A transmission electron microscope study on the route of entry of triclabendazole into the liver fluke, *Fasciola hepatica*

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SUMMARY

Uptake of triclabendazole by the liver fluke, *Fasciola hepatica* has been studied by experiments designed to block either oral uptake of drug, by use of ligatures, or trans-tegumental diffusion, by allowing the drug to bind to an excess of bovine serum albumin (BSA) in the medium. Changes to the tegumental system, musculature and gut were assessed using transmission electron microscopy. Flukes were incubated *in vitro* for 24 h in TCBZ.SO (15 μg/ml). Disruption to the tegument and muscle was similar in ligatured and non-ligatured flukes, suggesting that closing the oral route did not affect drug uptake. The ultrastructure of the gastrodermal cells remained unchanged. Non-ligatured flukes were also incubated for 24 h *in vitro* in TCBZ.SO (15 μg/ml) in the presence of red blood cells (RBCs). Oral uptake of blood was demonstrated, but gut ultrastructure remained normal, whereas the tegument was severely disrupted. In separate experiments, ligatured and non-ligatured flukes were incubated in TCBZ.SO (15 μg/ml) in the presence of BSA (30 mg/ml) for 24 h *in vitro*. There was a marked decrease in the degree of tegumental disruption observed compared with TCBZ.SO action alone; again, the gut remained normal. The findings support previous morphological and pharmacological studies indicating that trans-tegumental uptake of triclabendazole predominates in the liver fluke.

Key words: *Fasciola hepatica*, triclabendazole, drug uptake, ligature, bovine serum albumin, red blood cells, transmission electron microscopy.

INTRODUCTION

Fascioliasis, the parasitic infection caused by the liver fluke, *Fasciola hepatica*, continues to threaten animal health and the sustainability of the livestock industry, for two reasons. The disease has shown a marked rise in recent years (possibly due to climate change) and has spread to areas not previously affected by fluke (Pritchard *et al*. 2005). Control is via the use of drugs, and triclabendazole (TCBZ) in particular, but increased reports of resistance to this drug put continued effective control measures at risk (Fairweather, 2005, 2009). In order to make best use of TCBZ and perhaps design strategies to deal with resistance, a better understanding of drug action is needed. There have been considerable advances in the last few years in relation to the pharmacokinetics of TCBZ absorption, plasma kinetics, tissue distribution, metabolism and elimination in the host (Hennessy *et al*. 1987; Virkel *et al*. 2006; Mestorino *et al*. 2008). Also, there has been the demonstration that the fluke itself is able to metabolize TCBZ (Mottier *et al*. 2004; Robinson *et al*. 2004). But before the metabolism and inactivation of TCBZ can occur within the parasite, the drug must first enter the fluke. Drug uptake is an important aspect of pharmacokinetics and one that requires clarification. The current paper attempts to resolve this issue for TCBZ and *F. hepatica*.

An anthelmintic drug can enter a parasite by the oral route, across the tegument (or cuticle, in the case of nematodes) or via a combination of both these routes (Thompson and Geary, 1995). TCBZ and its metabolites are known to bind strongly to plasma proteins in the host (Mohammed Ali *et al*. 1986) and, considering the blood-feeding nature of *F. hepatica*, it might be assumed that oral uptake of drug would predominate over trans-tegumental uptake. The tegument plays an important role in the uptake of nutrients such as glucose and amino acids into the liver fluke, in addition to roles in sensory perception, osmoregulation and protection against the host's immune response, for example (Fairweather *et al*. 1999). The tegument of trematode and cestode parasites has been shown to be the primary site of anthelmintic entry (Alvarez *et al*. 2000, 2001, 2004, 2007; Mottier *et al*. 2003, 2006*a*). Similarly, the cuticle has been demonstrated as an important interface of drug uptake in nematodes (Ho *et al*. 1990; Sims *et al*. 1996; Cross *et al*. 1998).

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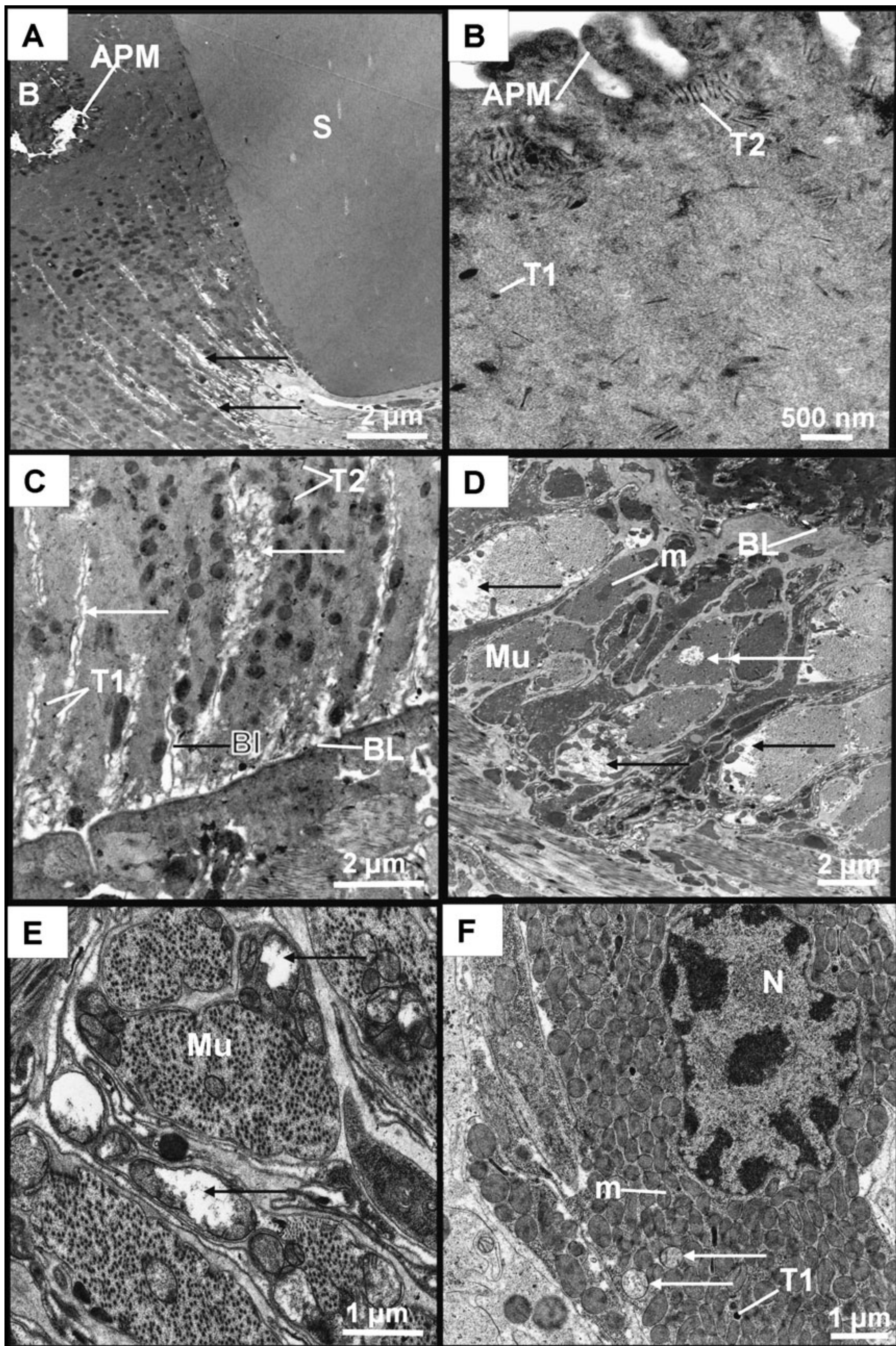

Fig. 1. For legend see opposite page.

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The aim of the present study was to determine whether the tegument or gut is the main route of entry of TCBZ.SO into *F. hepatica*. It is the second of a two-part investigation using a morphological approach: the first employed scanning electron microscopy (SEM) and has been published (Toner *et al*. 2009) and this second study utilized transmission electron microscopy (TEM). For these experiments, the importance of oral uptake was assessed by closing off the pharynx, thereby preventing oral ingestion of drug. This was achieved by securing a ligature around the oral cone of the fluke. To assess the contribution of trans-tegumental drug uptake, an excess of the plasma protein, bovine serum albumin (BSA) was added to the incubation medium, allowing drug to bind to it and so reduce the amount available for interaction with the fluke. In a further experiment, in order to clarify that oral feeding does occur *in vitro*, non-ligatured flukes were incubated in TCBZ.SO in the presence of red blood cells (RBCs) to stimulate oral uptake. Fine structural changes to the tegumental system and gastrodermal cells following the various drug treatments were evaluated by TEM and will be compared to the gross surface changes visualized by SEM.

MATERIALS AND METHODS

Isolate of F. hepatica

This study was carried out using the Cullompton isolate of *Fasciola hepatica*. It was obtained in 1998 as a field isolate from an abattoir in Cullompton, Devon, UK. It has been shown to be susceptible to albendazole (Buchanan *et al*. 2003), clorsulon (Meaney *et al*. 2003, 2004), triclabendazole (Robinson *et al*. 2002; McCoy *et al*. 2005; Halferty *et al*. 2008; McConville *et al*. 2009) and nitroxynil (McKinstry *et al*. 2003, 2007). Adult male Sprague-Dawley rats were each orally infected with 20 metacercarial cysts under light ether anaesthesia via a stomach tube.

In vitro *drug treatment*

Adult flukes were recovered from the bile ducts of the experimentally infected Sprague-Dawley rats under sterile conditions in a laminar flow cabinet. Flukes were washed several times in warm (37 °C) sterile NCTC 135 culture medium containing antibiotics (penicillin 50 IU/ml; streptomycin 50 μg/ml). Some flukes were ligatured by tying surgical thread just behind the oral sucker to prevent oral entry of triclabendazole. This technique has been used successfully in previous experiments on *F. hepatica* (Meaney *et al*. 2005*a*, *b*; Mottier *et al*. 2006*a*; Toner *et al*. 2009). Some flukes were left non-ligatured. All flukes were transferred to fresh NCTC 135 culture medium containing triclabendazole sulphoxide (TCBZ.SO) at a concentration of 15 μg/ml and incubated at 37 °C for 24 h. Ligatured and non-ligatured control flukes were incubated in NCTC 135 culture medium at 37 °C for 24 h. Controls at 0 h were also prepared. TCBZ.SO was used for the experiments as it is believed to be the principal metabolite of TCBZ and its concentration was chosen to correspond to maximum blood levels *in vivo* following a therapeutic dose of 10 mg/kg body weight (Hennessy *et al*. 1987).

In separate experiments, ligatured and non-ligatured flukes were washed several times in warm (37 °C) sterile NCTC 135 culture medium containing antibiotics (penicillin 50 IU/ml; streptomycin 50 μg/ml). Bovine serum albumin (BSA) (30 mg/ml) was added to fresh NCTC 135 culture medium containing TCBZ.SO at a concentration of 15 μg/ml and the solution left to stand for 5 min to allow the TCBZ.SO to bind. Ligatured and non-ligatured flukes were then incubated in the drug medium at 37 °C for 24 h. Ligatured and non-ligatured control flukes were incubated in NCTC 135 culture medium containing BSA (30 mg/ml) at 37 °C for 24 h. The concentration of BSA used was similar to that used by Mottier *et al*. (2006*a*) and equates to a physiological concentration of albumin in sheep plasma.

Fig. 1. Transmission electron micrographs (TEMs) of the tegumental syncytium, musculature and tegumental cells of adult non-ligatured and ligatured *Fasciola hepatica* treated *in vitro* with TCBZ.SO (15 μg/ml) for 24 h. (A) A bleb (B) is seen projecting from the apical plasma membrane (APM). Swelling of the mucopolysaccharide masses (arrows) surrounding the basal infolds is visible. S, spine. (B) There is an accumulation of secretory bodies below the apical plasma membrane (APM). Both T1 (T1) and T2 (T2) bodies are present, but there is a predominance of T2 secretory bodies. (C) Section through the basal region of the tegumental syncytium. The mucopolysaccharide masses (arrows) surrounding the basal infolds (BI) appear distended. A small number of T1 (T1) and T2 (T2) secretory bodies are present in the basal region. BL, basal lamina. (D) TEM showing muscle blocks (Mu) underlying the basal lamina (BL). The muscle bundles appear to be breaking down, with distinct areas of autophagic activity (arrows). In some muscle bundles the mitochondria (m) appear to have degraded (double arrow). (E) High-power micrograph of the muscle blocks (Mu). The muscle bundles appear to be breaking down, with distinct areas of autophagic activity (arrows). The muscle fibres appear more loosely-packed than normal. (F) TEM of a Type 1 tegumental cell in which there are few T1 secretory bodies (T1). Mitochondria (m) appear swollen and fill the cytoplasm of the cell. Some mitochondria are very rounded and electron-lucent (arrows). Golgi complexes are absent. N, nucleus.

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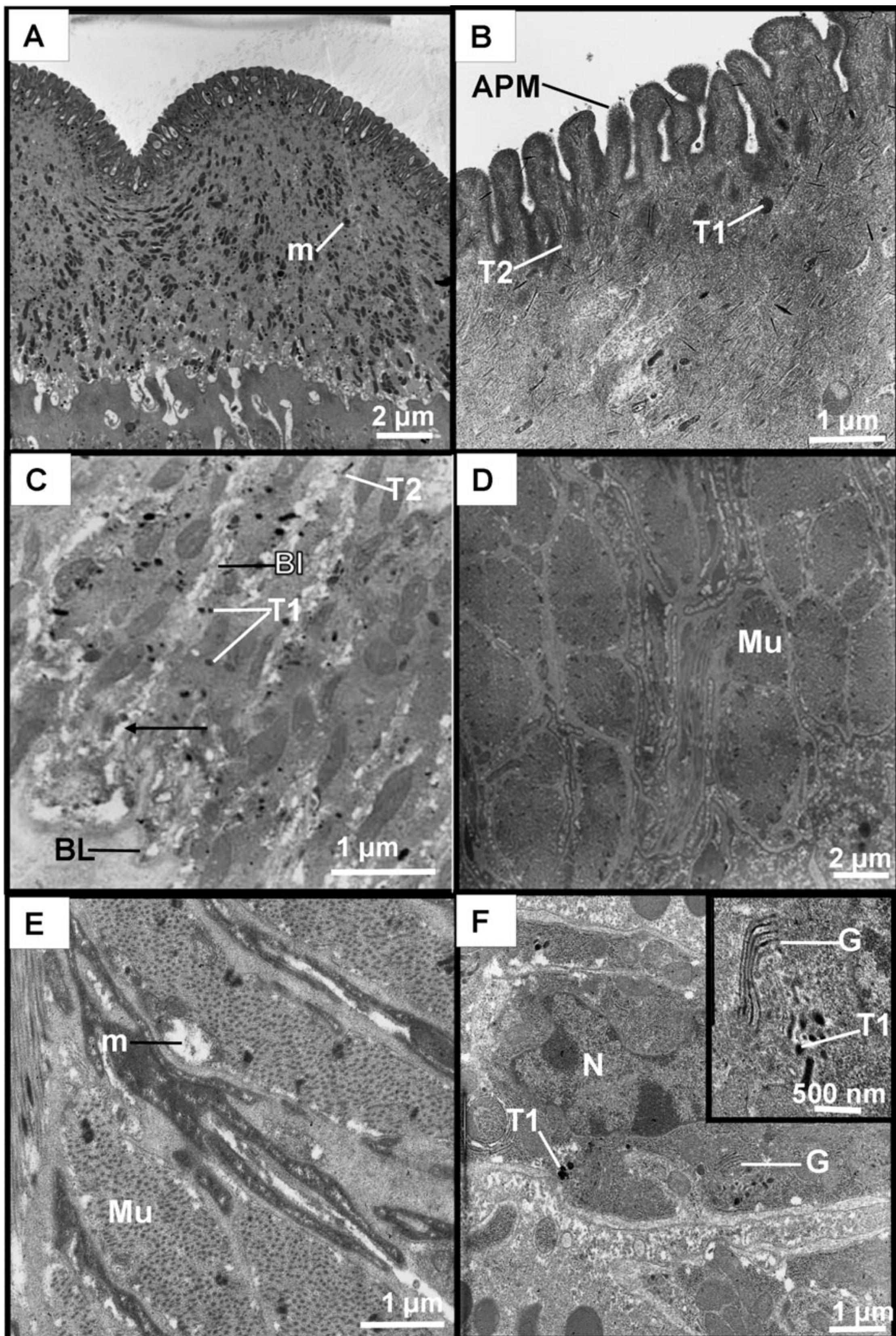

Fig. 2. For legend see opposite page.

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A further experiment involved the use of red blood cells (RBC's) to verify feeding in the flukes. Whole blood was collected from adult male Sprague-Dawley rats at necropsy using heparinized tubes to prevent clotting. Blood was centrifuged at 3354 ***g*** for 10 min and the supernatant discarded. From the remaining red blood cell fraction, 1·5 ml of RBC's were spread onto the bottom of sterile wells in a 6-well multi-well plate. Four ml of NCTC 135 culture medium containing triclabendazole sulphoxide (TCBZ.SO) at a concentration of 15 μg/ml was added to each well. Flukes displayed full gut contents on excision from the bile ducts of the rats (see Fig. 6A, Toner *et al*. 2009). Flukes were then washed in several changes of warm (37 °C) sterile NCTC 135 culture medium and allowed to regurgitate their gut contents (see Fig. 6B Toner *et al*. 2009). One fluke was then added to each well in the 6-well multi-well plate and allowed to graze on the RBC's for 24 h at 37 °C. At the end of the experiment, gut contents were present in all flukes, demonstrating that feeding had occurred (see Fig. 6C Toner *et al*. 2009). A control experiment was carried out in which flukes were allowed to graze on RBC's in the absence of TCBZ.SO for 24 h at 37 °C. A minimum of 6 intact flukes were fixed and processed for transmission electron microscopy (TEM) for each of the treatments.

Tissue preparation for transmission electron microscopy

Initially, the flukes were lightly flat-fixed for 30 min at room temperature in 4% (w/v) glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) containing 3% (w/v) sucrose. Flukes were then dissected into oral cone, anterior midbody, posterior midbody and tail regions, and transverse slices (3 mm in thickness) were taken from these sections. Specimens were then free-fixed for 4 h in 4% (w/v) glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) and subsequently washed in 0·1 M sodium cacodylate buffer (pH 7·4) containing 3% (w/v) sucrose at 4 °C overnight. Fluke sections were then post-fixed in 1% osmium tetroxide for 1 h and again washed in 0·1 M sodium cacodylate buffer (pH 7·4) at 4 °C overnight. Flukes were next dehydrated in an ascending series of ethanols, infiltrated and embedded in Agar 100 resin and polymerized for 48 h at 60 °C. Ultrathin sections (60–70 nm thick) were cut using a Reichert Ultracut E ultramicrotome, mounted on uncoated copper grids, double-stained with uranyl acetate and lead citrate, and viewed in a FEI CM 100 transmission electron microscope operating at an accelerating voltage of 100 keV.

RESULTS

Changes to the tegumental syncytium, the underlying tegumental cells and the somatic musculature

All the images are taken from the posterior midbody region of flukes as this was consistently the most affected region.

Non-ligatured and ligatured flukes treated with TCBZ.SO (15 μg/ml). There was no apparent difference in disruption to the ultrastructure of the tegumental syncytium, musculature or tegumental cell bodies between the ligatured and non-ligatured flukes and so the results will be discussed together (Fig. 1A–F). In the tegumental syncytium, there was some swelling of the mucopolysaccharide masses surrounding the basal infolds (Fig. 1A and C). Some blebbing of the apical plasma membrane (APM) occurred at the apical surface of the syncytium (Fig. 1A). There was an accumulation of T2 secretory bodies below the apical plasma membrane, with an apparent reduction in the number of T1 secretory bodies (Fig. 1B). A decrease in the number of secretory bodies at the base of the syncytium was also observed (Fig. 1C). In the subtegumental region, the musculature appeared extensively disrupted, in that the muscle bundles appeared to be breaking down, with distinct areas of autophagy and a loose arrangement of muscle fibres (Fig. 1D and E). Within the tegumental cells, mitochondria appeared swollen, they filled the cytoplasm and some seemed

Fig. 2. Transmission electron micrographs (TEMs) of the tegumental syncytium, musculature and tegumental cells of adult non-ligatured and ligatured *Fasciola hepatica* treated *in vitro* with TCBZ.SO (15 μg/ml) + bovine serum albumin (BSA) (30 mg/ml) for 24 h. (A) In the tegumental syncytium, there is minimal swelling of the mucopolysaccharide masses surrounding the basal infolds. Chains of mitochondria (m) and secretory bodies are distributed throughout the syncytium. (B) Both T1 (T1) and T2 (T2) secretory bodies are present below the apical plasma membrane (APM). (C) Basal region of the tegumental syncytium. The mucopolysaccharide masses (arrow) surrounding the basal infolds (BI) appear slightly swollen. T1 (T1) and T2 (T2) secretory bodies are present throughout the basal region. BL, basal lamina. (D) TEM showing muscle blocks (Mu). The muscle layers remain compact with no separation of the muscle bundles. (E) High-power TEM of the muscle blocks (Mu). The muscle fibres in the muscle blocks are tightly-packed. Blocks do not appear separate from each other. A mitochondrion (m) within one of the muscle bundles appears to be breaking down. (F) TEM of a Type 1 tegumental cell in which there are numerous T1 secretory bodies (T1). An active Golgi complex (G) is present. N, nucleus. *Inset* shows a Golgi complex, (G) producing T1 secretory bodies (T1).

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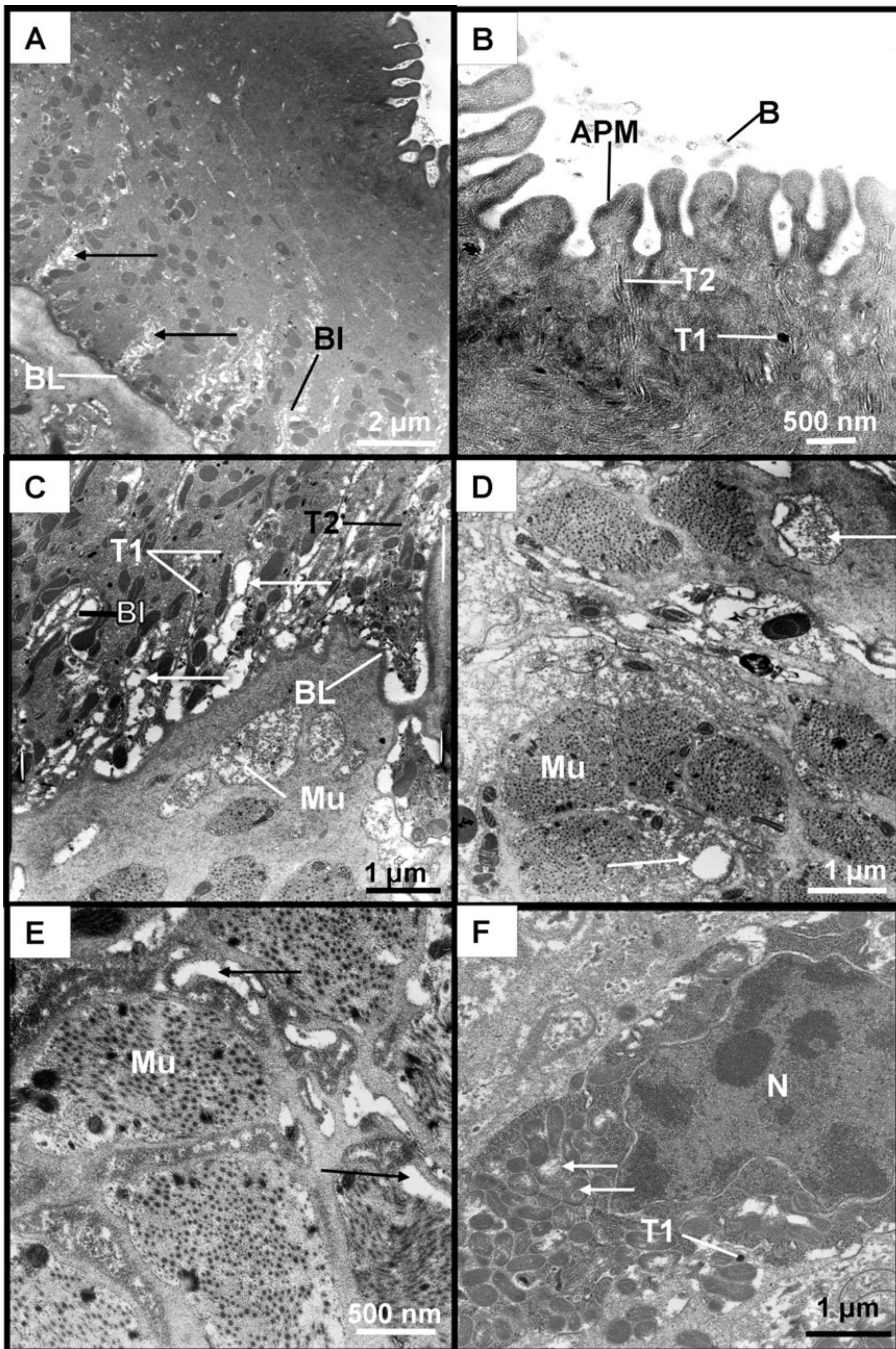

Fig. 3. For legend see opposite page.

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to be degrading (Fig. 1F). Secretory bodies were very sparsely distributed and there was a lack of Golgi complexes within the cell bodies (Fig. 1F).

Non-ligatured and ligatured flukes treated with TCBZ.SO (15 μg/ml)+BSA (30 mg/ml). Again, there was no apparent difference in disruption to the ultrastructure of the syncytium, musculature or tegumental cell bodies between the ligatured and non-ligatured flukes and so the results will be discussed together (Fig. 2A–F). Within the tegumental syncytium, there was very limited swelling of the mucopolysaccharide masses surrounding the basal infolds (Fig. 2A and C). T1 and T2 secretory bodies were present throughout the syncytium, both below the APM and in the basal region (Fig. 2B and C). The subtegumental musculature remained predominantly normal and the muscle fibres appeared tightly-packed (Fig. 2D and E). Within the tegumental cells, there were active Golgi complexes and reasonable numbers of secretory bodies produced by them (Fig. 2F).

Non-ligatured flukes treated with TCBZ.SO (15 μg/ml)+RBCs. Swelling of the mucopolysaccharide masses surrounding the basal infolds (Fig. 3A) was observed. There was an accumulation of T2 secretory bodies below the APM, and a reduction in the number of T1 secretory bodies present in this region (Fig. 3B). Some small blebs were observed projecting from the APM (Fig. 3B). A reduction in the number of secretory bodies at the base of the syncytium was observed compared to control specimens (Fig. 3C). The subtegumental musculature appeared very diffuse: the muscle blocks were more widely separated and contained fewer muscle fibres than normal (Fig. 3D and E). The tegumental cells contained very few T1 secretory bodies and Golgi complexes were absent. The mitochondria appeared swollen and rounded and seemed to fill the cytoplasm of the cell; some mitochondria showed signs of degeneration (Fig. 3F).

Non-ligatured and ligatured flukes incubated with BSA. There was no apparent difference in disruption to the ultrastructure of the tegumental syncytium, musculature or tegumental cell bodies between the ligatured and non-ligatured flukes and so the results will be discussed together (Fig. 4A–F). The tegumental syncytium showed little evidence of disruption: secretory bodies were present throughout the syncytium and the mucopolysaccharide masses and basal infolds remained unaffected (Fig. 4A–C). The muscle bundles appeared normal, with no indication of muscle breakdown (Fig. 4D and E). Within the tegumental cells, active Golgi complexes and numerous secretory bodies were present (Fig. 4F), and the mitochondria appeared normal.

Non-ligatured flukes incubated in the presence of RBCs. There was a normal distribution of T1 and T2 secretory bodies throughout the tegumental syncytium (Fig. 5A–C). The basal infolds, their associated mucopolysaccharide masses and the mitochondria appeared normal (Fig. 5C). Muscle blocks in the subtegumental region showed a typical organization with closely-packed muscle fibres (Fig. 5D and E). The tegumental cells appeared synthetically active and contained Golgi complexes and numerous secretory bodies (Fig. 5F). The mitochondria within the tegumental cells appeared normal.

0 h and 24 h non-ligatured and ligatured untreated controls. The tegumental syncytium, tegumental cells and somatic musculature of 0 h and 24 h non-ligatured and ligatured control specimens retained a normal ultrastructure, as described by Threadgold (1963, 1967) and Fairweather *et al.* (1999).

Changes to the gastrodermal cells

The results are presented in Fig. 6A–F. Following examination of all flukes in the present study, whether ligatured or non-ligatured, treated with TCBZ.SO, TCBZ.SO+BSA, TCBZ.SO+RBC, BSA or RBCs, the gut ultrastructure remained normal (Fig. 6A–F). Secretory bodies were present and the mitochondria and cisternae of granular

Fig. 3. Transmission electron micrographs (TEMs) of the tegumental syncytium, musculature and tegumental cells of adult non-ligatured *Fasciola hepatica* treated *in vitro* with TCBZ.SO (15 μg/ml)+red blood cells (RBCs) for 24 h. (A) In the tegumental syncytium, the mucopolysaccharide masses (arrows) surrounding the basal infolds (BI) appear swollen. BL, basal lamina. (B) There is an accumulation of T2s (T2) below the apical plasma membrane (APM), and an apparent decrease in the number of T1 secretory bodies (T1) present. Blebs (B) can be seen protruding from the surface membrane. (C) Basal region of the syncytium. The mucopolysaccharide masses (arrows) surrounding the basal infolds (BI) appear swollen. A few T1 (T1) and T2 (T2) secretory bodies are dispersed throughout the basal region. The underlying muscle blocks (Mu) appear to be breaking down. BL, Basal lamina. (D) TEM showing muscle blocks (Mu) containing distinct areas of muscle breakdown (arrows). (E) High-power TEM of the muscle blocks (Mu). There are notable areas of muscle degradation (arrows) and the muscle fibres are loosely arranged. (F) TEM of a Type 1 tegumental cell with few T1 secretory bodies (T1) and no Golgi complexes. The mitochondria appear swollen and rounded (arrows) and seem to fill the cytoplasm. N, nucleus.

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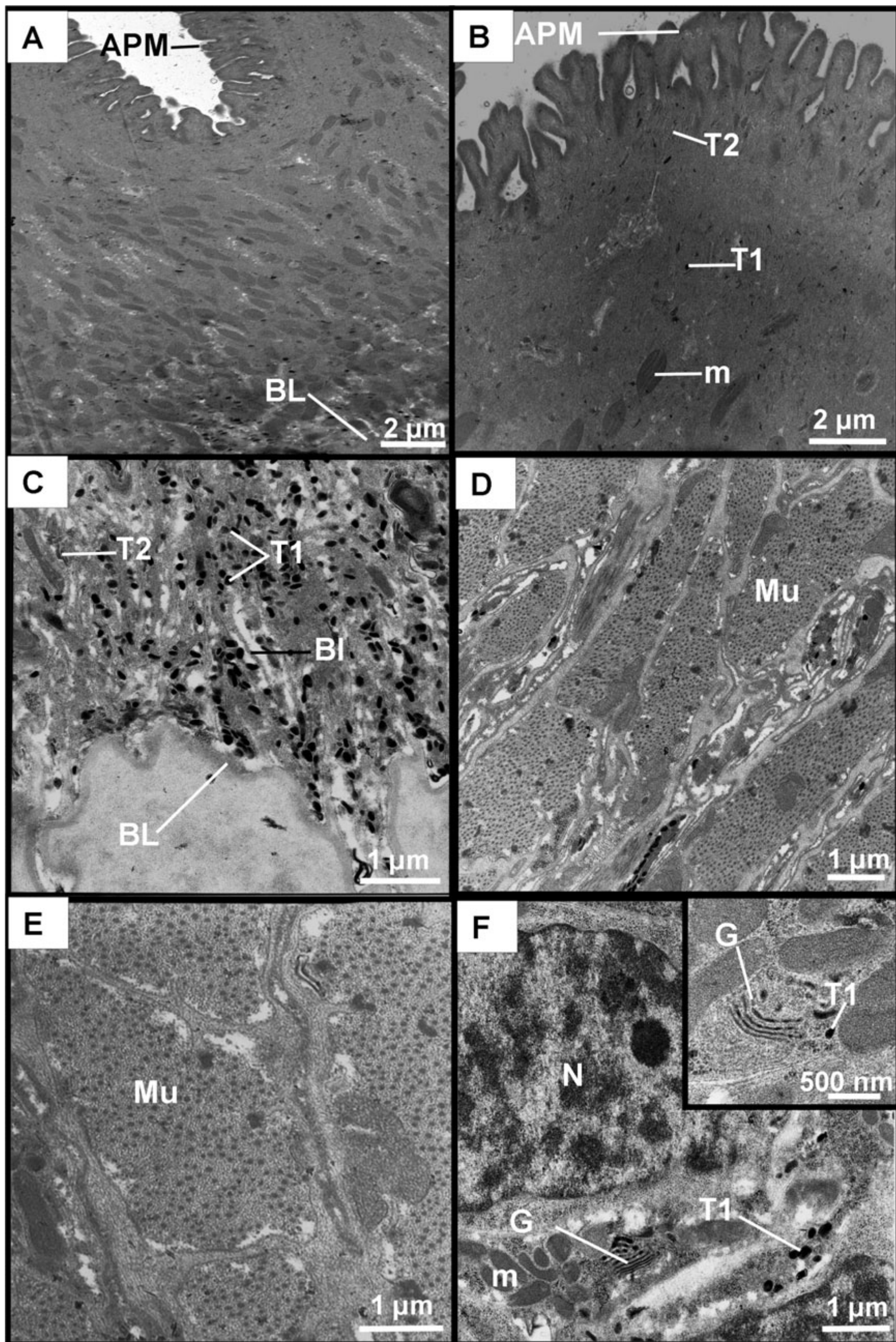

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endoplasmic reticulum were unaffected. The lamellae retained a normal morphology and projected into the gut lumen from the epithelial lining (Fig. 6A–F).

The gastrodermal cells of the 0 h and 24 h non-ligatured and ligatured control flukes also displayed a normal morphology, corresponding to that described by Robinson and Threadgold (1975).

Summary of results

The results of the various treatments are summarized in Table 1.

DISCUSSION

The aim of this study was to determine the major route of uptake of TCBZ.SO by the liver fluke via its effects on the fine structure of the tegument and gastrodermis. The importance of oral versus trans-tegumental drug uptake was investigated by isolating each route via a series of different experiments. Ultrastructural changes to the two tissues were evaluated by means of TEM. The tegument consistently appeared to be the most affected of the two surfaces, with the gut retaining its typical ultrastructure throughout the drug treatments. These observations will be discussed in relation to a parallel SEM study (Toner *et al*. 2009) and a previous pharmacological study (Mottier *et al*. 2006*a*) on drug uptake by *F. hepatica*.

Preventing the oral uptake of drug by means of a ligature did not affect the ability of the drug to enter the fluke and disrupt internal tissues, as evidenced by the similar level of disruption induced in both non-ligatured and ligatured flukes treated with TCBZ.SO. This is consistent with the result of a previous absorption kinetics study, which showed that statistically similar concentrations of TCBZ.SO were reached within non-ligatured and ligatured flukes (Mottier *et al*. 2006*a*). Moreover, in the present study there was no difference in disruption between TCBZ.SO-treated ligatured and non-ligatured flukes compared with those flukes incubated in the drug in the presence of RBCs. So, although feeding was seen to be taking place, allowing the oral uptake of drug, the gut remained unaffected.

The presence of an excess of BSA significantly reduced the morphological effects of TCBZ.SO: in fact, the tissues appeared relatively normal. Again, this is consistent with the uptake data, which showed that there was an 85% reduction in TCBZ.SO uptake following incubation in the presence of BSA (Mottier *et al*. 2006*a*). The current TEM results complement those of a parallel SEM study (Toner *et al*. 2009) and the combined data from the 3 studies highlight the importance of the tegument as the main route of entry of TCBZ.SO into the fluke.

In the present study it was the tegument which was most severely affected. The main changes seen within the tegumental syncytium were surface blebbing, an apical accumulation of T2 secretory bodies, a decrease of secretory bodies in the basal region and swelling of the mucopolysaccharide masses surrounding the basal infolds. In the tegumental cells, there was a decrease in secretory activity, as evidenced by the absence of Golgi complexes and lack of secretory bodies, together with some degenerative changes. The lowered production of secretory bodies would account for the decreased numbers seen in the syncytium, particularly of the T1 type. The accumulation of T2 bodies just beneath the apical plasma membrane is indicative of a stress response by the fluke and is typical of the reaction to fasciolicide drugs (Fairweather *et al*. 1986; Stitt and Fairweather, 1994; Buchanan *et al*. 2003; McKinstry *et al*. 2007, 2009; Meaney *et al*. 2004, 2005*b*, 2007; Halferty *et al*. 2009; McConville *et al*. 2006, 2008, 2009; O'Neill *et al*. 2009). The response is an attempt to maintain the integrity of the membrane, but the lack of T1 bodies suggests that this process cannot continue indefinitely. Ultimately, this would lead to the shedding of damaged membrane (by blebbing) and, indeed, this was seen.

The ultrastructural changes are compatible with the surface changes described previously (Toner *et al*. 2009). In that study, swelling and blebbing of the

Fig. 4. Transmission electron micrographs (TEMs) of the tegumental syncytium, musculature and tegumental cells of adult non-ligatured (A, B, D, E) and ligatured (C, F) *Fasciola hepatica* treated *in vitro* with BSA (30 mg/ml). (A) Within the tegumental syncytium, the mitochondria appear normal and there is no swelling of the mucopolysaccharide masses surrounding the basal infolds. APM, apical plasma membrane; BL, basal lamina. (B) Apical region of the syncytium. Both T1 (T1) and T2 (T2) secretory bodies are present below the apical plasma membrane (APM). m, mitochondrion. (C) Section through the basal region of the tegumental syncytium. The mucopolysaccharide masses surrounding the basal infolds (BI) appear normal. T1 (T1) and T2 (T2) secretory bodies are distributed throughout the basal region. BL, basal lamina. (D) TEM of the muscle blocks (Mu) beneath the basal lamina. The muscle exhibits a normal morphology. (E) High-power TEM of the muscle fibres in the muscle blocks (Mu): they appear normal. (F) TEM of a Type 1 tegumental cell containing many T1 secretory bodies (T1) and an active Golgi complex (G). The mitochondria (m) appear normal. N, nucleus. Inset shows a Golgi complex (G) producing T1 secretory bodies (T1).

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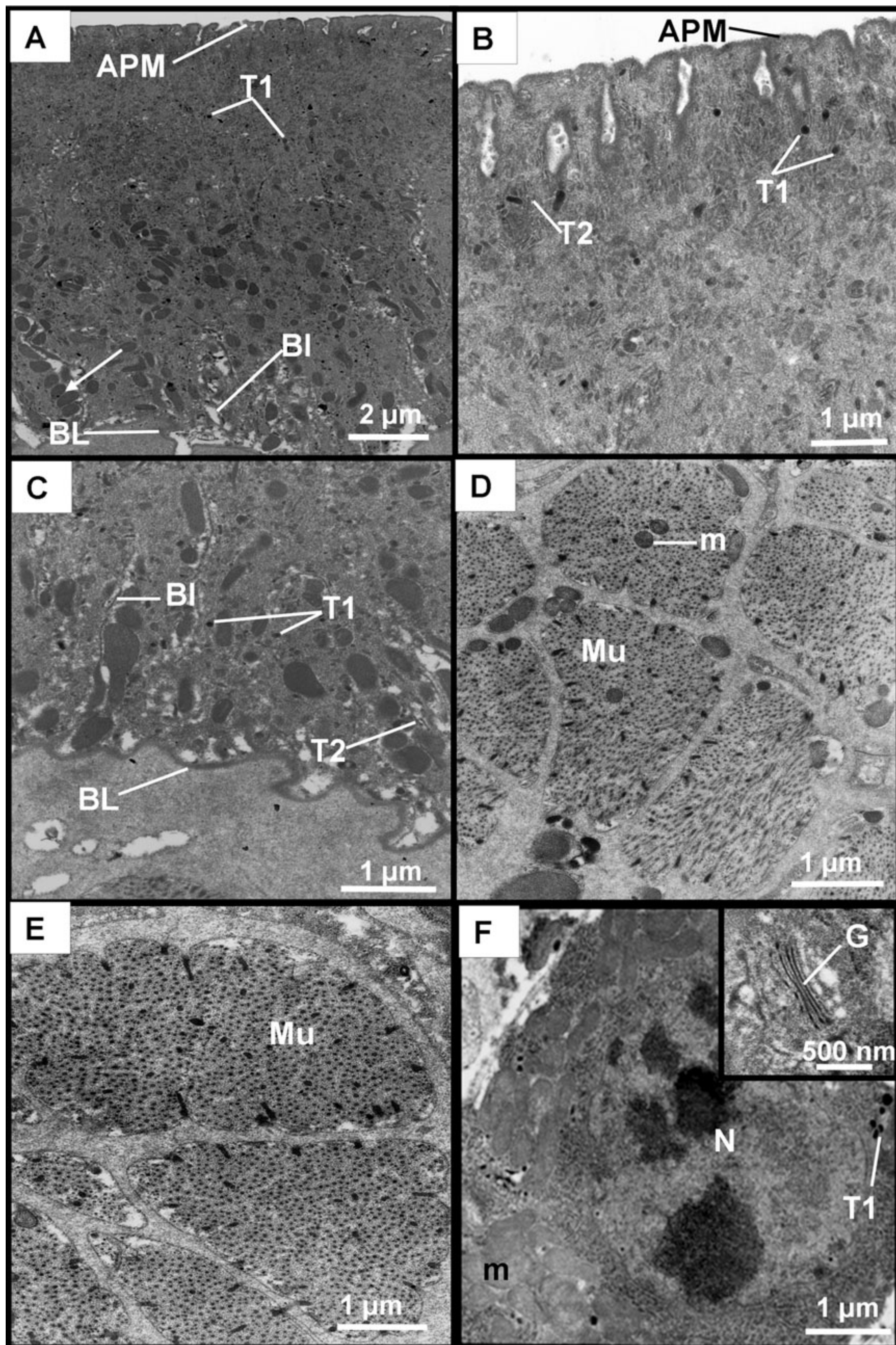

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tegument was observed in the same body region as examined in the present study (i.e. the posterior midbody). Swelling of the tegument can be linked to the swelling of the mucopolysaccharide masses in the syncytium; the masses, along with the basal infolds, play an important osmoregulatory role for the fluke (Threadgold and Brennan, 1978; Skuce *et al.* 1987). The cause of the blebbing has already been discussed. As in the SEM study, in which no gross changes to the gut surface were seen, the fine structure of the gut remained normal in non-ligatured flukes even though the oral route was open for drug uptake and even when the flukes were grazing on RBCs and taking in TCBZ.SO.

Despite the very strong binding of TCBZ and its metabolites to plasma proteins (>99%: Mohammed Ali *et al.* 1986), the evidence is overwhelmingly in favour of the tegument as the main route of uptake. This is also true for a derivative of TCBZ, namely, compound alpha (McConville *et al.* 2009). Monitoring changes over a 3-day period following *in vivo* treatment, there was severe disruption of the tegument, culminating in tegumental sloughing and degeneration of the tegumental cells. In contrast, the changes to the gastrodermal cells were relatively minor, and more likely due to the cessation of feeding accompanying the loss of motility of the flukes (McConville *et al.* 2009). Albendazole is able to induce disruption of the gut, as well as the tegument, whether the fluke is ligatured or not (Haughey, 2008). This may be due to its greater lipophilicity compared to TCBZ (Alvarez *et al.* 2004; Mottier *et al.* 2004).

While trans-tegumental entry is true for TCBZ and compound alpha, it is not a universal phenomenon among fasciolicides. For example, clorsulon binds to RBC carbonic anhydrase (Schulman *et al.* 1979) and has been shown to be taken up orally in experiments involving ligatures, as used in the current study (Meaney *et al.* 2005*a*, *b*). Nitroxynil, too, is probably taken up orally (McKinstry *et al.* 2007); it binds strongly to plasma proteins (Alvinerie *et al.* 1991 *a*, *b*). In experiments involving the artemisinin derivative, artemether, the gut was more severely affected than the tegument, suggesting that the drug is taken up by oral ingestion (O'Neill *et al.* 2009). For these compounds, it is believed that they are 'activated' by interaction with an iron-containing compound, such as haemoglobin, leading to the cleavage of the peroxide bridge in the drug molecule and the generation of free radicals. It is these free radicals which induce tissue damage, so drug action would necessitate oral ingestion, as has been suggested for the action of artemisinins against schistosomes (Xiao *et al.* 2003).

In addition to the severe changes induced in the tegumental system, disruption of the muscle bundles was observed in the present study. The muscle bundles were more widely-separated than normal and the bundles themselves were more loosely-packed, appearing to contain fewer muscle fibres. These changes would affect the motility of the fluke and this would exacerbate drug action, as reduced motility would impair the ability of the fluke to feed, thus triggering a state of starvation. Disruption to the somatic musculature was also noted in flukes treated *in vivo* with compound alpha (McConville *et al.* 2009).

In conclusion, then, the combined morphological and pharmacological data indicate that TCBZ enters the fluke predominantly across the tegument. The tissue changes observed in this paper complement and support those surface changes described previously (Toner *et al.* 2009) and provide an explanation for them. A better knowledge of the route of entry of TCBZ and its metabolites is important to understanding drug action, as their concentrations in bile provide ample opportunity for contact with the fluke (Hennessy *et al.* 1987). Moreover, it is relevant to the understanding of the mechanism of drug resistance, as uptake is reduced in TCBZ-resistant flukes (Alvarez *et al.* 2005; Mottier *et al.* 2006*b*).

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Fig. 5. Transmission electron micrographs (TEMs) of the tegumental syncytium, musculature and tegumental cells of adult non-ligatured *Fasciola hepatica* treated *in vitro* with RBCs. (A) Transverse section through the tegumental syncytium. T1 secretory bodies (T1) and chains of mitochondria (arrow) are distributed throughout the syncytium, APM, apical plasma membrane; BI, basal infold; BL, basal lamina. (B) Type-1 (T1) and Type-2 (T2) secretory bodies are present in normal numbers below the apical plasma membrane (APM). (C) Basal region of the tegumental syncytium. Type-1 (T1) and Type-2 (T2) secretory bodies are present. There appears to be no disruption to the basal infolds (BI) and the mucopolysaccharide masses surrounding them. BL, basal lamina. (D) TEM of the muscle blocks (Mu) which appear compact and contain normal mitochondria (m). (E) High-power TEM of two muscle blocks (Mu). They exhibit a normal morphology. (F) TEM of an active T1 tegumental cell in which T1 secretory bodies (T1) are abundant. The mitochondria (m) within the cell appear normal. N, nucleus. *Inset* shows a Golgi complex (G), which is well developed.

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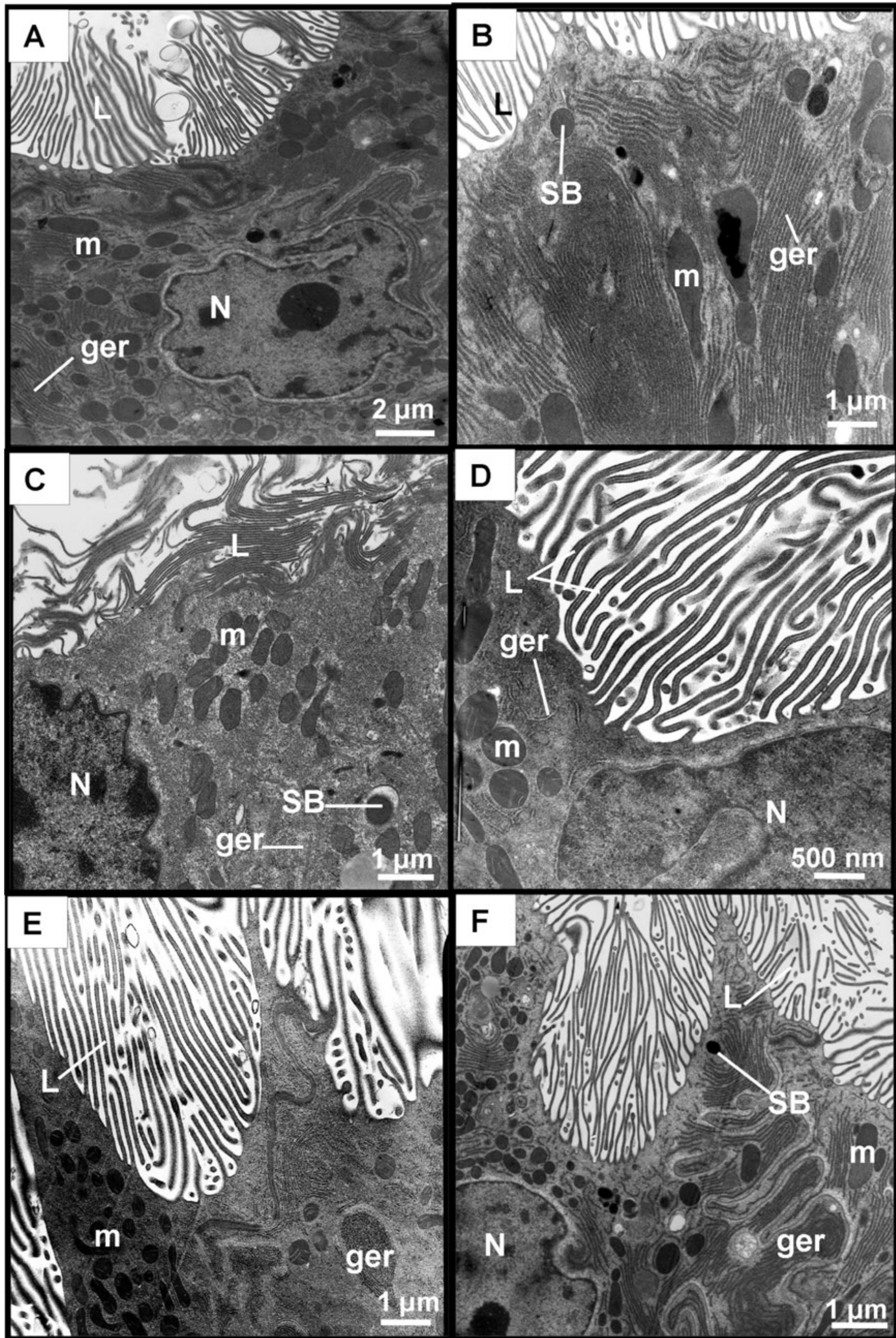

Fig. 6. For legend see opposite page.

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Table 1. Summary of TEM results

	Treatments 24 h *in vitro*								
	1	2	3	4	5	6	7	8	9
Disruption	Non-Ligatured TCBZ.SO	Ligatured TCBZ.SO	Non-Ligatured TCBZ.SO +BSA	Ligatured TCBZ.SO +BSA	Non-Ligatured BSA	Ligatured BSA	Non-Ligatured TCBZ.SO +RBC	Non-Ligatured RBC	Non-Ligatured & Ligatured 0 h and 24 h control
Blebbing	*xx*	*xx*	*x*	*x*	-	-	*xx*	-	-
Swelling of mucopolysaccharide masses surrounding the basal infolds	*xx*	*xx*	*x*	*x*	-	-	*xx*	-	-
Accumulation of T2 secretory bodies at apical plasma membrane	*xx*	*xx*	-	-	-	-	*xx*	-	-
Reduced numbers of T1 secretory bodies at apical plasma membrane	*xx*	*xx*	-	-	-	-	*xx*	-	-
Reduced numbers of secretory bodies in basal region	*xx*	*xx*	-	-	-	-	*xx*	-	-
Muscle disruption	*xxx*	*xxx*	*x*	*x*	-	-	*xxx*	-	-
Reduced number of secretory bodies in tegumental cells	*xxx*	*xxx*	-	-	-	-	*xxx*	-	-
Absence of Golgi complexes in tegumental cells	*xxx*	*xxx*	-	-	-	-	*xxx*	-	-
Disruption to mitochondria in tegumental cells	*x*	*x*	-	-	-	-	*x*	-	-
Disruption to gastrodermal cells	-	-	-	-	-	-	-	-	-
Totals	20	20	3	3	0	0	20	0	0

-, No noticeable disruption; *x*, mild disruption; *xx*, severe disruption; *xxx*, extremely severe disruption; TCBZ.SO, triclabendazole sulphoxide; BSA, bovine serum albumin; RBC, red blood cell.

Fig. 6. Transmission electron micrographs (TEMs) of the gastrodermal cells of adult non-ligatured and ligatured *Fasciola hepatica* treated *in vitro* with TCBZ.SO (15 μg/ml) (A, B), TCBZ.SO (15 μg/ml)+bovine serum albumin (BSA) (30 mg/ml) (C), BSA (30 mg/ml) (D), TCBZ.SO (15 μg/ml)+RBCs (E) or RBCs (F) for 24 h. (A) (Non-ligatured). The mitochondria (m) and cisternae of granular endoplasmic reticulum (ger) are normal. L, lamellae; N, nucleus. (B) (Ligatured). Secretory bodies (SB) are present, and the mitochondria (m) and cisternae of granular endoplasmic reticulum (ger) are normal. L, lamellae. (C) (Non-ligatured). The gastrodermal cell appears normal and contains secretory bodies (SB), cisternae of granular endoplasmic reticulum (ger) and mitochondria (m). L, lamellae; N, nucleus. (D) (Ligatured). The gastrodermal cell appears unaffected: it contains cisternae of granular endoplasmic reticulum (ger) and normal mitochondria (m). L, lamellae; N, nucleus. (E) (Non-ligatured). The cisternae of granular endoplasmic reticulum (ger) and the mitochondria (m) appear normal in this gastrodermal cell. L, lamellae. (F) (Non-ligatured). The cisternae of granular endoplasmic reticulum (ger) and the mitochondria (m) appear unaffected in this gastrodermal cell. Secretory bodies (SB) are present. L, lamellae; N, nucleus.

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