

Electron microscopical study to assess the *in vitro* effects of the synthetic trioxolane OZ78 against the liver fluke, *Fasciola hepatica*

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SUMMARY

Adult *Fasciola hepatica* were incubated for 48 h *in vitro* in the synthetic peroxide, OZ78 at a concentration of 100 µg/ml and then prepared for scanning and transmission electron microscopy. There was limited disruption to the external fluke surface, with only slight swelling and blebbing of the interspinal tegument in the midbody and ventral tail regions. By contrast, significant disruption was observed to the ultrastructure of the tegument and subtegumental tissues. There was severe swelling of the basal infolds in the tegumental syncytium and the flooding spread internally to affect the subtegumental tissues. In the tegumental system, there was swelling of the cisternae of granular endoplasmic reticulum and of the mitochondria, with the latter showing signs of breaking down. Autophagic vacuoles and lipid droplets were present and the synthesis of tegumental secretory bodies was much reduced. The gastrodermal cells were severely affected, with swelling and degeneration of the mitochondria and the presence of autophagic vacuoles and lipid droplets. The granular endoplasmic reticulum was swollen and vesiculated and the cells contained few secretory bodies. Both the vitelline and testis follicles showed evidence of extensive cellular disruption and degeneration. This study confirms previous data indicating the potential flukicidal activity of OZ78.

Key words: *Fasciola hepatica*, liver fluke, synthetic artemisinin derivative, OZ78, scanning electron microscopy, transmission electron microscopy, *in vitro* culture.

INTRODUCTION

The liver fluke, *Fasciola hepatica* is a major parasite of livestock in temperate regions of the world. There has been a dramatic rise in the incidence of disease in recent years, which has been attributed to climate change (Mitchell, 2002). Furthermore, fascioliasis is now recognized as a major zoonotic infection of increasing importance (Mas-Coma *et al.* 2005; WHO, 2007). Current control methods effectively centre round the use of anthelmintic drugs, of which the most commonly employed is the benzimidazole derivative, triclabendazole (TCBZ, 'Fasinex') (Fairweather, 2009). It is favoured due to its remarkably high efficacy against both adult and juvenile stages of *F. hepatica* (Boray *et al.* 1983). Unfortunately, due to the over-dependence on this drug and its continued use as a monotherapy, resistance is steadily on the increase. Resistance to triclabendazole was first noted on Australian farms in 1995 (Overend and Bowen, 1995). Since then, resistance

has been detected throughout much of Europe (Fairweather, 2005, 2009). Resistance in the UK and Ireland is believed to be spreading and is probably significantly underestimated (Wolstenholme *et al.* 2004). This means that there is an urgent need to find novel flukicidal compounds.

One group of drugs that has attracted considerable attention in the last few years is the artemisinins. The artemisinins are a family of natural or semi-synthetic compounds derived from the plant, *Artemisia annua* which is grown in eastern Asia (O'Neill, 2004) and have established uses as anti-malarial and anti-schistosomal drugs (Borstnik *et al.* 2002; Woodrow *et al.* 2005; Xiao, 2005; Utzinger *et al.* 2007). The main semi-synthetic derivatives of artemisinin are artemether, artesunate and the active metabolite, dihydroartemisinin (Woodrow *et al.* 2005). Artemether and artesunate have been shown to be effective fasciolicides in the treatment of rodent infections (Keiser *et al.* 2006a). Due to the unfavourable pharmacokinetic properties of the artemisinins, many synthetic anti-malarial peroxides have been prepared, including the synthetic 1,2,4-trioxolanes, such as OZ277 and OZ78 (Vennerstrom *et al.* 2004). Synthetic peroxides retain the peroxide pharmacophore essential for anti-parasitic activity, yet are more simple molecules, easier to synthesize and have improved

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pharmacokinetic properties (Jefford, 2007). Worm burden reductions of 100% have been achieved with both juvenile and adult *F. hepatica* following a single oral dose of 100 mg/kg OZ78 (Keiser *et al.* 2006*b*). Significantly, high efficacy has also been recorded for OZ78 and artemether in treatment of triclabendazole-resistant fluke infections in rats (Keiser *et al.* 2007*a*). These results suggest that OZ78 has a high potential as a flukicidal drug and warrants further evaluation. Relatively little work has been performed so far in relation to the morphological effects of artemisinin-type compounds on *F. hepatica* and it has been confined to surface changes (Keiser *et al.* 2006*a, b*; Keiser and Morson, 2008*a, b*).

The present study was carried out to determine the changes induced by treatment *in vitro* with the synthetic peroxide OZ78 on the surface morphology of adult *F. hepatica*, as visualized by scanning electron microscopy (SEM), and on the internal ultrastructure by means of transmission electron microscopy (TEM). The aim was to identify possible tissue targets of drug action.

MATERIALS AND METHODS

The experiment was carried out in 2 parts: the rat infections, *in vitro* incubation in OZ78 and initial fixation of fluke material was performed at the Swiss Tropical Institute, Basel and then the fluke material was sent to The Queen's University of Belfast for further processing and examination by scanning and transmission electron microscopy.

Female Wistar rats (purchased from RCC, Itingen, Switzerland) were experimentally infected with metacercarial cysts of the Cullompton isolate, shown in previous experiments to be susceptible to TCBZ *in vivo* and to its sulphoxide metabolite *in vitro* (Robinson *et al.* 2002; McCoy *et al.* 2005; Halferty *et al.* 2008, 2009; McConville *et al.* 2006, 2009*a*; Meaney *et al.* 2006, 2007; Devine *et al.* 2009; Toner *et al.* 2009). All animals were housed in environmentally-controlled conditions in groups of 4 and afforded 1 week to acclimatize. Aforementioned conditions were: temperature, 25 °C; humidity, 70%; 12 h light/dark cycle. Free access to water and rodent diet was also maintained. Twelve rats were infected intragastrically with 25 cysts each and, after a period of 9–10 weeks, adult flukes were recovered from the bile ducts at necropsy. Recovered flukes were then incubated in 6-well plates (Costar, Corning, USA), placing 2 fluke specimens per well. Each well contained 5 ml of NCTC 135 culture medium (Invitrogen, Carlsbad, USA) containing streptomycin and penicillin at concentrations of 50 µg/ml and 50 IU/ml, respectively. A stock solution of OZ78 (synthesized at the College of Pharmacy, University of Nebraska Medical Centre, USA) was prepared in 60% (v/v) DMSO and flukes were incubated with the drug for a period of 48 h at a concentration of 100 µg/

ml. The concentration used was based on the results of previous studies with OZ78 involving *F. hepatica*, *Echinostoma caproni* and *Clonorchis sinensis* (Keiser *et al.* 2006*b*, 2007*a, b*). Fluke motility was observed at regular intervals by way of a dissecting microscope. Specimens were judged dead if no movement was observed for 2 min. The experiment was terminated at 48 h, when the flukes were considered dead. Controls consisted of incubating flukes for 48 h at 37 °C in NCTC 135 culture medium in the presence of streptomycin and penicillin. The concentration of DMSO in the control medium (0.05%) was matched to that of the drug medium. The control flukes were still active and appeared normal after 48 h incubation. Controls at 0 h were also prepared. Following treatment, the flukes were fixed with 2.5% (v/v) glutaraldehyde in a PBS buffer (pH 7.3) for 24 h at room temperature. After rinsing and storing in PBS buffer, the specimens were sent to The Queen's University of Belfast for further processing and examination.

For scanning electron microscopy (SEM), 8 flukes were washed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose. Following a post-fixation in 1% osmium tetroxide for 1 h, the flukes were given 3 brief washes in 70% (v/v) ethanol and dehydrated in an ascending series of ethanol. After this, they were dried in hexamethyldisilazane, mounted on aluminium stubs and sputter-coated with gold-palladium. The specimens were viewed in an FEI Quanta 200 scanning electron microscope, operating at an accelerating voltage of 10 keV.

For transmission electron microscopy (TEM), 5 flukes were washed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose. The apical cones and tail regions of the flukes were discarded and the remaining midbody region was divided transversely into 2 parts; each part was further subdivided transversely into 6 pieces. The pieces were washed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose. Following post-fixation in 1% osmium tetroxide for 1 h, the tissue was washed in fresh buffer, dehydrated in an ascending series of ethanol and infiltrated and embedded in Agar 100 resin. Ultrathin sections, 60–70 nm in thickness, were cut on a Reichert Ultracut E ultramicrotome, mounted on bare 200-mesh copper grids, double-stained with alcoholic uranyl acetate (5 min) and aqueous lead citrate (3 min) and viewed in a FEI CM 100 transmission electron microscope operating at an accelerating voltage of 100 keV.

RESULTS

Scanning electron microscopy

The surface architecture of the control flukes appeared normal (Fig. 1A). It matched the images

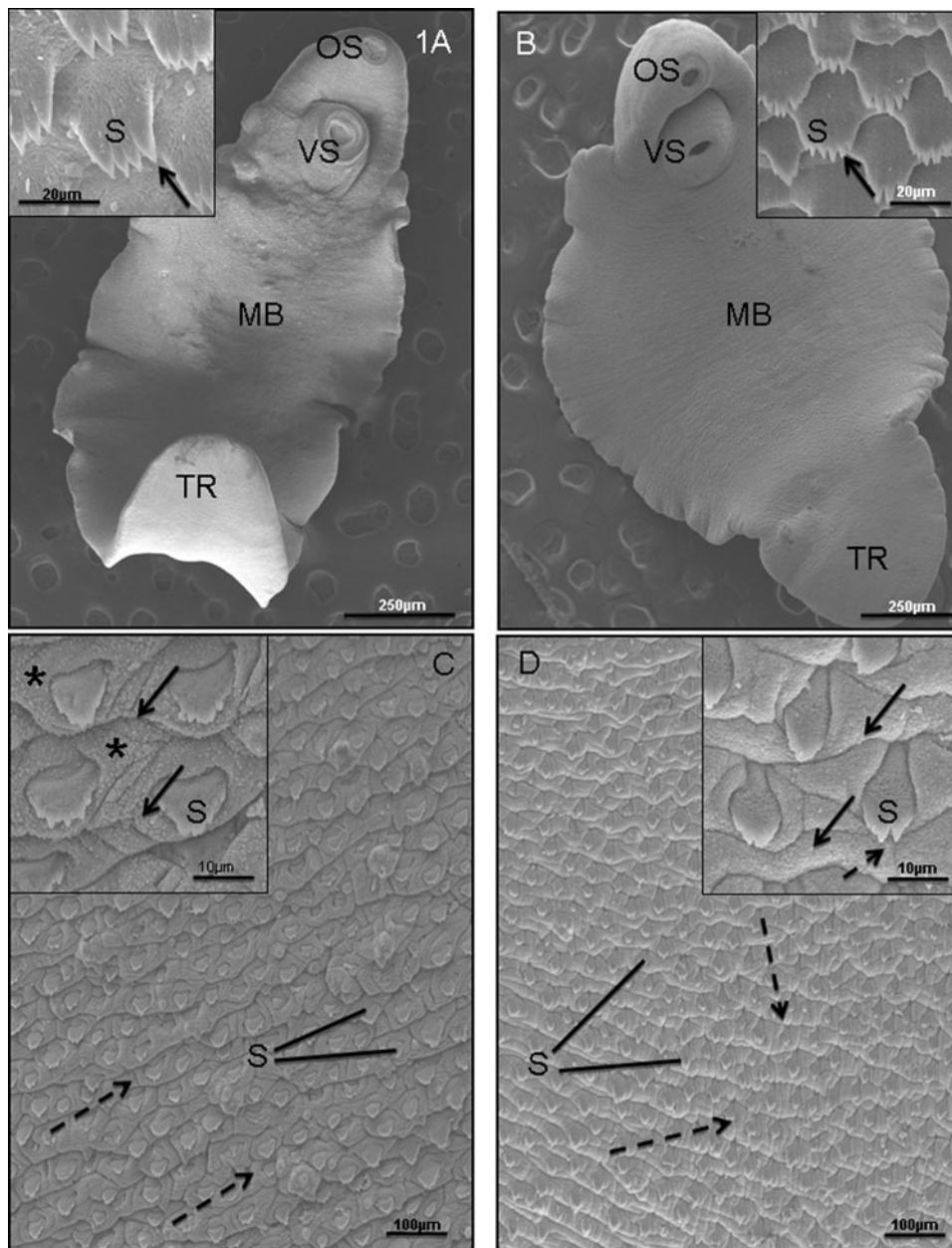


Fig. 1. Scanning electron micrographs (SEMs) of adult *Fasciola hepatica* incubated *in vitro* for 48 h. (A) Control, incubated in NCTC culture medium; (B–D) flukes treated with OZ78 at a concentration of 100 µg/ml. (A) Control fluke. Low-power image, showing the oral sucker (OS), ventral sucker (VS), midbody region (MB) and tail region (TR). *Inset* shows spines (S) from the oral cone region, which bear distinct spinelets (arrow). (B) Low-power image, showing the oral sucker (OS), ventral sucker (VS) midbody region (MB) and tail region (TR). *Inset* shows spines (S) from the oral cone region, which bear distinct spinelets (arrow). (C) Ventral midbody region. The inter-spinal tegument appears swollen (broken arrows). The arrangement of the spines (S) is more irregular than normal. *Inset* shows that the spines (S) are slightly sunken due to the swelling of the inter-spinal tegument (arrows). The tegumental surface has a roughened appearance (*). (D) Dorsal midbody region. Swelling of the inter-spinal surface is evident (broken arrows). The arrangement of the spines (S) appears irregular. *Inset* is a higher-power image showing that the spinelets (broken arrow) are less well-defined. The inter-spinal surface, although swollen (arrows), appears smooth. S, spine.

presented by Bennett (1975), Fairweather *et al.* (1999) and McConville *et al.* (2009b).

Following treatment with OZ78, little change was observed anteriorly (Fig. 1B). Spines were grouped in a regular, tight-knit arrangement and clearly protruded from the inter-spinal surface. The spinelets at the tips of the spines (Fig. 1B) were distinct and there was no spine disruption or swelling of the tegument.

The ventral midbody region showed slight swelling of the interspinal tegument (Fig. 1C). This swelling lent an uneven appearance to the surface of the fluke. At higher magnification (Fig. 1C inset), the spines were seen to be slightly sunken, indicating some swelling of the tegument surrounding them. The interspinal surface appeared rough and was covered with small blebs. The dorsal surface of the

midbody region exhibited general swelling of the interspinal tegument (Fig. 1D). The swelling again lent an uneven appearance to the surface of the fluke. Spines in this region appeared sunken, due to the swelling of the tegument around them (Fig. 1D inset) and the spinelets were less clearly defined (Fig. 1D inset).

The ventral surface of the tail region (Fig. 2A) showed a relatively normal morphology and the tegument was not swollen. At higher magnification (Fig. 2A inset), the interspinal surface was seen to be roughened. Spines were prominent and identical in shape and structure to those of control specimens. The dorsal tail (Fig. 2B) showed a regular pattern of spines protruding from a roughened tegumental surface. There was no evidence of swelling of the tegument.

Figures 2C and D demonstrate unusual features seen in 2 of the specimens. A bulbous swelling was observed in the dorsal anterior midbody region of 1 specimen, the identity of which is not clear (Fig. 2C and inset). Apart from this swelling, the surface of the fluke appeared normal. More dramatic disruption was evident in another specimen, in which the posterior end of the fluke was covered with large blebs, many of which had burst (Fig. 2D). One of the burst blebs was seen to contain a spine (Fig. 2D inset).

Transmission electron microscopy

The ultrastructure of the tegumental syncytium, tegumental cells and reproductive structures in the control specimens remained normal. For images, the reader is referred to the papers by Threadgold (1963, 1967) for tegument and parenchyma; Irwin and Threadgold (1970) and Colhoun *et al.* (1998) for vitellaria; and Stitt and Fairweather (1990) for testis.

Following drug treatment, severe alterations were evident throughout the tegumental syncytium, subtegumental region, gut and reproductive tissues, in sections taken from the midbody region and as illustrated in Figs 3 and 4. In the syncytium, the basal infolds were severely swollen and occupied the entire basal region of the syncytium (Fig. 3A and C). Blebbing of the apical plasma membrane was observed (Fig. 3B) and autophagic vacuoles were present in the syncytium (Fig. 3B). The numbers of T1 and T2 secretory bodies in the syncytium were considerably less than normal. The mitochondria were swollen and electron-lucent (Fig. 3C). The crystalline structure of the spines was seen to be disrupted: it had a rippled and striated appearance (Fig. 3B).

Beneath the basal lamina, large spaces separated the muscle blocks belonging to the circular and longitudinal muscle layers (Fig. 3D). Large spaces surrounded the tegumental cell bodies, too (Fig. 3E and F). Branches of the excretory ducts contained

many lipid droplets (Fig. 4A). Within some of the tegumental cell bodies themselves, there were large numbers of lipid droplets as well (Fig. 3E). In all of the cells, the cytoplasm was filled with swollen and electron-lucent mitochondria, with distinct cristae and some of the mitochondria appeared to be in the process of breaking down (Fig. 3F). The cisternae of granular endoplasmic reticulum (ger) were swollen as well (Fig. 3F). Autophagic vacuoles were present in the cells (Fig. 3F). Type 1 and type 2 tegumental secretory bodies were generally absent from the cells and there were no discernable Golgi complexes present.

There was severe disruption to the gastrodermal cells. Large spaces were present between adjacent cells, the cells only being held together by the tight junction at their apices (Fig. 4A). Within the cells themselves, there were large empty vacuoles, together with autophagic vacuoles and lipid droplets, but few secretory bodies (Fig. 4A and B). The cisternae of granular endoplasmic reticulum were swollen and vesiculated (Fig. 4B).

The condition of the vitelline follicles is shown in Fig. 4C and D. Within the follicles it was difficult to distinguish any stem and early immature cells and the follicle was loosely-packed. The predominant cell type was the mature cell, although it displayed a number of abnormalities. That is, the shell globule clusters were either loosely-packed or the shell protein globules appeared to have coalesced (Fig. 3C). The cisternae of the ger were swollen and a number of lipid droplets were present (Fig. 3C). In some cells, the cytoplasm appeared to be breaking down (Fig. 3C). In more extreme cases, the vitelline follicles were greatly reduced in size and contained no recognizable cell types, as the cells had broken down and were full of autophagic vacuoles (Fig. 3D). In these situations, the surrounding tissue was very disorganized and empty looking (Fig. 3D).

The testis follicles are pictured in Fig. 4E and F. Only spermatogonia and primary spermatocytes were present. The spermatogonia were rounded, the space between the inner and outer nuclear membranes was swollen and vacuoles were present in the cytoplasm (Fig. 4E). The spermatocytes appeared to have undergone nuclear division without undergoing cytoplasmic division, giving rise to multinucleate cells (Fig. 4E). In the most extreme cases, the follicle was almost totally devoid of spermatogenic cells and the tissue that remained was very disorganized and autophagic (Fig. 4F).

DISCUSSION

Following treatment *in vitro* of *F. hepatica* with the synthetic peroxide OZ78 for 48 h at a concentration of 100 µg/ml, there was a marked difference between what disruption was evident externally and what disruption occurred internally. That is, changes to

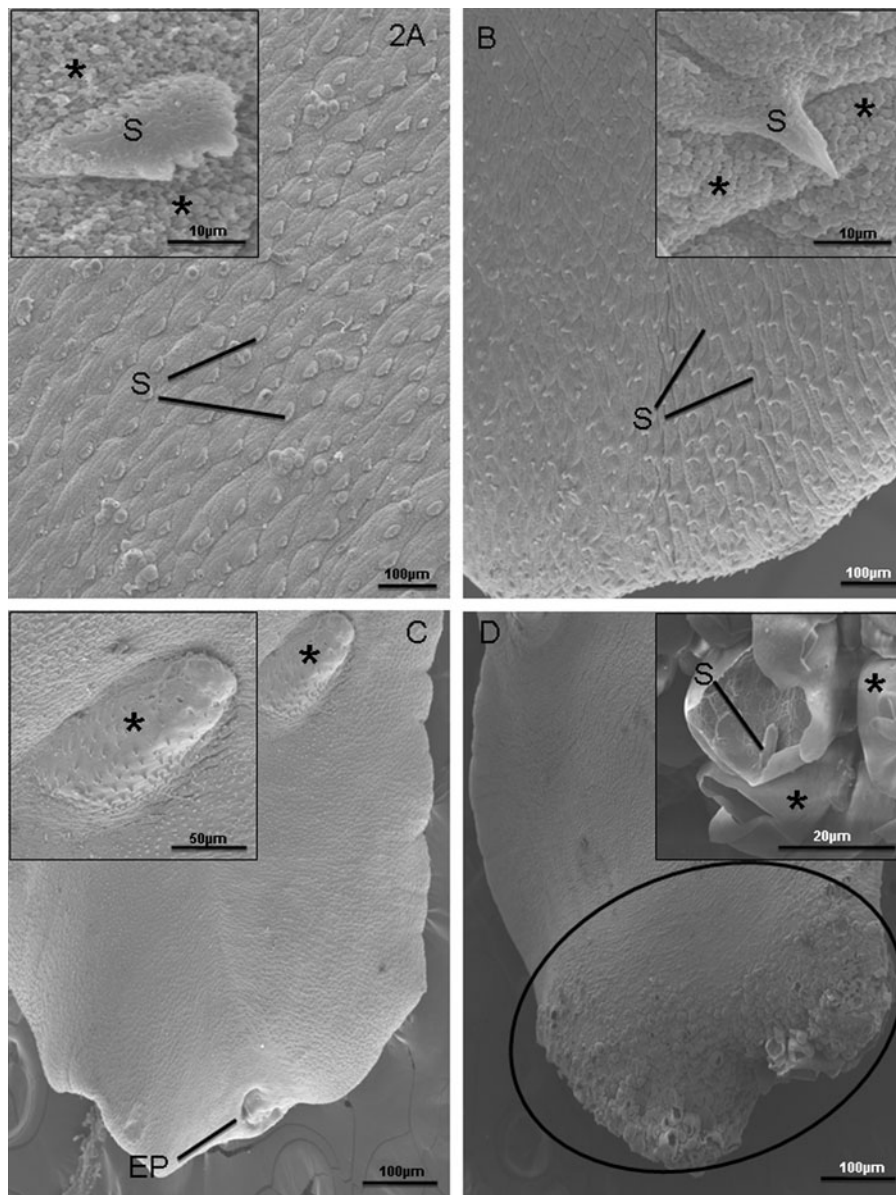


Fig. 2. Scanning electron micrographs (SEMs) of the posterior region of adult *Fasciola hepatica* following 48 h treatment *in vitro* with OZ78 (100 µg/ml). (A) Ventral tail region. The spines (S) project from the surface of the tegument, which is not swollen. *Inset* shows the roughened appearance of the inter-spinal surface, due to the covering of small blebs (*). S, spine. (B) Dorsal tail region. The spines (S) are regularly-arranged and protrude from the surface. *Inset* shows that the tegumental surface appears roughened (*) at a higher magnification. S, spine. (C) The main image shows the presence of a bulbous swelling (*) on the ventral surface in the posterior midbody region. EP, excretory pore. *Inset* shows the swelling (*) at higher magnification. (D) Severe disruption to the tail end of the fluke, which appears to be covered with large blebs, many of which have burst (oval outline). *Inset* shows the large and burst blebs (*) at high magnification. One of the burst blebs contains a spine (S).

the surface morphology were quite limited. In stark contrast to the SEM observations, however, there was severe internal disruption of the tegumental, subtegumental and reproductive tissues. The basal infolds of the tegumental syncytium were swollen, flooding the basal region of the syncytium. Flooding was also evident internally, with large spaces separating the subtegumental cells. The gastrodermal cells were severely affected, with evidence of metabolic disruption and breakdown. In both the vitelline and testis follicles, the normal development of the cells

was disrupted and, in more extreme cases, the vitelline follicles appeared to be in the process of breaking down. These findings will now be discussed in detail and compared with the findings of previous, related studies.

Previous morphological studies *in vitro* and *in vivo* with OZ78 and other artemisinins have concentrated solely on the tegument of *F. hepatica* using SEM (Keiser *et al.* 2006*a, b*, 2007*b*; Keiser and Morson, 2008*a, b*; O'Neill *et al.* 2009). While the changes seen can vary between flukes, they are generally relatively

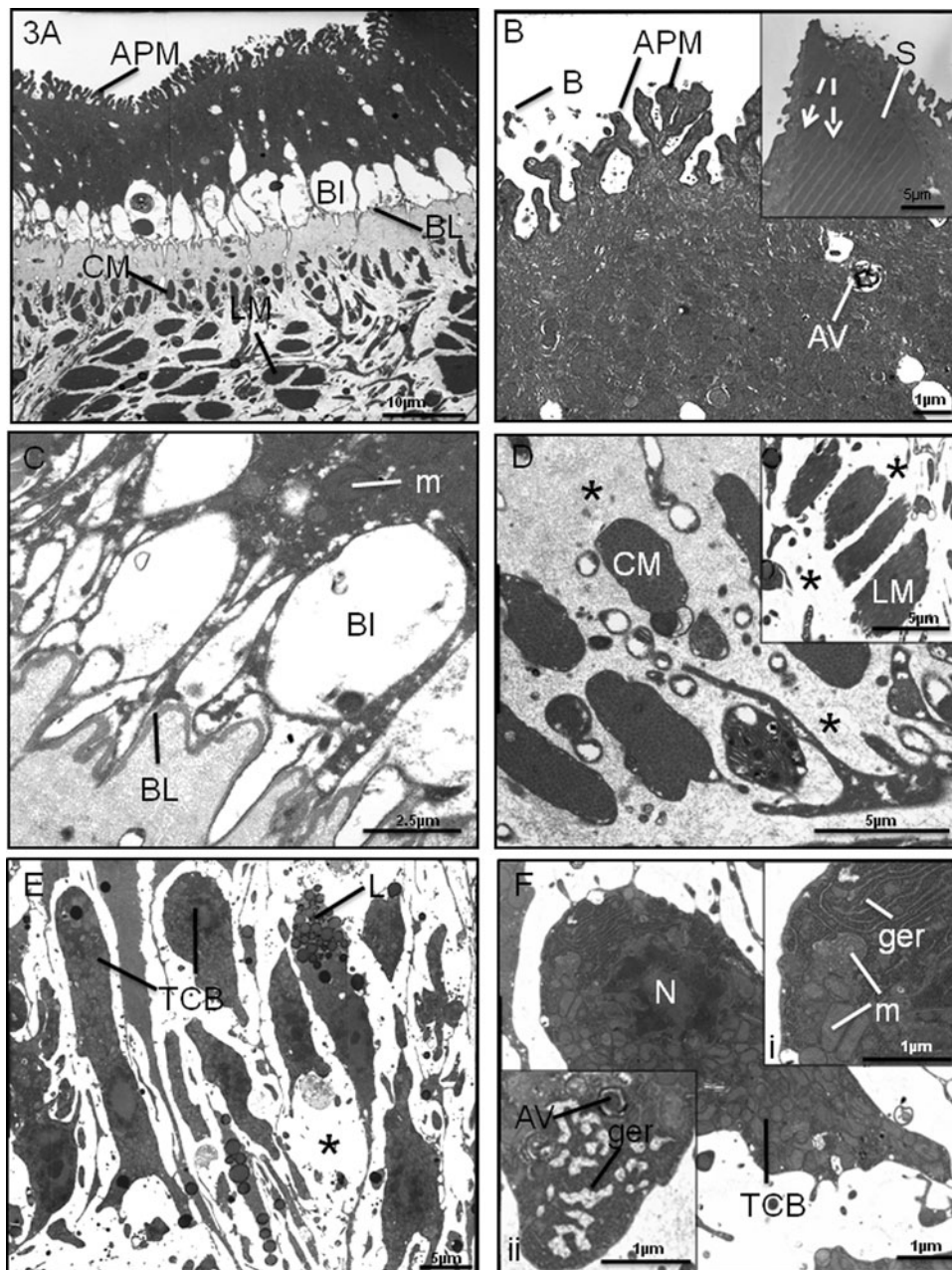


Fig. 3. Transmission electron micrographs (TEMs) of the tegumental syncytium and subtegumental region of adult *F. hepatica* following 48 h treatment *in vitro* with OZ78 (100 µg/ml). (A) The basal infolds (BI) are severely swollen. Beneath the basal lamina (BL), the circular (CM) and longitudinal (LM) muscle layers can be seen. APM, apical plasma membrane. (B) High-power image of the apical region of the tegumental syncytium. Small blebs (B) can be seen on the surface. An autophagic vacuole (AV) is present. APM, apical plasma membrane. *Inset* shows disruption (broken arrows) to the crystalline structure of a spine (S). (C) High-power image of the swollen basal infolds (BI) above the basal lamina (BL). The mitochondria (m) are swollen and electron-lucent. (D) Circular muscle (CM) layer showing the muscle bundles surrounded by large spaces (*). *Inset* shows the large spaces (*) between the longitudinal muscle (LM) bundles. (E) Low-power image of the subtegumental region. The tegumental cell bodies (TCB) contain large numbers of lipid droplets (L) and are separated from each other by large spaces (*). (F) High-power image of a tegumental cell body (TCB). The irregularly-shaped nucleus (N) can be seen located centrally within the cell. *Inset i* shows the swollen and electron-lucent appearance of the mitochondria (m). The cisternae of the granular endoplasmic reticulum (ger) are also distended. *Inset ii* shows the severe swelling of the cisternae of the granular endoplasmic reticulum (ger) at higher magnification. An autophagic (AV) vacuole is present in the cell.

mild, with swelling, localized areas of blebbing and occasional sloughing. The results of the present study were consistent with this pattern, with swelling and limited blebbing over part of the body surface.

There was not the severe and extensive disruption seen with other fasciolicides (e.g. Fairweather *et al.* 1987; Suce and Fairweather, 1990; Stitt and Fairweather, 1993; McKinstry *et al.* 2003; Meaney

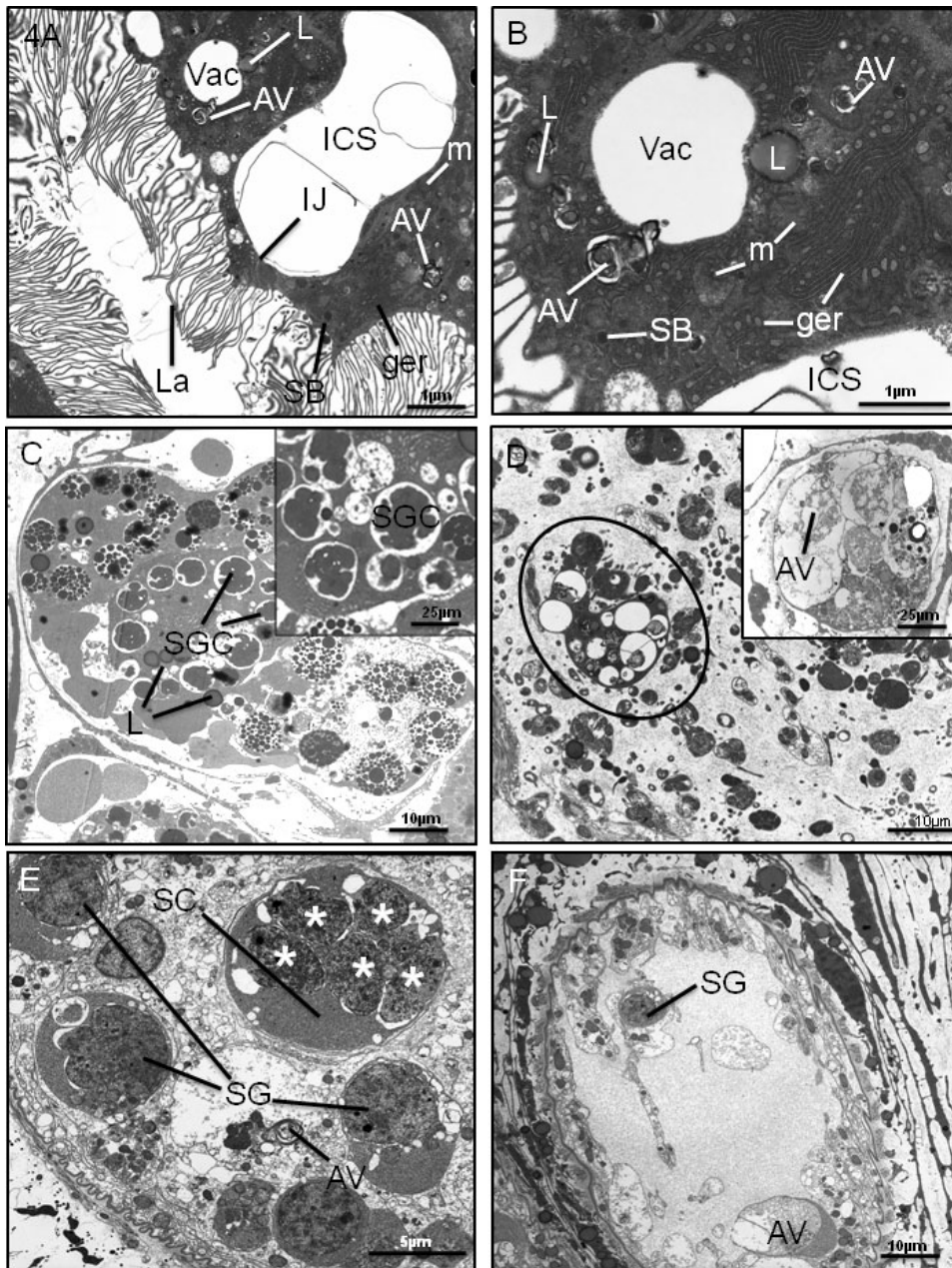


Fig. 4. Transmission electron micrographs (TEMs) of adult *Fasciola hepatica* following 48 h treatment *in vitro* with OZ78 (100 µg/ml). (A) Gastrodermal cells, showing a large intercellular space (ICS) between two adjacent cells. Within the cells, are vacuoles (Vac), autophagic vacuoles (AV), lipid droplets (L) and a small number of secretory bodies (SB). The cisternae of granular endoplasmic reticulum (ger) are swollen. The mitochondria (m) are swollen and electron-lucent. La, gut lamellae. (B) Gastrodermal cell, containing empty vacuoles (Vac), autophagic vacuoles (AV) and lipid droplets (L). Few secretory bodies (SB) are present. The mitochondria (m) are swollen and some are electron-lucent and appear to be breaking down. The cisternae of granular endoplasmic reticulum (ger) are swollen and vesiculated. ICS, intercellular space. (C) Vitelline follicle. The follicle is heavily-disrupted, with no definable early developmental stages present. The shell protein globules in the shell globule clusters (SGC) show signs of coalescence. Lipid droplets (L) are present in the cells. *Inset* is a high-power image showing the abnormal shell globule clusters (SGC). (D) Vitelline follicle (oval outline) is severely disrupted and is surrounded by widespread degeneration of the parenchymal tissue. *Inset* is a high-power image showing a follicle in the final stages of breakdown. Almost no discernible structures remain, and autophagic vacuoles (AV) are present throughout. (E) Testis follicle, showing disruption of the spermatogenic cells. The spermatogonia (SG) appear rounded and abnormal and the primary spermatocytes (SC) show division of the nuclei (*) without cytoplasmic division of the cells themselves. General degeneration of the follicular tissue is evident throughout the follicle and autophagic vacuoles (AV) are present. (F) Testis follicle, which is almost completely devoid of spermatogenic cells. There is widespread disruption throughout. SG, spermatogonia; AV, autophagic vacuole.

et al. 2003; McConville *et al.* 2006; Halferty *et al.* 2009). More severe changes were seen in 2 of the flukes. The large swelling observed in one of them is similar to that described previously (Keiser *et al.* 2006*b*), but it is not known what internal structure it relates to. Such individual variation in response to artemisinin treatment seems to be typical of *F. hepatica*, as it has been described in other studies (Keiser *et al.* 2006*b*; Keiser and Morson, 2008*a, b*). Surface changes with artemisinins are greater when haemin is included in the *in vitro* culture medium (Keiser and Morson, 2008*a, b*) and following *in vivo* treatment, which may be related to proposals concerning the activation of the drug following its ingestion by the fluke, as has been suggested for schistosomes (Xiao *et al.* 2003). That is, interaction with haemin or another iron-containing compounds derived from the haemoglobin in the blood that the worms feed upon may lead to activation of the artemisinin compound. Activation leads to cleavage of the peroxide bridge in the artemisinin molecule (which is the essential moiety required for activity) and generation of free radicals which are damaging to the parasite (Xiao *et al.* 2003). In relation to the role of haemin in drug action, an interesting observation has been made that, although disruption is greater in the presence of haemin, it does not accelerate the 'death' of the fluke (Keiser and Morson, 2008*b*). A similar observation has been made for artemether and artesunate (Keiser and Morson, 2008*a*). This suggests that cleavage of the peroxide bridge is not an absolute pre-requisite for drug action and that an iron-independent mechanism may be involved, views expressed by Xiao *et al.* (2007) and Keiser and Morson (2008*a*). Indeed, it has been suggested that the trioxolanes have different actions from the semi-synthetic artemisinins (Uhlemann *et al.* 2007).

In a separate *in vivo* study on *F. hepatica* involving artemether, the gut was consistently more severely affected than the tegument, lending support to the notion of oral uptake of the artemisinin compounds (O'Neill *et al.* 2009). In the present study, the gut was severely disrupted and this would make the condition of the fluke worse. There was little secretory activity and the ger was swollen and vesiculated. Vesiculation of the ger has been linked to a state of starvation or stress in the fluke (Robinson and Threadgold, 1975; Skuce and Fairweather, 1990; Meaney *et al.* 2004, 2005; McKinstry *et al.* 2007). Moreover, there were signs that the cells were beginning to break down, as evidenced by the presence of autophagic vacuoles and degenerating mitochondria.

In the tegumental system, the main changes seen were a severe swelling of the basal infolds, surface blebbing, swelling of the mitochondria and cisternae of ger, the presence of autophagic vacuoles and lipid droplets and a lack of secretory bodies in the tegumental cells. Similar changes to the tegument,

together with the widespread internal oedema, have been observed in schistosomes following treatment with artemether (Xiao *et al.* 2002*a, b*). In the present study, the blebs present on the apical plasma membrane were also seen with SEM and the swelling of the basal infolds (together with the internal flooding) will explain the tegumental swelling visible externally. Greater swelling of the basal infolds would lead to the sloughing of the tegument, as has been observed following treatment with other fasciolicides (e.g. Fairweather *et al.* 1986; Skuce and Fairweather, 1990; Stitt and Fairweather, 1993; Anderson and Fairweather, 1995; Meaney *et al.* 2004). Clearly, there is disruption of the tegumental osmoregulatory system, which spreads internally to affect the sub-tegumental tissues. This may be due to inhibition of the energy-dependent ion pumps on the tegumental membranes and is evidenced by the swollen mitochondria and cisternae of ger and the presence of lipid droplets, together with the swelling of the basal infolds. Similar changes have been seen following treatment with fasciolicides that are believed to target energy metabolism, including clorsulon, closantel and nitroxynil (Skuce and Fairweather, 1990; Meaney *et al.* 2004, 2005, 2007; McKinstry *et al.* 2007, 2009). Alternatively, the flooding may stem from damage to the surface membrane as a result of impairment of production of secretory bodies in the tegumental cells. Maintenance of the apical membrane is dependent on the continual production and release of secretory bodies (Fairweather *et al.* 1999). The lack of secretory bodies in the tegumental cells and reduced numbers in the syncytium suggest that they are no longer being produced, with the consequence that the surface membrane will be affected, leading to the changes observed. Similar morphological changes have been seen with fasciolicides that are not believed to affect energy metabolism, such as TCBZ and diamphenethide (Fairweather *et al.* 1986; Skuce *et al.* 1987; Stitt and Fairweather, 1994; Halferty *et al.* 2009). These two drugs have been shown to inhibit protein synthesis in the fluke (Anderson *et al.* 1993; Stitt *et al.* 1995), an action also attributed to artemisinin in the malarial parasite (Gu *et al.* 1983).

The testes and vitelline follicles were severely disrupted by treatment with OZ78. In the testes, only spermatogonia and primary spermatocytes were present and in low numbers. The fluke isolate used for this experiment was the Cullompton isolate: in this isolate, spermatogenesis is known not to proceed beyond the primary spermatocyte stage, with the consequence that this isolate is aspermic and parthenogenetic (Fletcher *et al.* 2004; Hanna *et al.* 2008). Following OZ78 treatment, the spermatogonia were abnormal and the primary spermatocytes were multinucleate, as a result of nuclear, but not cytoplasmic, division. Eosinophilic and apoptotic multinucleate bodies equivalent to aberrant

secondary spermatocytes have been described by Hanna *et al.* (2008), but were not seen in the present study. So, even within the limited amount of sperm development that this isolate undergoes, there was disruption of the two early stages and the follicle became very empty.

In those vitelline follicles that could still be recognized, there appeared to be a preponderance of mature cells, suggesting that the normal developmental sequence from stem to mature cell had been disrupted. A similar shift in cell population within the follicle was observed after treatment with TCBZ and diamphenethide (Fairweather *et al.* 1988; Stitt and Fairweather, 1996). Within the mature cells, the shell globule clusters were loosely-packed; in some clusters the shell protein globules had coalesced; the cisternae of ger were swollen; lipid droplets were present and the cytoplasm appeared to be breaking down. Again, similar changes have been observed in the vitelline cells of *Schistosoma mansoni* following treatment with artemether (Xiao *et al.* 2002*b*). The presence of lipid and the disruption of the ger could be indicative of metabolic disruption by OZ78, whilst the loosely-packed clusters could be the result of a decrease in shell protein synthesis, as occurred with TCBZ and diamphenethide (Fairweather *et al.* 1988; Stitt and Fairweather, 1996). Premature fusion of the shell protein globules has been described in drug-treated *F. hepatica* before, but may be related more to pH changes than to alterations in calcium ion levels, as it did not occur after treatment with the calcium ionophore, lasalocid (Colhoun *et al.* 1998). A potential target of artemisinin action in malarial parasites is the sarco(endo)plasmic reticulum Ca^{++} -ATPase (SERCA), a membrane-bound enzyme that regulates calcium levels within the parasite (Eckstein-Ludwig *et al.* 2003; Jung *et al.* 2005; Uhlemann *et al.* 2007): swelling of the cisternae of ger in the vitelline (and tegumental) cells may be linked to this action, but comparable changes to the sarcoplasmic reticulum in the muscle cells was not seen. The disruption of the ger would severely affect the synthetic activity of the cells.

In extreme instances, the vitelline follicles were greatly reduced in size, contained very few cells (which were in the process of breaking down) and were barely recognizable. There is an old maxim, first advanced by Dawes (1968) that, in order to survive, flukes will shut down non-essential organs (such as reproductive organs) when under stress – such as that induced by drug treatment. It is possible that the changes to the testes and vitelline follicles simply reflect that maxim. The vitelline follicles occupy a large proportion of the body of the fluke and the vitelline cells produce the shell protein that underpins the production of large numbers of eggs (~25 000 eggs per fluke per day: Happich and Boray, 1969); the cells have a high rate of turnover and are very sensitive to drug action (Fairweather *et al.* 1988;

Skuce and Fairweather, 1990; Stitt and Fairweather, 1996; Colhoun *et al.* 1998). However, the changes seen were considerable and may be indicative of a more general action of OZ78. A severe degeneration of the vitelline follicles and atrophy (along with almost complete disappearance) of the testes and ovaries have been described following treatment of *Schistosoma mansoni* with artemether (Xiao and Catto, 1989; Xiao *et al.* 2004). Remarkably, the changes were reversible. The changes seen in the current study could be attributed simply to prolonged incubation *in vitro*, but this is unlikely as they were not observed in the control flukes.

So far, the morphological changes seen following treatment with OZ78 have been discussed largely in relation to those observed with existing fasciolicides. As indicated above, it is believed that artemisinins may act – at least in part – via the production of free radicals and it may be possible to explain some of the changes on this basis. For example, artemisinins and free radicals are known to induce the peroxidation of membrane lipids and oxidation of membrane proteins and membrane-bound enzymes, such as ion pumps (Rohn *et al.* 1996; Berman and Adams, 1997; Mason *et al.* 1997; Sumegi *et al.* 2005). Na^+/K^+ -ATPase pumps are associated with the apical and basal tegumental membranes (Skuce *et al.* 1987) and their inhibition by OZ78 could lead to the severe swelling of the basal infolds and the internal tissue flooding observed. The swelling of the ger cisternae could be due to a similar mechanism. Treatment of other organisms and tissues with artemisinins and free radicals also causes collapse of the membrane potential of mitochondria, leading to their swelling and inhibition of electron transfer and oxidative phosphorylation (Dubin and Stoppani, 2000; Wakabayashi and Karbowski, 2001; Li *et al.* 2005). This action could explain the mitochondrial changes seen and would have a major impact on energy production by the fluke. Such changes contribute to the process leading to cell death, or apoptosis (Dubin and Stoppani, 2000; Wakabayashi and Karbowski, 2001; Li *et al.* 2005). The degenerative changes in the tegumental and vitelline cells (and the vitelline follicles) may be indicative of this phenomenon.

Finally, the present study has highlighted an interesting anomaly between the SEM and TEM data: indeed, the lack of significant surface changes belies the widespread disruption evident internally. A similar observation has been made by O'Neill *et al.* (2009). The severe disruption of the tegumental syncytium and subtegumental tissues might contribute to the efficacy of this particular synthetic peroxide against *F. hepatica* (Keiser *et al.* 2006*b*, 2007*a*; Keiser and Morson, 2008*b*). It is difficult to see how the fluke could survive such major impairment to the tegumental system that protects the fluke against immunological, enzymatic, bile and drug attack. Moreover, it is unlikely that the affected flukes could

produce any viable eggs, given the marked degeneration of the vitellaria. The present results have revealed some of the morphological changes induced by OZ78 and some of its tissue targets. It will be interesting to see if similar changes take place when haemin is incorporated into the culture medium *in vitro* and following drug treatment *in vivo*. Further research on the efficacy and actions of artemisinins and synthetic peroxides against *F. hepatica* is warranted, to consolidate and extend the promising results obtained to date.

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