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In vivo assessment of closantel ovicidal activity in *Fasciola hepatica* eggs

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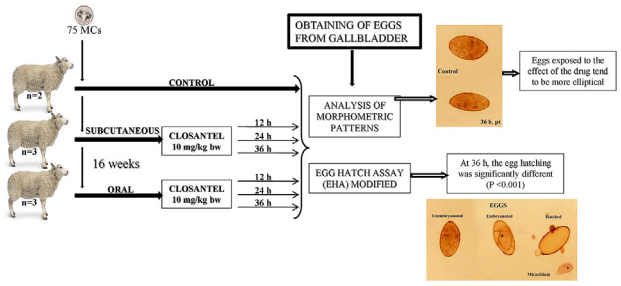
HIGHLIGHTS

- The in vivo ovicidal activity of closantel, an option to triclabendazole in fasciolasis treatment, is yet unknown.
- Sheep were treated with closantel and eggs collected at different times for morphometric studies and eggs hatch assay.
- Significant differences were observed in the morphology and hatchability between control and treated sheep.
- 67,5% of recovered eggs post closantel treatment was unembryonated differing significatively with the control group.
- Results obtained confirm that closantel affects in vivo the normal development of the eggs.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthelmintic resistance in livestock parasites is currently a worldwide problem.

Fasciola hepatica is a cosmopolitan parasite which causes considerable loss in sheep and cattle production systems all over the world. Chemotherapy is currently the main tool available for its control. The intensive use of triclabendazole, the drug of choice for more than 20 years, has resulted in the development of resistant strains. The therapeutic options are adulticides such as closantel (salicylanilide anthelmintic that binds extensively to plasma albumin) to treat chronic fascioliasis in sheep, and cattle. In the present work, an Egg Hatch Assay (EHA) and morphometric studies were used to evaluate in vivo the ovicidal activity and morphology *F. hepatica* eggs, recovered from closantel treated sheep collected at different time intervals post treatment.

Statistically significant differences ($p < 0.0001$) were observed in egg morphometry between the control and the treated groups in all the parameters studied. Eggs recovered from treated animals tend to be narrower and longer. Significant differences were found in the embryonation and hatching of eggs between 36 h post treatment (32, 5%) vs. approximately 85% in control, 12 h and 24 h post treatment. Our results confirm that closantel affects in vivo the normal development of the eggs.

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As one of the first effects, this drug affects the performance of the trematode's reproductive physiology. Even though closantel treated animals may still eliminate eggs in the first days post treatment, these are not viable.

1. Introduction

Fasciola hepatica is a cosmopolitan parasite which causes considerable loss in sheep and cattle production systems all over the world estimated at USD 2000–3000 billion (Boray, 1994; Fairweather, 2011c). Since its introduction in 1983, the fasciolicide triclabendazole (TCBZ) has been extensively used worldwide to control liver fluke infections in sheep and cattle, mainly because it possesses a uniquely wide spectrum of activity, killing not only adult *Fasciola* spp., but also immature and juvenile flukes as young as 2 days post-infection (Boray et al., 1983; Fairweather et al., 1999). In the absence of an effective vaccine against the fluke, control is achieved mainly by chemotherapy.

Triclabendazole (TCBZ) has been the drug of choice for treating liver fluke infections in livestock for over 20 years. More recently, it has been used successfully to treat cases of human fascioliasis (WHO, 2006). Anthelmintic resistance in livestock parasites is currently a worldwide problem. The intensive use of TCBZ has resulted in the development of resistant liver flukes, which was first described in farm animals in Australia in the mid-1990s (Overend and Bowen, 1995) and since then has been reported in, Europe and South America (Fairweather, 2011a; Olaechea et al., 2011). On those farms where the local fluke population is resistant to TCBZ, the therapeutic options are to use adulticides such as closantel to control chronic fascioliasis in sheep and cattle, in an attempt to minimize pasture contamination with fluke eggs and reduce the risk of acute infection in the next (Hanna et al., 2015). Closantel is a salicylanilide anthelmintic that binds extensively to plasma albumin (Michiels et al., 1987). As a result, its activity is mainly directed against blood-feeding internal parasites such as *F. hepatica*, *Haemonchus contortus*, *Oestrus ovis* and *Oesophagostomum* larvae.

The primary action of salicylanilides has been associated with the uncoupling of oxidative phosphorylation in mitochondria. Early *in vitro* studies, using houseflies as well as rat liver mitochondria, showed that several salicylanilides were potent inhibitors of electron transport-associated phosphorylation (Williamson and Metcalf, 1967). *In vitro* inhibition of electron transport-associated phosphorylation in *F. hepatica* and *Ascaris lumbricoides* was later reported for oxyclozanide, rafoxanide and closantel (Corbett and Goose, 1971; Kane et al., 1980).

Several forecasting systems exist for disease surveillance and will assist in monitoring any changes in the pattern of fascioliasis (Fox et al., 2011; McCann et al., 2010). Diagnosis has to operate at two other levels: diagnosis of drug efficacy and diagnosis of drug resistance. There is no standard protocol for the determination of drug efficacy, so interpretation of results can be difficult and there are fewer tests available. The Faecal Egg Count Reduction Test (FECRT) is probably most often used, with drug treatment being regarded as successful if there is a 95% reduction in fluke egg counts by 14 days post-treatment (pt). However, it is known that eggs can be stored in the gall bladder for several weeks (Chowaniec and Darski, 1970), so they may still be present, even though the flukes have been successfully removed; this can lead to false positive results.

The Egg Hatch Assay (EHA) test (Coles et al., 2006; von Samson-Himmelstjerna et al., 2009). It could be applied to fluke as well. Flukicides can affect not only egg formation and production, but also egg development and hatching (i.e. viability) (Maes et al., 1988; Malone et al., 1984; Toner et al., 2011). The EHA may have some potential to detect anthelmintic resistance in flukes. This test, used as a diagnostic method for the detection of benzimidazole resistance in nematodes (Coles et al., 2006).

In the present work, a modified EHA was used to evaluate in vivo the ovicidal activity and morphology in fluke eggs, recovered from closantel treated sheep collected at different time intervals post treatment, and immediately incubated without further addition of the drug.

2. Materials & methods

2.1. Experimental infection

Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar), and to internationally accepted Animal Welfare Guidelines (A.V.M.A, 2001). Eight parasite-free Corriedale weaned lambs were inoculated orally with 200 metacercariae of *F. hepatica* contained in a gelatin capsule. The isolate used for this experiment was the Cullompton isolate, which is TCBZ-susceptible. Details of its provenance were reviewed by Fairweather (Fairweather, 2011b). The presence of liver fluke in the lambs was confirmed 16 weeks after infection by the finding of eggs in the faeces, and liver damage was estimated indirectly by measurement of serum Glutamate Dehydrogenase and Gamma Glutamyl Transferase activities, as described previously (Solana et al., 2001). Sixteen weeks after oral inoculation, the animals were assigned to three experimental groups, based on their clinical condition and body weight, and were treated orally or by subcutaneous injection with closantel as detailed in Table 1.

2.2. Collection of F. hepatica eggs

Treated animals in groups 2 and 3 were stunned and exsanguinated at 12 h, 24 h and 36 h post-treatment, following the W.A.A.V.P. guidelines for evaluating antiparasitic treatments in ruminants (Wood et al., 1995). The eggs were directly recovered from the bile of each infected sheep by puncture of the gallbladder. After several washes with tap water, eggs were suspended in water (500 eggs/ml) and conserved in darkness at 4 °C until used.

2.3. Analysis of morphometric patterns

Eggs (45–60 per group) were measured using an ocular micrometer attached to 10X optical microscope with 10X objective. In all analyzed eggs the following measurements were made: I) WIDTH, the central area perpendicular to the axial axis, II) LENGTH, a centerline from one pole to the other, III) SIZE, was determined by multiplying the length by the width of each egg and IV) SHAPE was

Table 1
Summary of treatments administered to groups of experimental animals.

Group	Number of animals	Dose	Days post-infection before treatment
1	2	No treatment	–
2	3	10 mg/kg b.w. oral	90
3	3	10 mg/kg b.w. subcutaneous	90

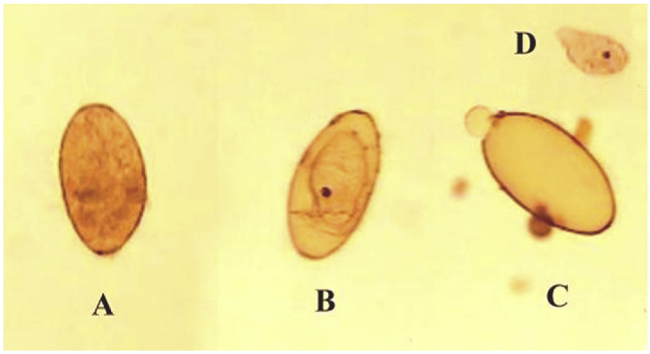

Fig. 1. *F. hepatica* Eggs. A: Unembryonated eggs; B: Unhatched embryonated eggs; C: Hatched eggs; D: Unstained miracidium (100X), direct observation.

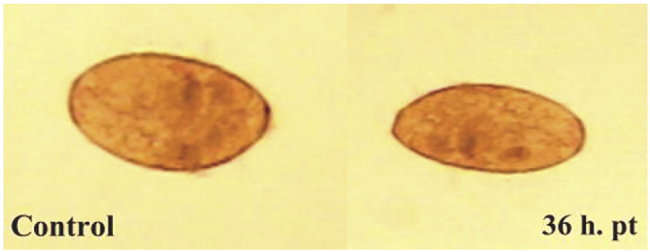

Fig. 2. *F. hepatica* Eggs. Effect of closantel (10 mg/kg bw) 36 h post treatment. Eggs recovered from treated animals tend to be more elliptical.

obtained by dividing the length by the width (Abrous et al., 1998; Poulin, 1997; Valero et al., 2001). Data processing was carried out with Infostat®. From the collected data, arithmetic means and standard deviations were obtained. The comparison of the egg measurements from the different groups was carried out with the one way Analyses of Variance and Tukey test. Values were considered significant if $p < 0.05$.

2.4. Egg hatch assay (EHA)

Untreated and treated eggs were kept in darkness at 25 °C for 15 days. After this period, the trematode eggs were exposed to daylight for 2 h. Afterwards, 1 ml of 10% (v/v) buffered formalin was added to each tube in order to stop egg hatching. Eggs were evaluated using an optical microscope (40× magnification). Approximately 80–90 eggs were counted to estimate the proportion of hatched eggs in each tube. Upon observation, eggs were classified in two groups:

1) Embryonated eggs (E): including hatched and unhatched embryonated eggs, with inner miracidium identified by the eyespots.
2) Unembryonated eggs (U): with no identifiable miracidium or increase in the number of cells, similar to freshly collected eggs (Abrous et al., 1998) (Fig. 1). The percentages of egg hatch are reported as the arithmetic mean ± standard deviation (SD). One way Analyses of Variance and Tukey test was used for the statistical comparison of the egg hatch data obtained from each experiment. A value of $P < 0.05$ was considered statistically significant. The statistical analysis was performed using Infostat®.

3. Results

3.1. Morphometric analysis

Results of the morphometric analysis of eggs can be seen in Table 2. Statistically significant differences ($p < 0.0001$) were observed between the eggs of the control group and the rest in all the parameters evaluated. Eggs exposed to the effect of closantel tend to be narrower and longer (Fig. 2).

3.2. EHA

The test ($n = 3$) was expressed as percentage of total eggs analyzed. The result obtained for control was 89, 5% ± 2.12 for (E) and 10.5% ± 2.12 (U). The results obtained of the oral and subcutaneous (SC) routes were not significantly different (data not shown), so it was decided to use the mean of both as a unique result for each group. The mean of both ways of administration at 12 h pt was 84.5% ± 0.71 (E) and 15.5% ± 0.71 (U); at 24 h pt was 86.75 ± 3.18 (E) and 12.75% ± 3.18 (U) and 36 h pt was 32.5% ± 3.54 (E) and 67.5% ± 3.54 (U) (Table 3). At 36 h, the egg embryonation and hatching was significantly different ($p < 0.001$).

Table 2
Morphometric analysis of *F. hepatica* eggs. Effect of closantel (10 mg/kg bw) subcutaneous (SC) and oral at 12, 24 and 36 h post treatment.

		Mean ± SD			
Eggs		Length (μ)	Width (μ)	Size (μ^2)	Shape
Control (n = 44)		149.78 ± 6.98	94.66 ± 10.67	14,204.95 ± 1917.58	1.60 ± 0.18
12 h	SC(n = 50)	152.68 ± 6.80	88.74 ± 7.10	13,561.04 ± 1379.62	1.73 ± 0.14
	Oral(n = 61)	151.88 ± 5.46	91.36 ± 8.61	13,875.66 ± 1420.21	1.68 ± 0.16
24 h	SC(n = 60)	154.55 ± 4.60	88.38 ± 3.61	13,655.61 ± 628.11	1.75 ± 0.10
	Oral(n = 59)	148.48 ± 5.36	86.18 ± 5.19	12,798.44 ± 932.35	1.73 ± 0.12
36 h	SC(n = 62)	152.99 ± 7.34	89.36 ± 5.63	13,677.98 ± 1174.60	1.72 ± 0.13
	Oral(n = 62)	153.12 ± 6.70	87.75 ± 5.94	13,442.10 ± 1152.01	1.75 ± 0.13

Table 3
Percentage (%) of embryonated and hatched eggs (E) and unembryonated eggs (U). Effect of closantel (10 mg/kg bw) subcutaneous (SC) and Oral at 12, 24 and 36 h post treatment (pt).***: Significantly different.

Stage	Eggs hatch assay (EHA) (%)								
	Control	Experimental							
		12 h pt			24 h pt			36 h pt***	
		Oral	Sc	Mean ± SD	Oral	Sc	Mean ± SD	Oral	Sc	Mean ± SD
E	89.5 ± 2.12	86.5	82.5	84.5 ± 0.71	86.5	87	86.75 ± 3.18	34	31	32.5 ± 3.54
U	10.5 ± 2.12	13.5	17.5	15.5 ± 0.71	13.5	13	13.25 ± 3.18	66	69	67.5 ± 3.54

4. Discussion

The basic aim of this study was to determine ovicidal activity and morphology in fluke eggs recovered from closantel treated sheep. The results showed clearly that the aim of the study was achieved.

The effect of closantel on the fluke's reproductive system can explain the observed changes in egg embryonation and hatching. Bearing in mind that each egg assembled in the ootype of *F. hepatica* incorporates about 30 vitelline cells, and that each fluke produces some 25,000 eggs each day, cell division and differentiation in the vitelline follicles consumes the majority, possibly the largest, proportion of the energy generated by intermediary metabolism. Therefore it is not surprising that drug induced restriction of available ATP had a marked effect on the functional activity in the vitelline tissue, which causes a decrease production and quality of eggs (Hanna et al., 2006). It is likely that defects in the quality of the vitelline cells would prevent successful shell formation and therefore render the eggs non-viable. The release of fertile eggs of *F. hepatica* into the environment is one of the main ways available to the fluke to perpetuate their species and maintain their active life cycle. Our results confirm that closantel affects in vivo the normal development of the eggs. As one of the first effects, this drug affects the performance of the trematode reproductive physiology.

The results of the time-course studies, reinforce the idea that any eggs present in successful efficacy trials represent eggs stored in the gall bladder prior to drug treatment and may not properly reflect what is happening to the actual flukes themselves, which are eliminated within 3–4 days pt ((Toner, E. et al. 2011)). Our results confirm that closantel affects in vivo the normal development of the eggs. As one of the first effects, this drug affects the performance of the trematode's reproductive physiology.

On the other hand, these results have an impact on the interpretation of the Faecal Egg Counts in efficacy studies and may be useful to further understand the mechanisms underlying the drug activity in target helminths parasites. Finally a curious data was obtained from de morphometric analysis, the size of the Cullompton strain is considerably greater than descriptions of field isolates described in the literature (Abrous et al., 1998; Fantozzi et al., 2011, 2012; Larroza and Olaechea, 2008; Valero et al., 2001). These reports describe eggs of approximately 130μ in length and 72μ in width, quite smaller than what we have observed (Table 2).

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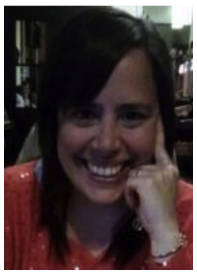

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