fluke infections and the management of flukicide resistance.

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REFERENCES

1. Boray, J.C., 1967, Studies on experimental infections with Fasciola hepatica, with particular reference to acute fascioliasis in sheep. *Ann Trop Med Parasitol* 61, 439-450.
2. Boray, J.C., 1994. Chemotherapy of Infections with Fasciolidae. In: ICOPA VIII, Round Table Conference: Immunology, Pathobiology and Control of Fasciolosis, Izmir, Turkey, pp. 83-97.
3. Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983, Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole. *Vet Rec* 113, 315-317.

4. Colegrave, A.J., 1968, Fascioliasis: Field trials of Nitroxynil in sheep. *Vet Rec* 82, 343-348.
5. Coles, G.C., Stafford, K.A., 2001, Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole-resistant Fasciola hepatica. *Vet Rec* 148, 723-724.
6. Fairweather, I., 2011a, Liver fluke isolates: a question of provenance. *Vet Parasitol* 176, 1-8.
7. Fairweather, I., 2011b, Raising the bar on reporting 'triclabendazole resistance'. *Vet Rec* 168, 514-515.
8. Fairweather, I., Boray, J.C., 1999a, Fasciolicides: efficacy, actions, resistance and its management. *Vet J* 158, 81-112.
9. Fairweather, I., Boray, J.C., 1999b, Mechanisms of Fasciolicide Action and Drug Resistance in Fasciola hepatica, In: Dalton, J.P. (Ed.) Fasciolosis. CABI, United Kingdom, pp. 225-276.
10. Flanagan, A.M., Edgar, H.W., Forster, F., Gordon, A., Hanna, R.E., McCoy, M., Brennan, G.P., Fairweather, I., 2011, Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in Fasciola hepatica. *Vet Parasitol* 176, 34-42.
11. Geary, T.G., Hosking, B.C., Skuce, P.J., von Samson-Himmelstjerna, G., Maeder, S., Holdsworth, P., Pomroy, W., Vercruysse, J., 2012, World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Guideline: Anthelmintic combination products targeting nematode infections of ruminants and horses. *Vet Parasitol* 190, 306-316.
12. Hutchinson, G.W., Dawson, K., Fitzgibbon, C.C., Martin, P.J., 2009, Efficacy of an injectable combination anthelmintic (nitroxynil, clorsulon, ivermectin) against early immature Fasciola hepatica compared to triclabendazole combination flukicides given orally or topically to cattle. *Vet Parasitol* 162, 278-284.
13. Lucas, J.M., 1967, 4-cyano-2-iodo-6-nitrophenol, M&B 10,755. I. Activity against experimental fascioliasis in rabbits, sheep and calves. *Br Vet J* 123, 198-211.
14. MAFF, 1986, Manual of Veterinary Parasitological Laboratory Techniques. Her Majesty's Stationary Office, London, 160 p.
15. McCoy, M.A., Fairweather, I., Brennan, G.P., Kenny, J.M., Ellison, S., Forbes, A.B., 2005, The efficacy of nitroxynil and triclabendazole administered synchronously against juvenile triclabendazole-resistant Fasciola hepatica in sheep. *Res Vet Sci* 78, 33.
16. McKinstry, B., 2008. Ultrastructural changes observed in Fasciola hepatica following treatment with nitroxynil and triclabendazole alone and in combination. PhD Thesis, Chapter 8, The Queen's University Belfast,
17. McKinstry, B., Halferty, L., Brennan, G.P., Fairweather, I., 2009, Morphological response of triclabendazole-susceptible and triclabendazole-resistant isolates of Fasciola hepatica to treatment in vitro with nitroxynil (Trodax). *Parasitol Res* 104, 645-655.
18. McKinstry, B., Mohan, G., Brennan, G.P., Forbes, A.B., Fairweather, I., 2005. The morphological effects of a combination of triclabendazole and nitroxynil in vitro against immature triclabendazole-resistant Fasciola hepatica. In: Abstract, British Society of Parasitology, Nottingham.
19. Meaney, M., Allister, J., McKinstry, B., McLaughlin, K., Brennan, G.P., Forbes, A.B., Fairweather, I., 2006, Fasciola hepatica: morphological effects of a combination of triclabendazole and clorsulon against mature fluke. *Parasitol Res* 99, 609-621.
20. Meaney, M., Allister, J., McKinstry, B., McLaughlin, K., Brennan, G.P., Forbes, A.B., Fairweather, I., 2007, Fasciola hepatica: ultrastructural effects of a combination of triclabendazole and clorsulon against mature fluke. *Parasitol Res* 100, 1091-1104.
21. Mitchell, G.B., Maris, L., Bonniwell, M.A., 1998, Triclabendazole-resistant liver fluke in Scottish sheep. *Vet Rec* 143, 399.
22. Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000, Resistance of Fasciola hepatica against triclabendazole in cattle and sheep in the Netherlands. *Vet Parasitol* 91, 153-158.
23. Mooney, L., Good, B., Hanrahan, J.P., Mulcahy, G., de Waal, T., 2009, The comparative efficacy of four anthelmintics against a natural acquired Fasciola hepatica infection in hill sheep flock in the west of Ireland. *Vet Parasitol* 164, 201-205.
24. Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011, Resistance of Fasciola hepatica against triclabendazole in cattle in Patagonia (Argentina). *Vet Parasitol* 178, 364-366.
25. Overend, D.J., Bowen, F.L., 1995, Resistance of Fasciola hepatica to triclabendazole. *Aust Vet J* 72, 275-276.
26. Parr, S.L., Gray, J.S., 2000, A strategic dosing scheme for the control of fasciolosis in cattle and sheep in Ireland. *Vet Parasitol* 88, 187-197.
27. Rapic, D., Dzakula, N., Sakar, D., Richards, R.J., 1988, Comparative efficacy of triclabendazole, nitroxynil and rafoxanide against immature and mature Fasciola hepatica in naturally infected cattle. *Vet Rec* 122, 59-62.
28. Reid, J.F., Armour, J., Jennings, F.W., Urquhart, G.M., 1970, The efficacy of nitroxynil in the treatment of naturally-occurring ovine fascioliasis. *Vet Rec* 86, 41-42.
29. Richards, R.J., Bowen, F.L., Essenwein, F., Steiger, R.F., Buscher, G., 1990, The efficacy of triclabendazole and other anthelmintics against Fasciola hepatica in controlled studies in cattle. *Vet Rec* 126, 213-216.
30. Sargison, N.D., Scott, P.R., 2011a, Anthelmintic resistance: potential benefits of 'over-diagnosis'. *Vet Rec* 168, 646-647.
31. Sargison, N.D., Scott, P.R., 2011b, Diagnosis and economic consequences of triclabendazole resistance in Fasciola hepatica in a sheep flock in south-east Scotland. *Vet Rec* 168, 159.
32. Siegel, S., Castellan Jr, N.J., 1998, Nonparametric Statistics for the Behavioral Sciences McGraw-Hill, New York, 384 p.