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Tegumental alterations of *Fasciola gigantica* due to *in vitro* treatment with Ro-354

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Abstract. Triclabendazole is the drug of choice against *Fasciola* infections in humans and animals. However, parasite resistance against triclabendazole is spreading in veterinary field, and there are no drugs of comparable activity currently available for the treatment and control of fascioliasis. The efficacy of a new rhodanine derivative Ro-354 against adult *Fasciola gigantica in vitro* was investigated. One hour post incubation, scanning and transmission electron microscopic examination revealed an evident disruption of the tegument of *F. gigantica* as blebbing, swelling and furrowing. Moreover, an increase in severity of tegumental damage as sloughing and absence of spines was observed. In conclusion, Ro-354 shows potent activity against *F. gigantica in vitro*, and, the authors recommend carrying out more studies to detect its efficacy *in vivo*.

INTRODUCTION

Fascioliasis is a disease caused by the parasitic trematode *Fasciola hepatica* or *Fasciola gigantica* and is prevalent in temperate regions of the world. For a number of years it has posed a major problem in ruminant livestock production, but recently, largely due to climate change, there has been a worrying upsurge in the incidence and spread of the disease (Michell, 2002; Halferty *et al.*, 2008). Moreover, fascioliasis is thought to be an emerging serious human health problem in some countries (Mas-Coma *et al.*, 2005). In Egypt, infections are reported from several governorates (Ali *et al.*, 1974; Farag *et al.*, 1979; Haridy *et al.*, 1999; Esteban *et al.*, 2003). Prevalence of fascioliasis in Egypt is currently reported to be 2-17% (Robert & Tolan, 2006). In a study of approximately 3000 severely anaemic Egyptian children, 3% were infected with *F. gigantica* and 14-82% had peripheral eosinophilia (Mansour-Ghanaei *et al.*, 2003). Among individuals who presented with fever of unknown origin to an Egyptian hospital, 4% had *F. hepatica*. Fascioliasis is now considered epidemic throughout the Nile valley in Egypt with scattered endemic foci along the river (Helmy *et al.*, 2008).

F. hepatica and *F. gigantica* are the causative agents of fascioliasis. Both parasites were recorded in Egypt, one native and the other came with imported cows and sheep. In livestock, a multitude of problems occur, including blood component changes, hepatic pathogenesis and liver trauma, inflammatory responses, weight loss, decreased milk production and even death (Behm & Sangster, 1999; Schweizer *et al.*, 2005; Keiser *et al.*, 2007). The costs in the nineties of these symptoms to the livestock industry were put at approximately $ 3 billion dollars per year (Boray, 1994); that figure is likely to be much higher now.

Triclabendazole (TCBZ) is a benzimidazole derivative and is the current drug of choice for treating fascioliasis. Alternative

drugs, which are presently not available and there is considerable concern of drug resistance development. Triclabendazole resistance has been first reported from sheep and cattle in Australia in the mid 1990s and has been spreading over Europe, the Netherlands, UK, Ireland or Spain (Keiser *et al*., 2005; Alvarez-Sanchez *et al*., 2006; Brennan *et al*., 2007; Keiser & Morson, 2008). There is an increasing awareness that novel trematocidal drugs should be identified.

Ro-354 is a derivative of rhodanine, which belongs to an important group of heterocyclic compounds known as thiazolidinones. Ro-354 was previously investigated as having antischistosomal activity by Taha & Soliman (2007), Taha (2007) and Soliman (2008).

The data presented here is scanning and transmission electron microscope observations of tegumental changes in the flukes due to incubation *in vitro* with Ro-354. Examination of tegumental changes is important because the tegument is the interface with the host environment, it is the principal route by which Ro-354 can pass through the fluke and it plays an essential role in protecting the fluke against immune attack and anthelmintic action (Fairweather *et al*., 1999; Mottier *et al*., 2006).

MATERIALS AND METHODS

Drug

Ro-354 was synthesized at Chemistry department, Faculty of Science, Ain Shams University (Cairo-Egypt).

Parasite collection and preparation

Cattle isolate of *F. gigantica* was used for this study. It was originally obtained as a field isolate from an abattoir in El-Basatin slaughtered-house, Cairo-Egypt.

Adult flukes were removed from the bile duct and washed in several changes of warm (37ºC), sterile physiological saline. Whole flukes were transferred to fresh culture medium (RPMI 1640) containing RO-354 at a concentration of 100 µg/ml. RO-354 was initially prepared as a stock solution in dimethyl formamide (DMF) and was added to the culture medium to give a final solvent concentration of 0.1% (v/v). The controls were prepared by incubating whole flukes for one and three hours at 37ºC in RPMI 1640 culture medium containing 0.1% (v/v) DMF. Five flukes from each group were processed for scanning and transmission electron microscopy (SEM &TEM).

SEM observations

Adult flukes were rinsed with 0.9% NaCl (w/v) and fixed with 2.5% (v/v) glutaraldehyde in a phosphate buffer (pH 7.4) for 24 h at room temperature. After rinsing with PBS buffer, the specimens were washed with distilled water, dehydrated with ethanol and critical-point dried with liquid CO_2. The flukes were mounted on aluminium stubs, sputter-coated with gold at 20 nm thick, and observed in a high-resolution SEM (Joel SEM) at an accelerating voltage of 5 kV.

TEM observations

Immediately following incubation, all flukes (control and treated) were fixed overnight in 2.5% (w/v) glutaraldehyde in phosphate buffer (pH 7.4). The body regions were sliced transversely into strips l-2mm wide and the slices transferred to fresh fixative for the rest of the fixation period. The material was washed in phosphate buffer (pH 7.4) then post-fixed in 1.0 % osmuim tetroxide in phosphate buffer, dehydrated through ethanol and embedded in spurr low-viscosity resin. Ultrathin sections were cut on ultra-microtome, mounted on uncoated copper grids, double-stained with alcoholic uranyl acetate (5 min) and aqueous lead citrate (8 min) and viewed in a Joel transmission electron microscope operated at 100 kV.

RESULTS

SEM examination

Control flukes

The control specimen exhibited a normal surface morphology, both ventrally and dorsally. The tegument of the fluke is flat and smooth and the spines project clearly from the surface (Figs. 1-3). The oral and ventral

suckers showed normal structures with thick rims covered with transverse folds (Fig. 1). The ventral surface of the control flukes showed numerous broad spines with characteristic serrate tips (Figs. 2 and 3). Also, in the tail region, the surface is smooth and the peg-like spines are distributed sparsely over the surface (Fig. 4).

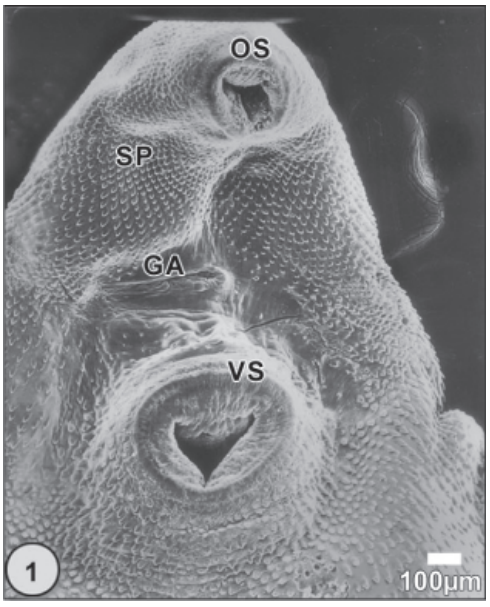

Figure 1. The apical surface showing smooth oral (OS) and ventral (VS) suckers with thick rims covered with transverse folds. Note also genital atrium (GA) and numerous sharp spines (SP). Bar =100µm

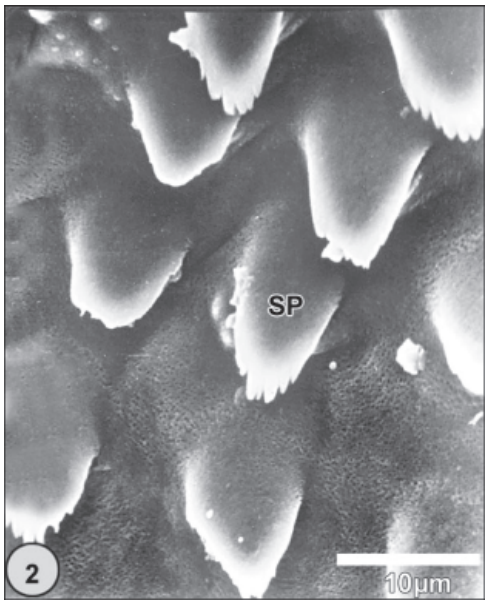

Figure 2. ridged tegument with numerous broad and flat spines (sp) projecting beyond the surface of the tegument with characteristic serrate tips. Bar=10µm

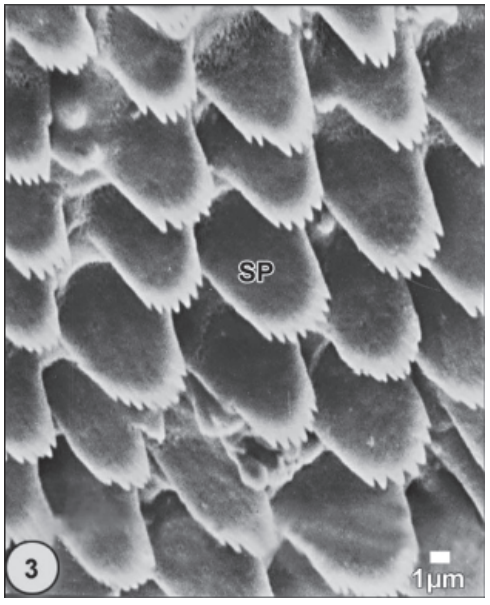

Figure 3. Showing another part of the ventral surface of normal untreated fluke with closely spaced spines (sp) in alternative pattern (behind and around ventral sucker). Bar= 1µm

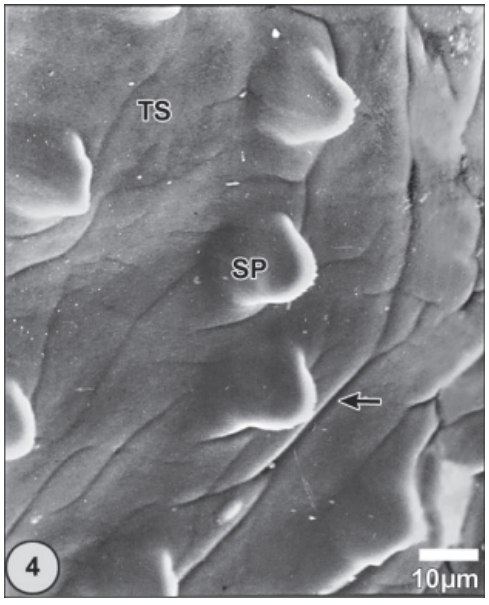

Figure 4. The most posterior ventral region of the tegument the distance between spines (sp) increase with tegumental surface appears folded. Spines are shorter with fewer tips. Bar= 10µm

Ro-354 treated flukes

The tegument of Ro-354-treated flukes showed a great damage everywhere (Figs. 5-14). The treated flukes revealed severe damage of the oral and ventral sucker. The damaged oral sucker appeared with swollen rim and the interior tegument has sloughed off to expose the basal lamina (Figs. 5 and 7). The area around sucker is peppered with blebs (Fig. 7).

The ventral sucker showed severely damaged rim. In some worms, the rim of the ventral sucker lost the transverse folds while the margin appeared abnormal and hazy (Fig. 6). In other treated worms, removal of the tegumental covering, sloughing and blebbing of the interior tegument of sucker had occurred. In some area near to the rim, the tegument has completely sloughed away, revealing the basal lamina (Fig. 8).

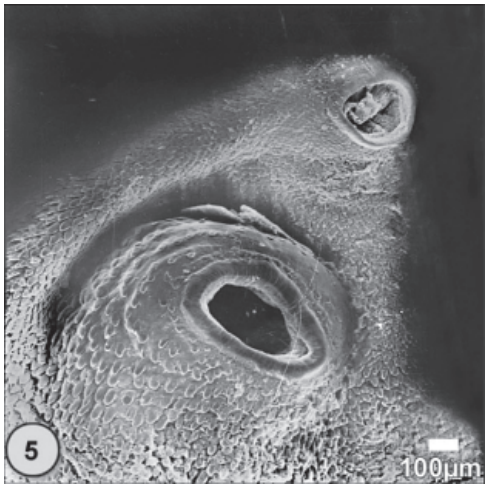

Figure 5. The apical cone surface showing distortion of the oral and ventral suckers. Bar= 100µm

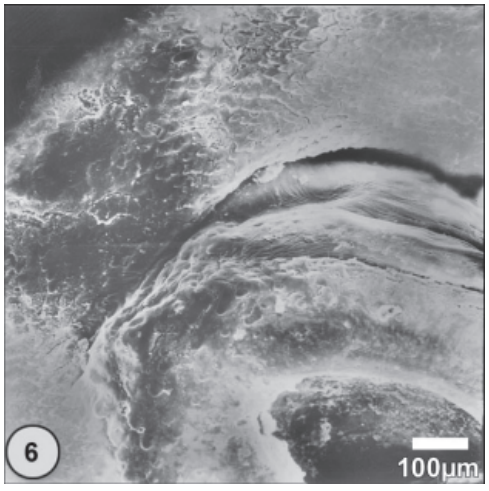

Figure 6. Showing smooth, flattened areas (arrows) on the surface along the lateral margins of the fluke. Note severe swelling of the tegument causing disappearance of spines, note also tremendous effect on the ventral sucker (VS). Bar=100µm

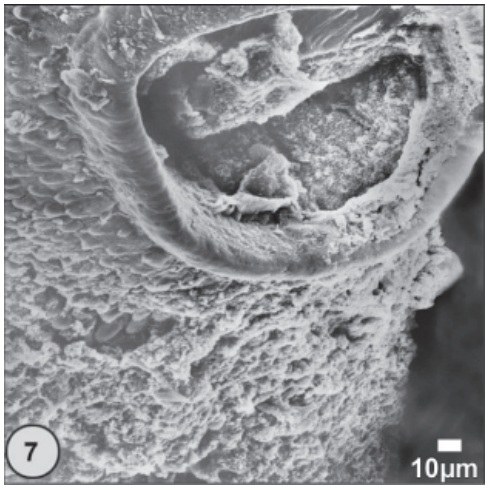

Figure 7. Showing damaged oral sucker with swollen rim and interior tegument has been sloughed off to expose the basal lamina (BL). Note also the surface around oral sucker is peppered with blebs. Bar=10µm

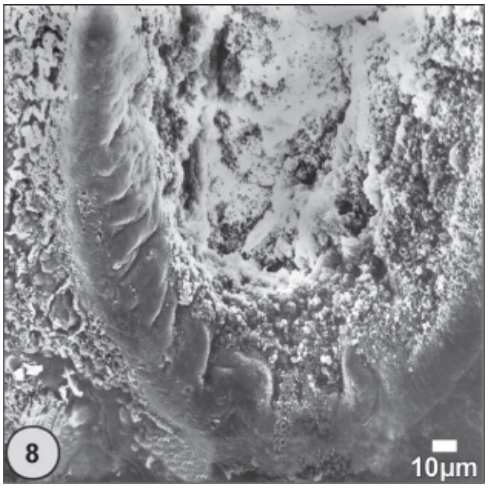

Figure 8. Ventral sucker with blebbing interior severe erosion of the muscular rim (arrow). Bar=10µm

Moreover, the tegument was swollen and the spines appeared to be submerged by the swollen tegument between them (Figs. 9 and 10). Extensive blebbing carpeted the tegumental surface which was largely evident on the lateral margins (Fig. 9). In some regions, the tegument was severely swollen to the extent that the spines were no longer visible (Figs. 6 and 11).

Also, in the tail region, the surface destruction was noted everywhere, from blebbing to sloughing and erosion in both specimens collected after one or three hours post treatment (Figs. 12-14).

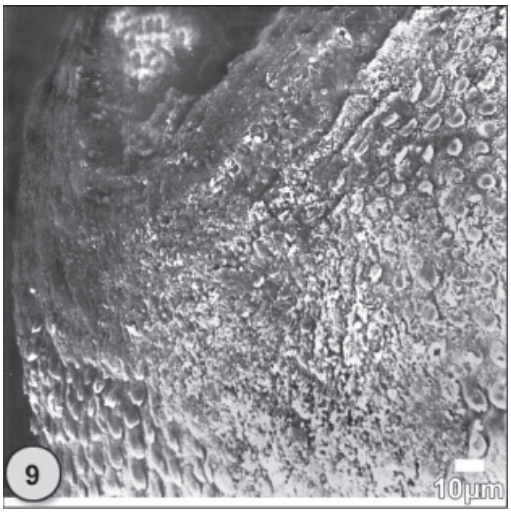

Figure 9. The region around oral sucker (OS) showing extensive blebbing. Bar=10µm

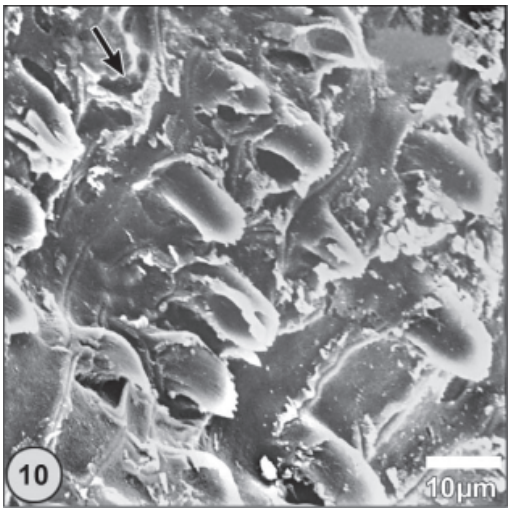

Figure 10. Showing severe disruption of the tegumental surface. Some spines (sp) are partially submerged by the swollen tegument around it and some empty spine sockets (ESS). Bar=10µm

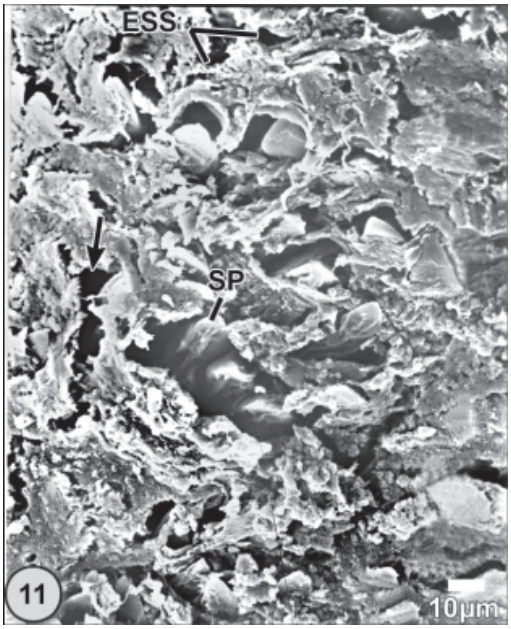

Figure 11. Spines completely submerged, empty spine sockets (ESS). Bar=10µm

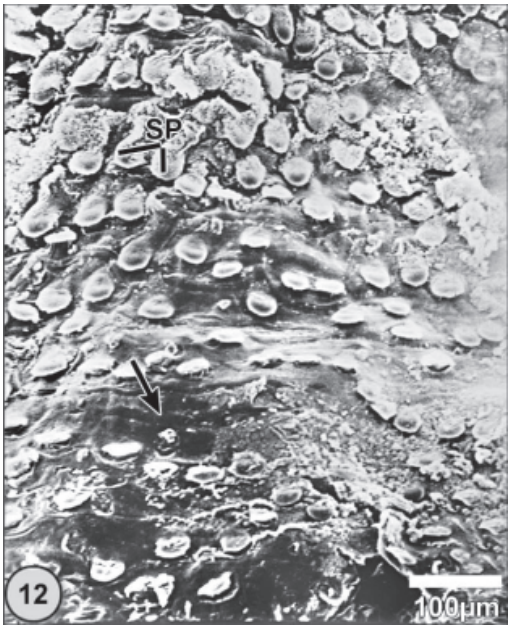

Figure 12. Tremendous effect on the tegumental surface in tail region. Note swelling Sloughing and spines submersion. Bar=100µm

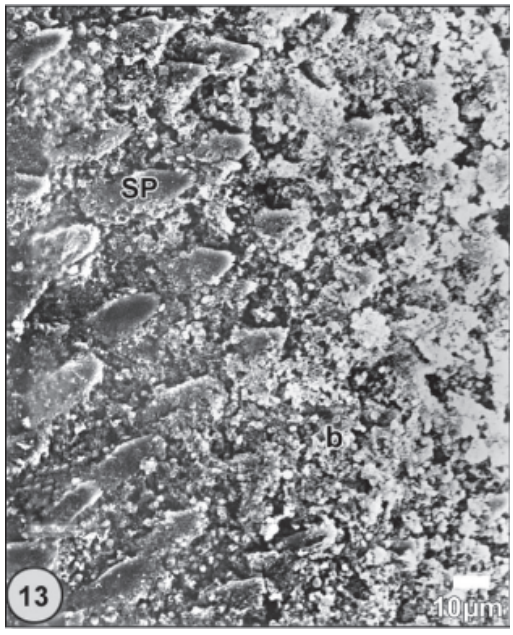

Figure 13. Severe blebbing on the posterior ventral region. Bar=10 µm

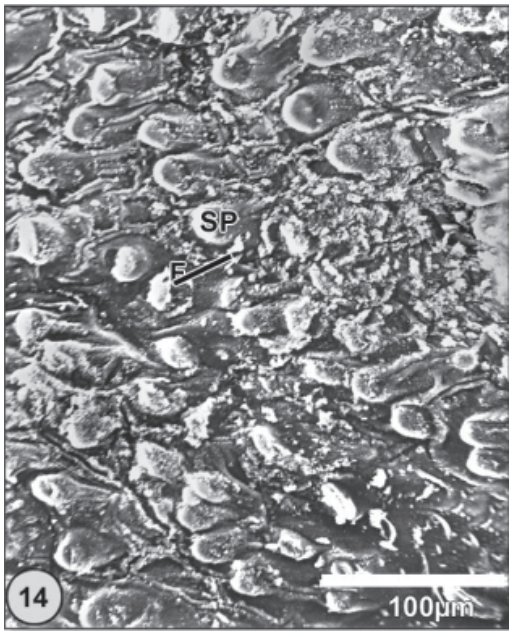

Figure 14. Showing erosion of the surface to such extent no tegument remained, only a mass of fibrous structures. Bar=100 µm

The changes are more noticeable ventrally than dorsally. The lateral margins of the flukes are more heavily disrupted than the central region of the body. The order of severity of the disruption caused by the compound varied from region to region.

TEM examination of control flukes

The tegumental syncytium is bounded distally by the apical plasma membrane (APM). The basal plasma membrane is invaginated to form long basal enfolds, which reach almost to the tegumental apex. Mitochondria are present throughout the syncytium, but are concentrated basally. Two types of secretory bodies are present: T_1 and T_2 types. Circular and longitudinal muscle layers are situated beneath the syncytium: the muscle blocks are closely packed; though they are permeated by cytoplasmic connections between the cell bodies and syncytium. (Fig. 15)

Treatment with Ro-354

The tegumental syncytium was severely disrupted, with the basal infolds were swollen

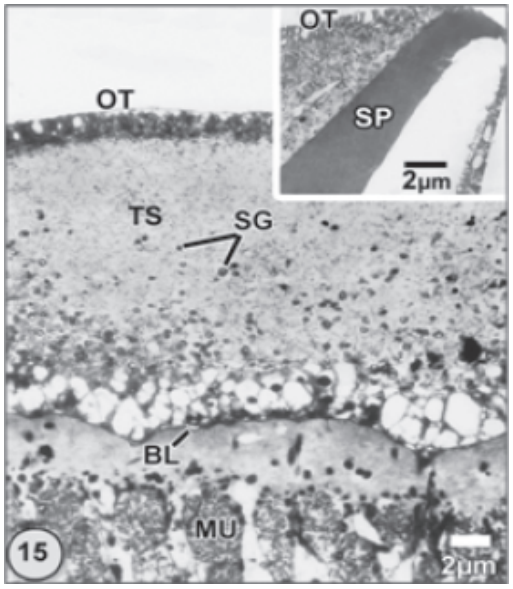

Figure 15. Low magnification of the syncytium showing normal morphology. The basal lamina (BL) and secretory granules (SG). T1, T2 secretory granules – APM apical plasma membrane. Inset shows the apical region of syncytium, with spines (sp). Mu, the muscle blocks are tightly-packed, with little space between them. Bar=2 µm

and were filled with a flocculent materials. Vacuolation increased in the outer tegumental regions. The apical regions of the syncytium were eroded in many regions and blebs were present (Figs. 16 & 17). The mitochondria were more electron-dense than normal, as well as being swollen and rounded. Also the number of type 2 secretory bodies was reduced. Beneath the syncytium, small spaces were present between the muscle bundles and the tissues and were more loosely-packed and less well-organized than normal (Fig. 18), subtegumental muscles obviously undergo lysis and severe disruption.

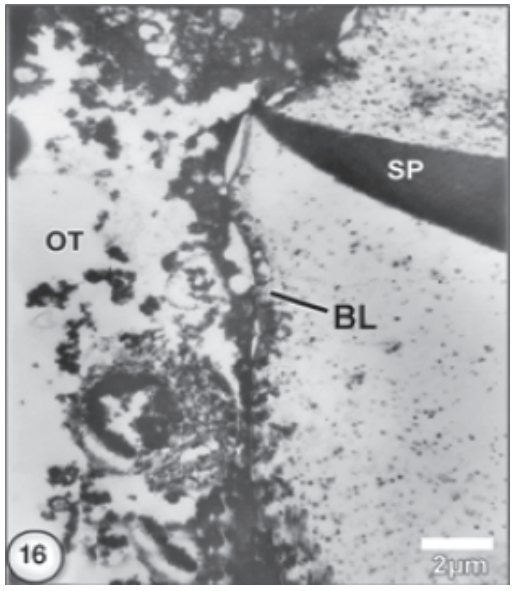

Figure 16. Swelling of the tegument causing complete submersion of the spines (SP). Note APM erosion or sloughing. Bar=2 µm

DISCUSSION

The use of SEM in this study enabled an overview of the surface changes induced by the new rhodanine derivative Ro-354 to be made. Regional differences in response to drug action were observed, in that the ventral surface was more severely disrupted than the dorsal. These regional differences are similar to those described previously after the treatment of both *F. hepatica* and *F. gigantica* with TCBZ*SO (Stitt & Fairweather, 1993; Meaney *et al.*, 2002; Halferty *et al.*, 2008). In previous studies with other fasciolicides (Fairweather *et al.*, 1987; Anderson & Fairweather, 1988; Skuce & Fairweather, 1990; Buchanan *et al.*, 2003; Meaney *et al.*,

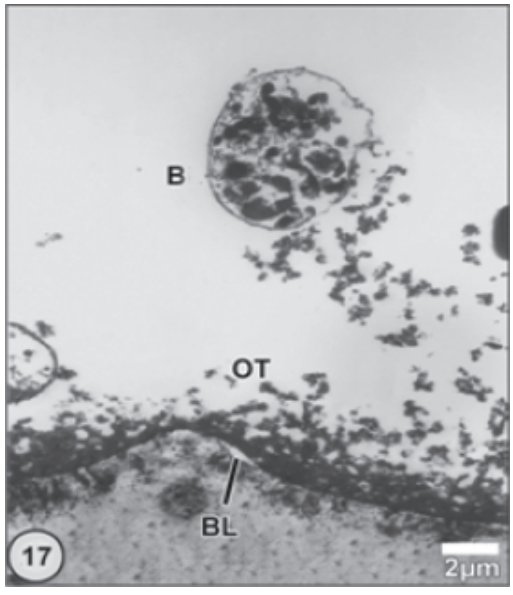

Figure 17. Sloughing of the outer tegumental layer with blebbing (B) in the form of large membrane bound vacuoles containing numerous electron dense granules. Bar=2 µm

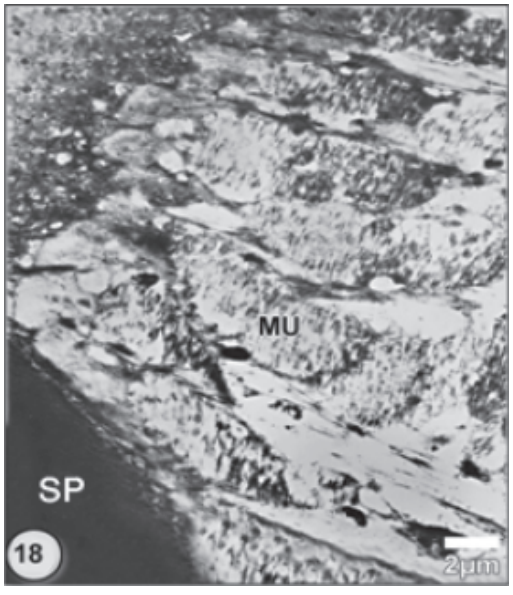

Figure 18. Generally affected muscle blocks gained pale appearance due to damage of the muscle fibers, spine (SP). Bar=2 µm

2003, 2006; McConville *et al.*, 2006, 2007, 2008; Toner *et al.*, 2008). Blebbing is believed to be a device for shedding surface membrane damaged by drug action and allowing to be replaced by fresh membrane.

The swelling of the tegument probably has an osmotic basis: it is reflected in the swelling of the basal infolds in the syncytium and is due to the damage caused to the tegumental membrane and associated ion pumps. The swollen condition of the mitochondria may have a similar basis. Large spaces appear between the cells and latter lose connections with the surrounding parenchyma. A similar condition was noticed after incubation with the deacetylated (amine) metabolite of diamphenthide (Fairweather *et al.*, 1986; Anderson & Fairweather, 1995) and with a combination of half-strength Clorsulen plus TCBZ*SO (Meaney *et al.*, 2007).

Surface changes induced by Ro-354 induced swelling and furrowing of the tegument, blebbing and some disruption to the spines, sloughing and spine loss. These changes are typical to drug action, as they have been seen.

A decrease in the number of secretory bodies was observed in the syncytium. This is most probably due to a decline in their synthesis in the tegumental cell bodies. A similar reduction of secretory bodies has been observed with TCBZ (Stitt & Fairweather, 1994; Meaney *et al.*, 2007) and with two other benzimidazole derivatives, ABZ.SO and compound alpha SO (Buchanan *et al.*, 2003; McConville *et al.*, 2006, 2007). The consequences of this are that the fluke is unable to maintain the integrity of the apical membrane osmotic changes are likely to ensure and, in the extreme cases, the tegument is sloughed off.

In conclusion, the present study has shown that RO-354 causes severe disruption of the tegument in liver flukes. The high damage occurs after 3 hours of treatment. The results confirmed previous SEM and TEM microscope observations on liver flukes using other drugs. Finally the results of the present study should be confirmed by more investigation on the effects of RO-354 on liver flukes *in vivo*.

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