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Reproductive disruption in *Fasciola hepatica* associated with incomplete efficacy of a new experimental formulation of triclabendazole

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ABSTRACT

The objective of the present study was to analyse the reproductive viability (using histopathologic studies) of *Fasciola hepatica* from cattle artificially infected and treated subcutaneously with a new experimental formulation of triclabendazole (8 mg/kg b.w.). The results of the efficacy controlled test, which only takes into account the presence of live adult flukes, indicated that, whilst in the control group ($n = 7$) 533 live specimens were recovered, in the test groups (doses of 8 and 12 mg/kg b.w.) only 195 and 47 adults were recovered, respectively. These numbers indicate efficacies of 69% and 95.6%, respectively. It was observed in that dose of 8 mg/kg b.w. some specimens remained viable, but they were infertile, which severely compromises the biological cycle of the trematode. In the testis tubules of flukes treated with the low dose of TCBZ (8 mg/kg), very few cells were present and the vitelline follicles were markedly reduced in size and each follicle contained very few cells. This would have direct implications for the pathogenesis of the parasitosis since the remaining parasites would produce little clinical–productive manifestations, would stimulate the immune response and would find it difficult to establish future re-infestations/re-infections. Consequently, these observations will also prompt a review of certain methodological and interpretative aspects related to efficacy tests, where the only discriminative factor is the reduction of the adult parasite load. On one hand, histopathological studies could be complementary to the efficacy controlled test for TCBZ or other BZD formulations.

1. Introduction

Fasciolosis is a world-wide parasitic zoonosis (World Health Organization, 2006, 2007; Lewin, 2007) produced by the parasite *Fasciola hepatica* which affects mainly sheep, cattle, goats, swine, horses, other herbivores and, accidentally, man (Carrada Bravo, 2003). In cattle, fasciolosis manifestations are weight loss, decrease in milk production

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and quality (Reinhard, 1957) and impaired reproductive and feed efficiency (Malone, 1982, 1986).

F. hepatica is a parasite prevalent in ruminants raised in temperate regions of the world. It is economically important since losses due to its infection are estimated in U$S 2000–3000 million yearly (Boray, 1994). Also, fasciolosis has become an emergent zoonosis in many countries (Mas-Coma et al., 2009a): it is estimated that 17 million people are currently infected and 180 million are at risk of infection (World Health Organization, 2007). In the last few years, a rise in cattle fasciolosis cases has been reported (Mitchell, 2002), which is believed to be due to weather changes determining a different distribution of the snail, *Galba truncatula* which is a required intermediate host (Kenyon et al., 2009; Mas-Coma et al., 2009b). In the absence of an efficacious vaccine, chemotherapy remains the main tool in treating fasciolosis. Even though other alternatives exist, current measures of controlling fasciolosis are based on the use of drugs such as triclabendazole (TCBZ) (Fairweather, 2005, 2009; Keiser et al., 2005).

At recommended therapeutic doses, most of the fasciolicidal compounds available present good efficacy against adult stages of *F. hepatica*. Albendazole (ABZ) is a benzimidazole anthelmintic (BZD) utilised to control adult *F. hepatica* (older than 12 weeks), but it is not efficacious against immature stages (Solana et al., 2009). The anthelmintic TCBZ is an halogenated BZD showing an excellent efficacy against both juvenile (immature) and adult stages of *F. hepatica* (Boray et al., 1983), rendering it as the drug of choice to treating fasciolosis (Boray et al., 1983; Smeal and Hall, 1983). The anthelmintic activity of the different BZD compounds is based on their binding to the β-tubulin of parasites. This modifies the tubulin-microtubule dynamic equilibrium, producing loss of cellular function and detachment and death of endoparasites.

In ruminants, the efficacy controlled test (ECT) is the most widely recommended and reliable test to evaluate anthelmintic activity. Also, it is used in dose titration and confirmation assays. In the ECT, experimental animals with a controlled parasitosis are randomly assigned to different groups (control and treated) and, after a pre-established time, sacrificed in order to identify and count the number of parasites present. However, the viability of recovered specimens is not checked over, except for a macroscopic observation. The use of a placebo group is necessary (Wood et al., 1995). Previous studies with TCBZ in cattle have established an effective fasciolicidal oral dose at 10–12 mg/kg body weight (b.w.) which results in an efficacy of higher than 90% whilst, with an oral dose of 8 mg/kg b.w., more than 40% of adult flukes remained. Even though these results established the therapeutic dose of TCBZ as 10–12 mg/kg b.w., they provided no information about the viability of surviving specimens when an 8 mg/kg b.w. dose was administered.

The aim of the present study was to analyse the reproductive viability (by determining the presence or absence of histopathologic lesions and egg output) in adult *F. hepatica* recovered from artificially infected cattle and treated subcutaneously with an experimental formulation of TCBZ at a dose rate of 8 mg/kg b.w. The study focussed on the vitellaria and the testes, as these tissues are essential components of egg formation and are known to be highly sensitive to drug action (Halferty et al., 2009; McConville et al., 2010).

2. Materials and methods

2.1. Drugs

For all the treatments was used a new experimental injectable formulation of TCBZ at 25%. The animals were treated subcutaneously and the doses were calculated on an individual weight basis and injected in the distal region of the neck, immediately cranial to the scapula.

2.2. Egg count reduction test (ECRT)

Prior to the efficacy controlled test (ECT) an in order to evaluate the anthelmintic capacity of this formulation of TCBZ an ECRT was performed.

From a commercial herd, fifteen (15) cows, aged 2–3 years old, were chosen on the basis of the *F. hepatica* egg counts and were randomly assigned to two experimental groups. The test group ($n = 8$) was treated subcutaneously with the experimental formulation of TCBZ (25%) at a dose of 12 mg/kg b.w., whilst the animals in the control group ($n = 7$) remained untreated.

2.2.1. Faecal sampling

Faecal samples were collected from each animal on day 0 and day 20 post-treatment.

2.2.2. Sample processing

Using a sedimentation technique, the number of parasite eggs per 5 g of faeces was determined (Parffit, 1970).

2.3. Efficacy controlled test (ECT)

Twenty-one animals were used: they were 8–10 months old, without any history of fasciolosis, and came from a farm in the south-west of the province of Buenos Aires, Argentina. Animals were identified (by ear-tag), weighed and treated with one dose of ivermectin (0.2 mg/kg b.w.) in order to treat possible infections with trichostrongylid nematodes and lung parasites.

2.4. Production of metacercariae and experimental infection

Metacercariae were bred in snail populations (*Lymnaea viatrix*) kept on a bed of algae (*Obscillatoria obscura*). Eggs of *F. hepatica* were incubated for 10 days at 27 °C. Miracidia were obtained and counted and were used to infect snails. Infected snails were kept for 30 days at 25 °C, before collection of metacercariae. About 25,000 metacercariae were produced. This number ensured that enough metacercariae were available for the inocula needed to infect the experimental animals. Each experimental animal was orally inoculated with 400 metacercariae.

Table 1
Egg count reduction test (ECRT) (expressed as eggs per 5 g of faeces).

Control without treatment			TCBZ 12 mg/kg b.w.		
Animal	Ep5 g		Animal	Ep5 g	
	1st sampling	2nd sampling		1st sampling	2nd sampling
1	13	17	1	19	0
2	27	27	2	20	0
3	28	45	3	31	0
4	48	32	4	40	5
5	68	120	5	51	0
6	71	120	6	57	0
7	135	53	7	187	4
			8	189	4
Average	55.7	59.1		74.3	1.6
Reduction					97.8%

2.5. Experimental design

Based on their clinical condition and body weight, animals were assigned to three experimental groups ($n = 7$), and treated as follows:

Group	Animals	Dose	Days post-infection before treatment
1	7	No treatment	–
2	7	8 mg/kg b.w.	90
3	7	12 mg/kg b.w.	90

An experimental formulation of TCBZ (25%) at two dose levels (8 and 12 mg/kg b.w.) was administered subcutaneously in the distal region of the neck, immediately before the shoulder. Total administered volume was calculated on an individual body weight basis.

Two weeks after treatment, experimental animals of all groups were sacrificed according to the W.A.A.V.P. guidelines for evaluating antiparasitic treatments in ruminants (Wood et al., 1995).

2.6. Histopathologic studies

2.6.1. Parasite collection

Flukes were detached from the biliary duct by soft digital pressure, picked with plastic forceps, and placed in 0.9% (w/v) NaCl solution at 37 °C for 30 min. After that time, they were transferred to Petri plates and fixed with 10% (v/v) neutral buffered formalin for 24 h. Histological processing and embedding in wax was carried out by conventional techniques. Sections 3 μm in thickness were cut from each block face and stained with haematoxylin and eosin using standard histological protocols. Sections were examined and the reproductive tissues photographed using a Leica DM LBZ microscope with a Nikon Coolpix 5000 camera system.

2.7. Statistical analyses

Data obtained from adult counts were compared by an analysis of variance test (ANOVA). Statistical analyses were performed with commercial software Instat 3, 00 (Graphic Pad Software, Inc.).

3. Results

3.1. ECRT data

Using a dose rate of 12 mg/kg, the ECRT showed a marked reduction in egg elimination, which decreased from 74.3 eggs per 5 g to 1.6 eggs per 5 g at the second sampling point post-treatment (Table 1). These results (the efficacy was 97.8%) confirmed the effectiveness of the formulation and of the dose and justified carrying out an efficacy controlled test.

3.2. ECT data

The results of the ECT (which only takes into account the presence of live adult flukes) indicated that, whilst in the control group ($n = 7$) 533 live specimens were recovered, in the test groups (doses of 8 and 12 mg/kg b.w.) only 195 and 47 adults were recovered, respectively. These numbers indicate efficacies of 69% and 95.6%, respectively (Table 2).

3.3. Histology

The testis tubules of control flukes were filled with spermatogenic cells and all stages of spermatogenesis and spermiogenesis were present: that is, spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 1A and B). The histological features of the testis were consistent with the descriptions given by Hanna et al. (2008). In the testis tubules of flukes treated with the low dose of TCBZ (8 mg/kg), very few cells were present. They probably represented spermatogonia and sustentacular cell nuclei; later stages of spermatogenesis were absent. Several eosinophilic bodies were present: they represent apoptotic spermatocytes (Hanna et al., 2008). Much of the lumen of the tubules was occupied by cell debris and the tubules appeared to be very vacuolated (Fig. 1C and D). The number of spermatogonia in control and TCBZ-treated flukes is presented in Figs. 2 and 3.

The vitelline follicles of control flukes were well-developed and appeared to contain the usual complement of stem, immature and mature vitelline cells in the usual

Table 2
Efficacy controled test of TCBZ (experimental injectable formulation).

Control group		TCBZ 12 mg/kg group. 14 days post-treatment		TCBZ 8 mg/kg group. 14 days post-treatment	
Animal	*F. hepatica* adults	Animal	*F. hepatica* adults	Animal	*F. hepatica* adults
1	51	1	1	1	65
2	73	2	1	2	8
3	160	3	1	3	25
4	141	4	1	4	31
5	79	5	13	5	17
6	9	6	29	6	1
7	20	7	1	7	48
Total	533	Total	47	Total	195
Mean	76.14	Mean	6.7	Mean	27.86
Geometric mean	53.13	Geometric mean	2.33	Geometric mean	16.47
Efficacy			95.6%		69%

proportions (Fig. 4A and B). For a description of the different vitelline cell types, see Hanna et al. (2010). In the flukes treated with the low dose of TCBZ (8 mg/kg), the vitelline follicles were markedly reduced in size and each follicle contained very few cells. The cells were predominantly mature in type and appeared to be quite fragmentary (Fig. 4C and D). The number of vitelline cells in control and TCBZ-treated flukes is presented in Fig. 5.

4. Discussion

The ECRT is an efficacious method which offers an estimation of the anthelmintic efficacy in natural infections by comparing egg counts per gram of faeces from animals before and after treatment (Dorsman, 1956). Currently, to evaluate the anthelmintic efficacy of a new formulation of a probable anthelmintic drug, the ECT is

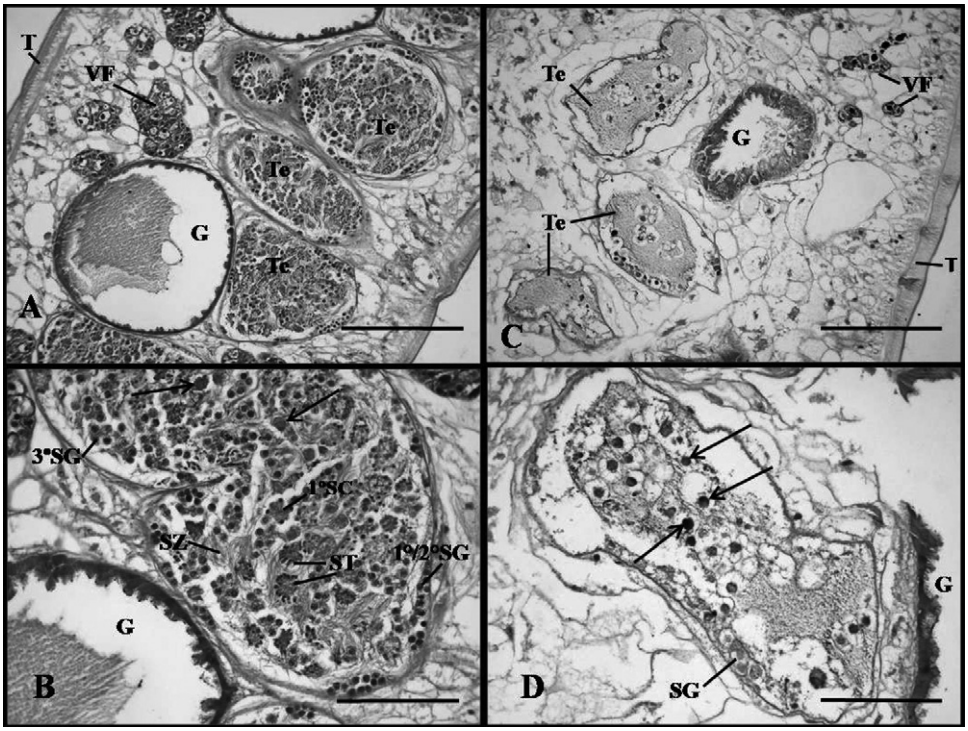

Fig. 1. Micrographs of testis tubules in control (A and B) and TCBZ-treated (8 mg/kg b.w.) (C and D) *Fasciola hepatica*, stained with haematoxylin and eosin. (A) The testis tubules (Te) are well-developed and are filled with spermatogenic cells of all stages of development. G, gut; T, tegument; VF, vitelline follicles. Scale bar = 200 μm. (B) The testis tubule contains all stages of sperm development: 1°/2° spermatogonia (1°/2° SG), 3° spermatogonia (3° SG), 1° spermatocytes (1° SC), spermatids (ST) and spermatozoa (SZ). A small number of eosinophilic bodies (arrows) are present. G, gut. Scale bar = 100 μm. (C) The testis tubules (Te) are very vacuolated and contain few cells. Much of the lumen is occupied by cytoplasmic/cellular debris. The vitelline follicles (VF) are very reduced in size. G, gut; T, tegument. Scale bar = 200 μm. (D) The testis tubule is very vacuolated and contains much cellular debris. A number of cells, probably spermatogonia (SG), are located at the periphery of the tubule. Several eosinophilic bodies (arrows) are present. G, gut. Scale bar = 100 μm.

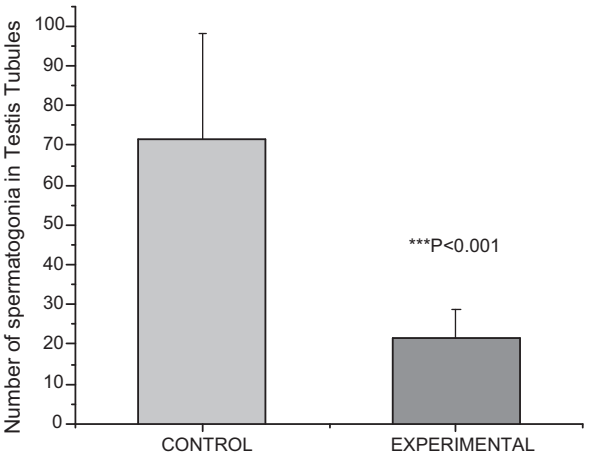

Fig. 2. Total number of spermatogonia present in testis tubules of *Fasciola hepatica* from control and TCBZ-treated (8 mg/kg b.w.) (experimental) animals. Data are expressed as mean ± SD (n = 15).

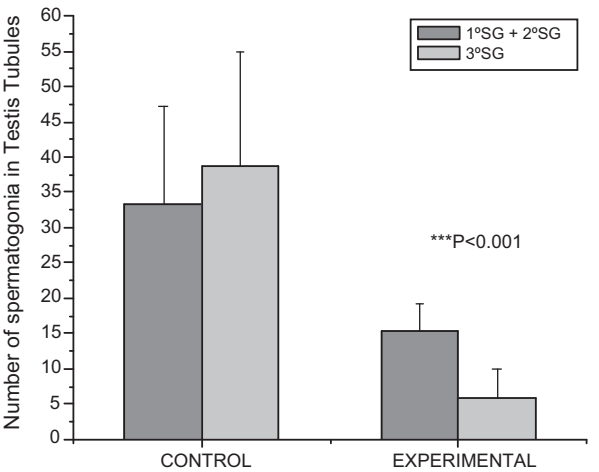

Fig. 3. Different stages of spermatogonia (1°, 2° and 3° SG) present in testis tubules of *Fasciola hepatica* from control and TCBZ-treated (8 mg/kg b.w.) (experimental) animals. Data are expressed as mean ± SD (n = 15).

the most reliable method. It is based on the counting of parasites in animals of treated and untreated experimental groups, making it possible to determine the efficacy against both adult and immature stages (Presidente, 1985).

4.1. Importance of trematode reproduction on the maintenance of the infection

To complete its biological cycle, *F. hepatica* needs two hosts, one intermediate (snail) and another definitive

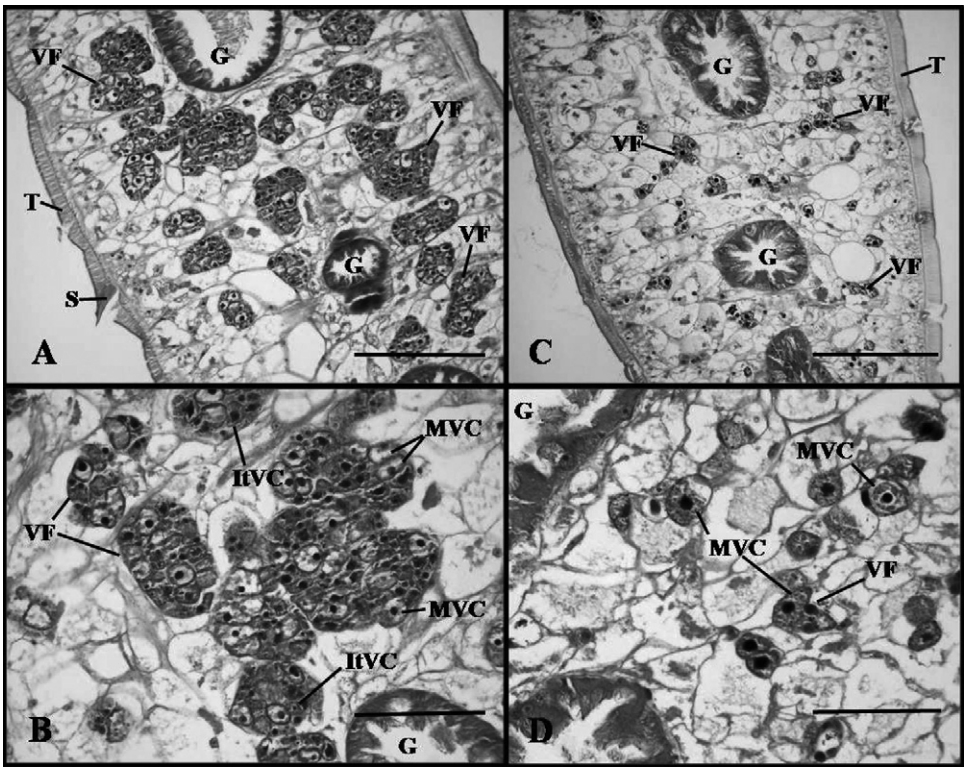

Fig. 4. Micrographs of vitelline follicles of control (A and B) and TCBZ-treated (8 mg/kg b.w.) (C and D) flukes, stained with haematoxylin and eosin. (A) Micrograph showing the distribution of vitelline follicles (VF) in the fluke. The follicles are well-developed and each follicle contains several cells, at different stages of development. G, gut; S, spine; T, tegument. Scale bar = 200 μm. (B) High-power micrograph showing the presence of immature (ItVC) and mature (MVC) vitelline cells in the vitelline follicles (VF). G, gut. Scale bar = 100 μm. (C) Micrograph showing the scattered distribution of vitelline follicles (VF) in the fluke. Each follicle is very small and contains very few cells. G, gut; T, tegument. Scale bar = 200 μm. (D) High-power micrograph of the vitelline follicles (VF), each of which contains only a few cells and the cells are predominantly mature vitelline cells (MVC). G, gut. Scale bar = 100 μm.

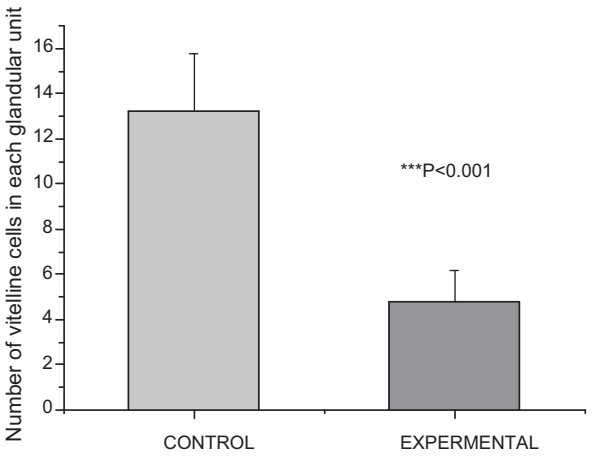

Fig. 5. Total number of vitelline cells present in each glandular unit of *Fasciola hepatica* from control and TCBZ-treated (8 mg/kg b.w.) (experimental) animals. Data are expressed as mean ± SD ($n = 20$).

(mammal) thus, the presence of *F. hepatica* will always rely on the presence of a snail population that will serve as the intermediate host (Olaechea, 2007). A parasitized sheep can eliminate about 2–2.5 million eggs per day, which will be deposited in the environment and will contaminate wild ruminants and lagomorphs (rabbits), contributing, along with the multiplication of the intermediate host, to perpetuate the life cycle of the fluke (Carrada Bravo, 2007). This high oviposition rate indicates that the parasite has serious difficulties to overcome in order to complete its cycle and, hence, requires a reproductive system working perfectly in order to preserve the species.

The extension of the prepatent period of *F. hepatica* in infected animals following treatment with TCBZ with subtherapeutic doses have been described for Büscher et al. (1987). In the macroscopic analysis, these authors concluded that both the extension of the prepatent period and the smaller size of the flukes observed in animals treated are primarily caused by retarded fluke development perhaps in concert with selective killing of the more mature fraction of the fluke burden by the drug. Stunting, delayed maturation and sustained reduction of egg output have been observed in studies with other fasciolicides (Malone et al., 1984; Maes et al., 1990; Hanna et al., 2006). These results, together with the present data, could be of considerable epidemiological significance and could influence control programmes, for example, by allowing the interval between doses to be extended.

4.2. Histopathological confirmation of infertility in surviving adults

Triclabendazole is a BZD anthelmintic. By making an analogy with other anthelmintic BZDs, it is inferred that TCBZ binds to β-tubulin molecules and alters biological processes that are dependent on microtubules. There is evidence supporting this hypothesis. Morphological studies of the tegument, vitelline cells and testes of liver flukes after treatment with the active sulphoxide metabolite of TCBZ (TCBZ.SO) showed an inhibition of mitosis in the spermatogenetic and vitelline cells (Stitt and Fairweather, 1992, 1996). Along with these observations, alterations in the transport processes in the tegumental system led to severe disruption of the tegumentary surface, ending in a complete loss of the tegument (Stitt and Fairweather, 1993, 1994). Immunolocalization studies showed the loss of tubulin staining in the tegumental syncytium after TCBZ.SO treatment (Robinson et al., 2002; McConville et al., 2006). These results suggest that the microtubules may depolymerise, which, in turn, would hinder the movement of secretory bodies to the tegumental surface (Brennan et al., 2007). This process is fundamental to maintain the integrity of the membrane surface and its interruption could explain the morphological changes observed. In the histopathological analysis in the present study following treatment with TCBZ with sub-therapeutic doses, due to the possible depolymerisation of microtubules and consequent inhibition of mitosis, there were marked changes to the testes and vitelline follicles. Changes were present at 14 days post-treatment, suggesting an irreversible change causing infertility, but not compromising the vitality of the flukes.

4.3. Effect of adult presence and host immunity

It has been postulated that the active presence of surface antigens is an important source of parasitic antigens available for the immune system. They can be taken up and processed by antigen-presenting cells, dendritic cells and macrophages, and later be presented to T helper cells. Also, the excretion–secretion products (ESP) of nematodes, trematodes and cestodes contribute to the immune-evasion strategies of parasites by different mechanisms (Lightowlers and Rickard, 1988). On the other hand, the ESP of *F. hepatica* can also exert direct immunesuppressive effects on anti-bacterial immunity through the activity of proteases on immune molecules independently of the Th2 cells (Brady et al., 1999). This mechanism is an obstacle to achieve an efficacious immunisation against bacterial and viral diseases in parasitized animals, since protection by Th1 cells is affected by Th2 induced by ectoparasites and helminths. A recent study reported that the immune response evoked by ESP from *Fasciola gigantica* was associated with a low but highly significant reduction in trematode counts ($P < 0.0001$); also, the size of the recovered flukes was small, suggesting that immunisation with ESP could be a safe and effective way to reduce the transmission of the infection (El Ridi et al., 2007). In this way, the results of the present work raise the possibility that the reduction of the number of flukes present after drug action can exert a constant stimulation of the immune response with little or no effect on animal production and can counteract future infections. An additional advantage is that the remaining flukes will be sterile, avoiding contamination of the environment and limiting future infections. Briefly, this effect could be similar to a live vaccine.

5. Conclusion

- Results of the present work are an interesting aspect of the anthelmintic effect of TCBZ on the trematode, *F. hepatica* and demand further studies of higher complexity in

order to evaluate aspects related to the reproducibility and the magnitude of such observations, as well as to the immune-productive and methodological consequences.

- Once the permanent and irreversible disruption of the reproductive system has been corroborated, it will be necessary to study aspects of parasite survival under such conditions in the liver of ruminants and their relation to immunological aspects that could determine a barrier to future infections. These observations represent a novel point of view and it is original in reference to therapy of parasitic diseases.
- Consequently, these observations will also prompt a review of certain methodological and interpretative aspects related to efficacy tests, where the only discriminative factor is the reduction of the adult parasite load. On one hand, histopathological studies could be complementary to the efficacy controlled test for TCBZ or other BZD formulations. This idea has also been put forward by Hanna et al. (2010). On the other hand, the possibility of developing a formulation that produces a substantial reduction of parasitic loads whilst, at the same time, the surviving specimens stimulate the host immune response and decrease the spreading of parasite eggs in the environment, offers an attractive alternative to the current treatment, which is based on the elimination of the parasites. Ultimately, the results of the present study offer a potential alternative to cases of drug failure.

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