**Fasciola gigantica: The in vitro effects of artesunate as compared to triclabendazole on the 3-weeks-old juvenile**

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1. Introduction

*Fasciola gigantica* remains one of the most important helminth parasites of livestock in tropical countries. In Thailand, it is endemic in cattle and water buffalo, with prevalence rates of 13.9% and 8.9%, respectively (Sukhapesna et al., 1990; Tantasuvan and Kitikoon, 1996). The incidence is high in the North and North-East, than 2000 years (Hien and White, 1993; Klayman, 1985). In most country, artesunate and artemether are the only two derivatives of artemisinin that have been licensed for treatment of *Plasmodium falciparum* malaria since 1990 (Kamchonwongpaisan and Meshnick, 1996). In addition to their antimalarial properties, artesunate and artemether are also effective against various species of trematodes, such as *Clonorchis sinensis* (Chen et al., 1983; Keiser et al., 2006b; Kim et al., 2009) when treated in vitro and in vivo. More recently, it has been discovered that artemisinin derivatives also display fasciociidal activities. A randomized controlled study showed a high
efficacy of artesunate in the treatment of symptomatic human fascioliasis in Vietnam (Hien et al., 2008). An oral administration of artesunate at a dose of 200 mg/kg resulted in 95% and 56.4% reduction of worm burden in rats harboring adult and juvenile Fasciola hepatica, respectively (Duthaler et al., 2010). A high oral dosage of 400 mg/kg artesunate could completely eradicate adult F. hepatica harbored in rats (Keiser et al., 2006a). Likewise, 72 h in vitro incubations in 50 and 100 μg/ml artesunate resulted in 75% and 100% mortality of adult and juvenile F. hepatica, respectively (Duthaler et al., 2010). As well, an in vitro incubation in 10 μg/ml of artemether or artesunate caused severe tegumental damage in adult F. hepatica (Keiser and Morson, 2008). Similar alterations of the surface were also observed in adult F. gigantica when treated in vitro with 10–30 μg/ml of artemether (Shalaby et al., 2009). Ultrastructural changes of the tegument and gut of the triclabendazole-resistant adult F. hepatica in the rats were observed following 24–72 h in vivo treatment with 200 mg/kg artemether (O’Neill et al., 2009). However, to our knowledge no study to date has assessed the effects of these drugs on juvenile stage of F. gigantica, and no detailed morphological changes, particularly on the ultrastructure of the tegument has been reported. This is important because the immature flukes causes the most damage and severe pathological changes in liver of definitive hosts, as they feed on the hepatic parenchyma while migrating through the liver before entering the bile duct, where they become adults. Hence, this experiment aims to investigate the effect of artesunate on the 3-week-old juvenile F. gigantica, and observe the surface alterations at both light and electron microscopic levels after the in vitro treatment, by comparing with triclabendazole.

2. Materials and methods

2.1. Parasites

Metacercariae of F. gigantica were obtained from laboratory-reared snails Lymnaea allula, infected with miracidia hatched from eggs of adult F. gigantica collected from the bile ducts and gall bladders of the cattle and buffaloes at a slaughter house in Pathum Thanie Province, Thailand. To obtain juvenile fluke male Golden Syrian hamsters were infected intragastrically with 30 metacecercariae each. Twenty-one days post infection, hamsters were sacrificed, juvenile flukes (8–12 worms per animal) were recovered from the liver parenchyma. They were washed several times with 0.85% NaCl and only actively motile parasites were selected for the experiment. The use of hamsters and experimental protocol were approved by the Animal Ethic Committee, Faculty of Science, Mahidol University.

2.2. Preparation of the drugs

Triclabendazole (Fasinex®, 10%, Ciba-Geigy Ltd., Switzerland) was a gift from Novartis Ltd., Thailand. Artesunate (Guilin No. 2 Pharmaceutical Factory, Guangxi, People’s Republic of China) was a gift from Novartis Ltd., Thailand. The drugs were dissolved in 5% sodium bicarbonate, yielding a concentration of 60 mg/ml, which was added to the M-199 medium (Sigma Co., St. Louis, MO, USA) containing antibiotics (penicillin, 50 IU/ml; streptomycin, 50 μg/ml, and 50 μg/ml haemin (Fluka, Buch Switzerland, Xiao et al., 2001) to give final concentrations of ATS at 20, 40, and 80 μg/ml. Triclabendazole (TCZ) was initially prepared as a stock solution in dimethyl sulphoxide (DMSO) and then added to the M-199 medium containing the antibiotics to give a maximal concentration of 0.5% (V/V). TCZ at concentrations 20, 40, and 80 μg/ml in M-199 medium was prepared from the stock solution and used as the positive controls.

2.3. In vitro treatment with the drugs

The 3-week-old juvenile flukes (10 per group) were incubated in the medium containing various concentrations of the drugs at 37°C with 5% CO2. For TCZ treatment, control flukes were incubated in M199 medium containing antibiotics and 0.1% (v/v) DMSO, while for artesunate treatment control flukes were incubated in M199 medium containing antibiotics and 50 μg/ml haemin. The motility of control and drug-treated flukes were observed at 1, 3, 6, 12, and 24 h of incubation. The motility of each parasite at each incubation period was scored using the criteria proposed by Kiuchi et al., (1987). The flukes with no motility were stained in 1% methylene blue (w/v, diluted in 0.85% NaCl) for 2 min, and the excess dye was washed out with 0.85% NaCl.

Score 1 = movement of only some parts of the body
Score 2 = movement of the whole body
Score 3 = movement of the whole body, but not dead (unstained with 1% methylene blue)
Score 4 = immobile and dead (stained with 1% methylene blue)

The efficacies of the drugs tested against the 3-week-old juveniles of F. gigantica were evaluated from the relative motility (RM) value (Kiuchi et al., 1987), calculated as followed:

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RM = \frac{MI_{test}}{MI_{control}} \times 100
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Motility index (MI) = \( \frac{\sum nN_n}{N_0} \)  
\( n = \text{score} \), \( N_n = \text{number of flukes with the score of n} \)

2.4. Scanning electron microscopy (SEM)

The flukes incubated in the drugs at concentrations of 40 and 80 μg/ml were fixed in 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4 at room temperature for 4 h. Then, they were washed three times with PBS, postfixed in 1% osmium tetroxide (OsO4) in 0.1 M PBS, pH 7.4, at 4°C for 1 h, and washed six times in cold distilled water, 15 min each. Subsequently, they were dehydrated through a graded series of ethanol (50%, 70%, 80%, 90%, 95%, 100%), for 30 min in each step. Thereafter, the worms were dried in a Hitachi HCP-2 critical-point drying machine using liquid carbon dioxide as a transitional medium. After drying, specimens were mounted on aluminum stubs and coated with platinum and paladium in an ion-sputtering apparatus, Hitachi, E-102, set at 10–15 mA for 6 min. The specimens were examined and photographed in a Hitachi scanning electron microscope S-2500, operating at 15 kV.

2.5. Transmission electron microscopy (TEM)

After several washings with 0.85% NaCl solution, the flukes were sliced into several thin strips then fixed in 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS, pH 7.4 at room temperature for 4 h, dehydrated by ethanol and embedded in Araldite 502. Ultrathin sections were cut using a glass knife and collected on 300-mesh copper grids, doubly stained with 1% methanolic uranyl acetate and lead citrate for 30 min each. The sections were viewed and photographed in a Hitachi H–300 transmission electron microscope, operating at 75 kV.
3. Results

3.1. The motility of the 3-week-old juveniles

3.1.1. Control groups

The juvenile flukes incubated in M199 medium with 0.1% DMSO or M199 medium containing 50 µg/ml haemin remained actively mobile with whole body movement (RM = 100) throughout the study period of 24 h.

3.1.2. Triclabendazole (TCZ)

At the 6 h examination time point, the juvenile flukes treated with TCZ started to exhibit decreased motility. All flukes treated with 80 µg/ml TCZ showed movement of only some parts of their bodies (RM = 67). Eighty percent of the flukes treated with 40 µg/ml TCZ showed movement of only parts of the body (RM = 73, 83). At 12 h incubation, 90% of flukes treated with 40 µg/ml TCZ exhibited movement of only parts of the body (RM = 73), whereas 20% of the flukes treated with 20 µg/ml TCZ showed no movement, but they were still alive and unstained with methylene blue (RM = 20). After 24 h post incubation, 100% of the flukes treated with 80 µg/ml TCZ and 30% of those treated with 40 µg/ml TCZ were dead (RM = 0, 23). In the group treated with 20 µg/ml TCZ, 20% of them became partially immobile and 80% were completely immobile but not dead (RM = 40) (Fig. 1).

3.1.3. Artesunate (ATS)

Twelve hours post incubation, the flukes treated with ATS started to show decreased motility. Eighty percent of the flukes treated with 80 µg/ml ATS were partially immobile (RM = 73). Fifty and thirty percent of the flukes treated with 40 and 20 µg/ml ATS showed movement of some parts of their bodies (RM = 83, 90), respectively. At the 24 h examination time point, 20% of the flukes treated with 80 µg/ml ATS became immobile and 80% were partially immobile (RM = 60). Seventy and eighty percent of flukes treated with 20 and 40 µg/ml ATS became partially immobile (RM = 76, 73), respectively. No fluke was found to be dead after being treated with ATS for 24 h. (Fig. 1).

3.2. Tegument alterations as observed by SEM

3.2.1. Control groups

The tegument of the untreated 3-week-old juvenile parasites and the control groups which were incubated in 0.1% DMSO or 50 µg/ml haemin showed similar surface topography at 24 h incubation. The key features of surface topography of the 3-week-old juvenile *F. gigantica* is briefly mentioned in order to differentiate the alterations resulting from treatments with the drugs. The 3-week-old fluke is flattened and leaf-like, measuring about 2.0–2.5 mm in length and 1.5 mm in width. The ventral surface contains two suckers: the oral sucker is located at the tip (Fig. 2A), whereas the ventral sucker is situated at one-third of the body from the anterior end. Tegument on the ventral surface is covered with numerous single-pointed spines except at areas around the suckers. The spines are more crowded on the cephalic cone and middle region, and reduced in number and size at the posterior and tail regions (Fig. 2A–C). Tegument surface between the spines folds into small ridges and pits (Fig. 2B, inset). On the dorsal surface, the spines are also single-pointed type, and they are reduced in number and size from the cephalic cone to the middle region (Fig. 2D and E), and are absent in the tail region (Fig. 2F).

3.2.2. Triclabendazole (TCZ) - treated groups

The sequences of changes observed in the tegument of the 3-week-old juveniles after treatment with TCZ were composed of the followings: an area of swollen ridges which appeared first in few small loci and later at multiple scattered loci (Fig. 3A). These edematous changes lead to bleb formation (Fig. 3B–D), and erosion of the tegument after these blebs were ruptured (Fig. 3E). At a later time period, the affected surface was eventually collapsed and lesions were formed (Fig. 3G and H). Grading of the tegumental changes and disruption in the 3-week-old juvenile flukes during the course of incubation with TCZ are summarized in Table 1.

Three hour post incubation with 40 µg/ml TCZ, slight swelling of the tegument was observed on the cephalic and middle regions, particularly along the lateral margins; the spines were partially submerged in the swollen ridges (Fig. 3A). Following 6 h treatment, several small blebs were observed inside the oral sucker. In addition, small areas of erosion were observed at the outer rim of the sucker (Fig. 3B). Tegument on ventral surface of the cephalic and middle regions showed moderate swelling of tegumental folds and ridges. The spines in this area were submerged in the swollen tegument. Numerous blebs covered the swollen tegument, especially along the lateral margins (Fig. 3C), where some blebs were ruptured, giving rise to lesions in this area (Fig. 3C, inset). Tegument swelling also appeared on the tail but there was no blebs. The dorsal surface of cephalic region showed severe swelling of tegumental ridges (Fig. 3D), which were also covered with numerous small blebs (Fig. 3D, inset), while the middle region appeared slightly swollen. Twelve hour post incubation, erosions and lesions appeared on all regions of the ventral surface, and at the tail region the underlying parenchymal tissues were exposed (Fig. 3E, inset). On the dorsal surface the tegument appeared slightly swollen. At the 24 h examination time point, the treated flukes exhibited deformity of their bodies, and the tegument on the middle and tail regions also appeared corrugated (Fig. 3F). The lesions were more pronounced throughout the ventral surface, especially on the rim of the ventral sucker. Tegument on the dorsal surface also appeared to be highly disrupted.

At the 3 h examination time point with 80 µg/ml TCZ, swelling of the tegument was observed on the cephalic and middle regions,

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**Fig. 1.** Relative motility (RM) values of the 3-week-old juveniles of *Fasciola gigantica* after in vitro incubations in triclabendazole (TCZ) and artemesunate (ATS) at various doses.
and the spines were partially submerged in the swollen ridges. At 6 h incubation in 80 μg/ml of TCZ, all of the flukes examined showed extensive tegumental changes. On the ventral surface, small blebs were formed inside the oral sucker as well as at its rim similar to that observed in 40 μg/ml TCZ treated-flukes at 12 h period (Fig. 3G, inset). Severe changes were observed on all regions of the ventral surface, consisting of swelling of the tegument and ruptured blebs and erosions, especially at the tail region (Fig. 3G, inset ii). In contrast, the dorsal surface was less affected and appeared only slightly swollen. At 12 h incubation, all flukes exhibited abnormal shape (Fig. 3H). The tegument around the oral and ventral suckers were extremely swollen (Fig. 3I). Severe blebbing, erosions, and lesions were observed on both the ventral and dorsal surfaces of the tegument (Fig. 3H, inset).

3.2.3. Artesunate (ATS) – treated groups

In ATS treated flukes, the sequence of tegument damages were similar to that induced by TCZ, and are summarized in Table 1.

Three hour post incubation in 40 μg/ml ATS, the ventral surface of cephalic region showed only mild swelling in the area below the ventral sucker and on lateral margins. The rim of the ventral sucker exhibited swelling and protrusion (Fig. 4A). The dorsal tegument appeared unaffected. At 6 h incubation, the ventral surface of the cephalic region exhibited blebbing and erosions, especially on the lateral margin. The tegument around the ventral sucker was severely swollen and covered with numerous blebs. Disrupted blebs were also present on the rim of the ventral sucker, with small patches of tegument sloughed off (Fig. 4B). Severe swelling and blebbing were prominent on ventral surface of the middle region where spines were completely submerged in the swollen tegument, with some detached, exposing empty sockets (Fig. 4C). The dorsal tegument appeared less affected with only mild swelling. At the 12 h examination time point, blebs occurred on both ventral and dorsal surfaces of the cephalic and middle regions, especially along the lateral margins with a few eroded areas (Fig. 4D). At 24 h post incubation, the treated flukes showed extensive destruction of the tegument on both surfaces. Tegument on the tail region was severely edematous and eroded (Fig. 4E, inset).

Tegument damages after treatment with 80 μg/ml of ATS was similar but occurred more rapidly and severely than those seen at the lower concentrations. After 3 h incubation, tegument on the ventral surface at the cephalic and middle regions exhibited moderate swelling whereas the dorsal tegument on the cephalic region showed mild swelling. At the 6 h examination time point, tegumental swelling and blebbing were more pronounced on both sides of cephalic and middle regions, with several patches of erosions and lesions exposing the underlying tissues (Fig. 4G). The tail region was less affected. At the 24 h examination time point, the tegument of cephalic region was severely damaged, with swelling and blebbing of the surfaces around oral as well as ventral sucker. The ventral...
surface showed a large area of tegument disruption (Fig. 3H), which was more severe on the middle and the tail regions (Fig. 4H, inset). The dorsal surface also showed severe disruption. There was extensive swelling with blebbing of ridges on all regions, and large patches of tegument along the lateral margins appeared eroded and completely sloughed off (Fig. 4I).

Fig. 3. Scanning electron micrographs of the 3-week-old juveniles of *F. gigantica* treated with 40 μg/ml (A–F) and 80 μg/ml (G–I) of TCZ in vitro. (A) Three hour post incubation with 40 μg/ml TCZ, the tegument on the ventral middle region shows swelling of ridges (arrow), the spines (Sp) are partially submerged in the swollen tegument. (B) Six hour post incubation, small blebs (Bl) occur on the muscular rim of the oral sucker (Os) with some appear disrupted and eroded (arrow). (C) At the 6 h examination time point, the tegument on the ventral middle region shows severe swelling of ridges (asterisk), some parts are covered with clusters of blebs (arrows). Inset exhibits the blebs (Bl) at higher magnification, and some are disrupted (arrow head). (D) At 6 h incubation, the dorsal surface in the cephalic cone shows tegument swelling, and blebs (inset, Bl) are distributed throughout this region. (E) Twelve hour post incubation, the ventral surface showing disruption at the tail region (arrow). Inset shows higher magnification of the damaged area with the exposed parenchymal tissues (arrow). Note the swollen tegument (inset, arrow head) at the rim of the affected area. (F) At the 24 h examination time point, the treated fluke shows deformity, as the tegument on the ventral surface is severely swollen (asterisks) and becomes corrugated (arrow) especially at the middle and tail regions. The ventral sucker (Vs) shows swollen rim. Os = oral sucker. (G) Six hour post incubation in 80 μg/ml TCZ, the ventral sucker (inset i, Vs) exhibits swollen rim which is also covered with small blebs (inset i, Bl). Erosions occur at the ventral surface of the middle and the tail regions (arrow), where the basal lamina is exposed (inset ii, arrow head). Os = oral sucker. (H) Twelve hour post incubation, the tegument on the cephalic cone region becomes swollen and appears corrugated (arrow head), with numerous blebs (inset, Bl). Os = oral sucker, Vs = ventral sucker. (I) Higher magnification of the cephalic cone region in Fig. 2H, the tegument as well as rims (arrows) of the oral (Os) and ventral (Vs) suckers are severely swollen and become corrugated.
3.3. Tegument alterations as observed by TEM

3.3.1. Control groups

The ultrastructure of the tegument of the 3-week-old juvenile *F. gigantica* in control groups which were incubated in 0.1% DMSO or 50 μg/ml haemin exhibited normal morphology at all incubation periods. After 24 h incubation, the control flukes showed intact tegument syncytium and sub tegumental muscles (Fig. 5A–C). The apical part of the syncytium exhibited many ridges and pits, while underneath there are numerous tegumental granules and mitochondria, and intact spines (Fig. 5A and B). There were two types of secretory granules the G1 is a dense ovoid granule about 90 × 180 nm in size and containing homogeneous dense matrix. The G2 is discoidal in shape and about 25 × 220 nm in size (Fig. 5B). The apical layer of the syncytium contained numerous G2 granules whereas the basal layer was filled with both types of granules. The mitochondria were distributed throughout the basal layer. The cytoplasm of the syncytium consisted of tightly packed microtrabeculae of very thin filaments. The basal layer was invaginated to form vertical basal in folds. The subt egumental muscle layers were composed of tightly packed muscle cells which were filled with thick and thin microfilaments (Fig. 5C). Clusters of tegumental cells were located underneath the muscular layers. These cells contained highly euchromatic nuclei with nucleoli, and their cytoplasm were filled with numerous mitochondria and tegumental granules (Fig. 5D).

3.3.2. Triclabendazole-treated groups

The severity of ultrastructural changes in the tegument of the 3-week-old juvenile *F. gigantica* after treatment with TCZ were dependent on doses and time of incubation. Three hour post incubation with 40 μg/ml TCZ, most tegumental ridges were swollen with deep invagination of the pits. Large areas of depolymerized microtrabecular network appeared throughout the width of the basal layer, which resulted in the formation of non membrane-bound vacuoles (Fig. 6A). G1 and G2 granules as well as mitochondria were decreased in numbers when compared to the control flukes. In addition, basal in folds became dilated in some parts of the tegument (Fig. 6A). Twelve hour post incubation, tegument swelling and blebbing were more extensive than at earlier times. There were fewer number of both G1 and G2 granules and mitochondria in the basal layer of the syncytium (Fig. 6B). Large areas of non membrane-bound vacuoles appeared throughout the width of the syncytium (Fig. 6B). The tegumental cells appeared necrosed as their nuclei contained clumps of chromatin. The nuclear membrane and the plasma membranes showed areas of discontinuity (Fig. 6D). There were fewer numbers of granules, and the electron lucent area occurred in the cytoplasm due to cytoskeletal depolymerization. At the 24 h examination time point, the apical layer exhibited severe swelling and blebbing. The vacuoles were increased in size and distributed throughout the width of the syncytium (Fig. 6C). There were marked drops in the numbers of tegumental granules and mitochondria in the syncytium. The basal layer of syncytium was almost completely lifted away from the basal lamina (Fig. 6C).

When the flukes were treated with 80 μg/ml TCZ, the ultrastructural changes of the tegument were similar to those observed at the lower concentrations. However, the damages occurred faster and covered more extensive area. The tegumental syncytium showed the most severe disruption after 12 h incubation. The apical layer was severely damaged with swelling and blebbing of tegumental ridges: so much that some regions broke down and were shed off. Most vacuoles were large and occurred throughout the width of the syncytium (Fig. 6E). Large areas of the tegument were sloughed off, exposing the basal lamina (Fig. 6F). The muscular layers underneath the syncytium also exhibited depolymerization of myofilaments (Fig. 6F). Tegumental cell bodies were severely disrupted, with few granules remaining (Fig. 6G). The cytoplasm of these cells were almost completely devoid of distinguishable organelles and appeared markedly electron lucent. The remaining mitochondria in the cytoplasm were swollen and became rounder as well as degenerated. Golgi complexes and rough endoplasmic reticulum became fragmented (Fig. 6H).

3.3.3. Artesunate-treated groups

The tegument of the juvenile flukes after treatment with ATS also showed similar changes to those described in the TCZ treated parasites.

Six hour post incubation in 40 μg/ml ATS, the apical layer of the tegument showed severe swelling and blebbing. In addition, most of the ridges contained several open bodies. Large areas of depolymerized microtrabecular occurred through the syncytium
At the 12 h examination time point, large vacuoles appeared in all layers of the tegumental syncytium. At the base of the syncytium, basal infolds were slightly dilated. There were reduced numbers of granules and mitochondria in the tegument (Fig. 7B).

Six hour post incubation with 80 μg/ml ATS, there were marked decreases in numbers of G1 and G2 granules, as well as mitochondria. Swelling and blebbing became prominent in apical layer of the syncytium. The cytoskeleton became depolymerized. The basal infoldings became dilated (Fig. 7C). The most extensive tegument

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disruption was observed after 24 h incubation. Several large membrane bound vacuoles occurred in the basal layer, and the syncytiotum was almost devoid of any tegumental granules (Fig. 7D and E). The remaining granules were mostly the G2 type, which were found just below the apical plasma membrane (Fig. 7E). Many spines appeared to be broken up (Fig. 7D). There were fewer number of mitochondria and most of them were swollen, with small vacuoles inside and disrupted outer membrane (Fig. 7E and F). Severe depolymerization of the microtrabeculae in the syncytium was widespread throughout the tegument (Fig. 7E). Such disruption was also prominent in the tegumental cells, thus most appeared vacuolated (Fig. 7G). The cytoplasm of most cells was almost completely lack of any organelles, and appeared markedly electron lucent (Fig. 7G). The subtegumental muscle layers showed extensive depolymerization of myofilaments (Fig. 7H).

4. Discussion

The fasciolicidal properties of artesunate (ATS) against the juvenile stage of F. gigantica has been evaluated using the relative motility value (RM) and changes of the tegument as observed by SEM and TEM. These findings were compared with those caused by triclabendazole (TCZ), the highly potent fasciocide against the immature and adult flukes. The RM of flukes increased significantly from 6 to 12 h incubation, with the highest motility value (RM) was observed after 24 h incubation with 25–50 μM TCZ, whereas the immature flukes were found to be more sensitive to the drug than adult parasites, as exposure for 24 h to 10 μM TCZ resulted in a complete immobilization and death (Bennett and Köhler, 1987).

Exposure of adult F. gigantica for 3 h to 175 μg/ml TCZ resulted in decreased motility of the flukes and death within 12 h of incubation (Saowakon et al., 2009). For the ATS-treated juvenile flukes the RM value began to decrease markedly at 12 h for all dosages, and complete immobilization was detected only in the flukes treated with 80 μg/ml ATS for 24 h. However, no fluke was found dead. These findings were similar to previous studies in F. hepatica (Keiser et al., 2006a; Duthaler et al., 2010), which employed the doses ranging from 1 to 100 μg/ml of ATS, and showed that adults treated with a lower dose of ATS at 10 μg/ml showed decreased motility but did not die. With increasing ATS concentration to 100 μg/ml, all of the treated juvenile and adult flukes died within 72 h of incubation (Keiser et al., 2006a; Duthaler et al., 2010). Furthermore, Keiser et al. (2009) reported the killing of juvenile F. hepatica in rats whose serum concentrations of ATS were between 10 and 100 μg/ml after receiving ATS at a dose of 10 mg/kg intravenously, or 100 mg/kg orally. This indicated that our doses (20–80 μg/ml) lied within the ranges of effective doses used both in vitro and in vivo for F. hepatica experiment. Our incubation time for F. gigantica may not be long enough to kill the parasites; however, the trend was clear that F. gigantica was also highly affected by ATS but to a lesser extent than TCZ.

When observed by SEM, it is evident that TCZ has a rapid and severe effect on juvenile F. gigantica. The sequences of pathological changes observed in the tegument was initially composed of the swelling of the tegumental folds and ridges which appeared first as a small patch which scattered in multiple loci. Subsequently, there were blebs formed at the surface which were later ruptured, resulted in the formation of small patches of lesions.

In ATS treatment, the surface alterations of the juvenile F. gigantica may not be long enough to kill the parasites; however, the trend was clear that F. gigantica was also highly affected by ATS but to a lesser extent than TCZ.

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In ATS treatment, the surface alterations of the juvenile F. gigantica exhibited similar sequence as that of TCZ but with less severity. Similar tegument alterations were noted after incubation of adult F. hepatica and F. gigantica in artemether (Keiser and Morson, 2008; Shalaby et al., 2009), and were also observed in the
Fig. 6. Transmission electron micrographs of the 3-week-old juveniles treated with 40 μg/ml (A–D) and 80 μg/ml (E–H) of TCZ in vitro. (A) Three hour post incubation in 40 μg/ml TCZ, the apical layer of the syncytium exhibits swollen ridges (Rd) and deeply invaginated pits (Pi). Blebbing (Bl) occurs on the swollen ridges. The basal layer of the syncytium contains many large non membrane-bound vacuoles (Va). The basal infolds show dilation (asterisk), G1 granules and mitochondria (Mi) are distributed throughout the syncytium. Sp = spine. Ba = basal lamina. Mu = muscle layers. (B) Twelve hour post incubation, the ridges (Rd) are severely swollen with blebbing (Bl). Several electron-lucent area (arrows) appear throughout the syncytium. Fewer numbers of mitochondria (Mi) and G1 granules are present in the syncytium. Sp = spine. Ba = basal lamina. (C) At the 24 h examination time point, some parts of the syncytium are separated (arrow) from the basal lamina (Ba). There are numerous blebs (Bl) on the apical layer and vacuoles (asterisks) in the syncytium. The mitochondria (Mi) are swollen. (D) A tegumental cells (Tc1) shows fewer G1 granules around its nucleus (Nu). Fewer of G2 are present underneath the plasma membrane, while there are few electron-lucent areas (arrow) in the cytoplasm of Tc2 cell. The plasma membrane between these cells shows disruption (arrow heads). (E) Twelve hour post incubation in 80 μg/ml TCZ, tegument shows numerous membrane-bound vacuoles (asterisk) in the basal layer of the syncytium. The apical layer exhibits severe blebbing (Bl) and shedding. Fewer numbers of G1 and G2 granules are present in the syncytium. There are also fewer mitochondria (Mi) remaining in the syncytium, all of them appear swollen, and some are lysed (arrow). Sp = spine. Ba = basal lamina. (F) At the 12 h examination time point, some parts of the syncytium are totally sloughed off, exposing the basal lamina (Ba), and muscular layers (Mu). (G) Twelve hour post incubation, tegumental cells (Tc1 and Tc2) showing lysis of the plasma membrane (arrows). The nuclear membrane of Tc2 exhibits disruption (arrow head). Fewer number of mitochondria is present in the cytoplasm of both cells, and most of them are swollen (Mi). Nu = nucleus. (H) At the 12 h examination time point, a tegumental cell (Tc1) contains fewer G1 granules, and most become necrotic. Mitochondria are severely swollen (asterisks), and show necrotic change (arrow). A few patches of plasma membrane lysis are observed (arrow head). Golgi apparatus (Gl) is fragmented.
When examined by TEM, the earliest changes in the tegument of the 3-week-old juvenile flukes after treatment with TCZ and ATS were the swelling and blebbing of the apical layer followed by the occurrence of numerous electron-lucent areas so called “open bodies” underneath the apical plasma membrane. At later time periods, the basal infolds became dilated, and some turned into membrane-bound vacuoles at the base of the tegument syncytium. Concurrently, the microtrabecular network in the syncytium...
became depolymerized, leading to the formation of non-membrane-bound vacuoles of various sizes throughout the syncytium, leading to its total disintegration and detachment. The sequence of ultrastructural changes was generally similar at all doses; however, the changes occurred faster at the higher doses and longer incubation times. The number of tegumental granules and mitochondria in the syncytium and the cell bodies decreased progressively. Eventually, tegumental cells also showed total disruption and necrotic changes. These alterations of tegument are similar to those observed in the tegument of adult triclabendazole-resistant *F. hepatica* treated with artemether, where following 72 h treatment with 200 mg/ml artemether in vivo, the tegument exhibited surface blebbing while the cell bodies produced fewer secretory granules (O’Neill et al., 2009).

From the SEM and TEM observations, it is evident that both TCZ and ATS cause severe damage to the tegument of *F. gigantica* as the primary target, perhaps because of causing disruption of the cytoskeleton and osmoregulatory system. The tegument of fasciolas has many of the characteristics of a transporting epithelium that is involved in ion and water controls (Threadgold and Brennan, 1978). The basal plasma membrane, in particular, is invaginated to form long, parallel-structures known as basal infolds. Associated with these membrane infolds, and with the apical plasma membrane, are mitochondria. The Na$^+$/K$^+$ ATPase is found to be localized in the apical plasma membrane and the basal infoldings, which is indicative of the presence of ion-pumps on the tegument membrane (Threadgold and Brennan, 1978). It seems likely that the initial swelling of the tegument is due to an osmotic effect of the drugs brought about by a change in the permeability of the apical plasma membrane. Thus, large influx of Na$^+$ ions might overcome the capacity of the Na$^+$/K$^+$-ATPase-driven ion pump situated along the apical plasma membrane (Threadgold and Brennan, 1978), and the Na$^+$ ions could penetrate deeper into the syncytium and being pumped into the basal infolds. The lumen of the infolds would become hypertonic with respect to the surrounding cytoplasm, and water would be drawn into the infolds from the cytoplasm, forcing them to swell and eventually detached from the basal lamina as seen in TEM after prolonged incubation with the drugs.

The blebbing observed in the treated flukes is a common feature observed in many species of drug-treated trematodes. In *F. hepatica*, surface blebbing has been observed following treatments with a number of anthelminitics, including compound alpha (Rivera et al., 2005), diamepantide (Anderson and Fairweather, 1988), closulon (Meaney et al., 2003, 2004), and triclabendazole (Stitt and Fairweather, 1993, 1994). It has been suggested that the blebbing occurs as a result of increased efforts on the part of the parasites to shed and then replace the damaged outer tegumental membrane (Bennett et al., 1980). In association with this membrane responses, a number of “open bodies” occurred in close contact with the apical plasma membrane. These were believed to be G$_1$ or G$_2$ secretory granules which had attached to the apical plasma membrane and opened to release their content for the repairment of the damaged surface membrane (Rogan and Threadgold, 1984).

Vacuolization of the tegument in this study consists of two types: the first is membrane- bound and the other is non membrane-bound vacuoles. The former arise from dilatation of the basal infolds as a result of a water and ion imbalance as mentioned earlier. The latter is a consequence of depolymerization of the cytoskeletal network and autophagy of the organelles in the syncytium. Similar vacuolation of the tegument has been described in a number of anthelmintic-treated schistosomes (*Xiao et al., 2002; Sobhon and Upatham, 1990;* O. viverrini (Apinhasamit and Sobhon, 1996), and also in *F. hepatica* (Halferty et al., 2009; Stitt and Fairweather, 1994).

It is known that TCZ blocks the polymerization of microtubules which, in turn, indirectly interrupts the transport of the tegumental G$_1$ and G$_2$ granules, thus causing a drop in numbers of the granules in the tegument while an accumulation of these granules in the cytoplasm of tegumental cells. Consequently, the turnover of tegument surface membrane and the integrity of the microtubeculae could not be maintained, and resulted in blebbing, vesiculation and sloughing of the syncytium. With increased incubation times, the tegumental cells became completely necrosed and lysed. These changes are similar to those observed in *F. hepatica* tegument after treatment with TCZ-SX (Stitt and Fairweather, 1994) and tubulozoic-C (Stitt and Fairweather, 1993).

Artemisinin and its derivatives, currently used against malaria parasite (Meshnick, 2002), also display potent antischistosomal activities in vitro and in vivo (Jiraungkoorskul et al., 2005, 2006; Xiao and Catto, 1989). The schistosomes are more susceptible to the drug than the adult stage. Recently, the fascioelastic property of artemisinins against both juvenile and adult *F. hepatica* and adult *F. gigantica* had been reported (Duthaler et al., 2010; Keiser et al., 2006a; O’Neill et al., 2009; Shalaby et al., 2009). It is well established that the mechanism of action of these compounds appears to involve the intraparasitic iron (Fe$^{3+}$), which catalyzes the cleavage of the endoperoxide bridge to produce carbon-centered free radicals (Meshnick, 2002). The actions of these radicals in malarial parasites are thought to be composed of: (1) alklylation of heme; (2) alklylation of parasite protein; (3) inhibition of a Ca$^{2+}$ transporting ATP-ases (SERCA); (4) oxidative damage to parasite plasma membrane; and (5) disruption of the electron transport chain in mitochondria (O’Neill et al., 2010). Li et al. (2005) demonstrated that mitochondria could be the primary target of artemisinin, since mitochondria are known to be a rich source of transition metals, including iron and copper (Kispal et al., 1999), and the mitochondria could consequently be damaged by the locally generated free radicals (Li et al., 2005). Artesunate might act against the fasciolas in the same manner as proposed in malaria parasite. The tegument of the fluke contains a large number of mitochondria in the basal layers as well as in the middle zone (Sobhon et al., 2000). After treatment with ATS, these mitochondria exhibited severe swelling, rupturing of the outer membrane and contained flocculent densities when viewed by TEM. As well swollen mitochondrial had been observed in tegument of adult triclabendazole-resistance *F. hepatica* treated with artemether (O’Neill et al., 2009). The disruption of these energy-providing organelles might result in the swelling of the ridges and basal infolds due to osmotic imbalance resulted from the impairment of energy-dependent Na$^+$/K$^+$-ATPase ion pump at the plasma membrane. Similar changes have been observed following treatment with closulon which disrupt energy production in the fluke (Meaney et al., 2004).

In this study, regional differences in response to both drugs’ action are similar, with the ventral surface tend to be more severely disrupted than the dorsal, and the middle part of the fluke more disrupted than the proximal and the tail. These regional differences are similar to those described previously after treatment of juvenile *F. hepatica* and adult *F. gigantica* with TCZ-SX (Meaney et al., 2002; Stitt and Fairweather, 1993) and TCZ (Saowakon et al., 2009). This different effect may reflect differences in the anatomy and physiology of various regions of the tegument (Anderson and Fairweather, 1988; Fairweather et al., 1986). It was previously reported that the tegument of both the juvenile and adult flukes could take up drugs (Bennett and Köhler, 1987). Usually, the ventral tegument of *F. gigantica* is thicker than the dorsal tegument. In addition the spines are more numerous, which in turn increase the area of drug absorption since the spines are also covered with the tegument. These differences can lead to the more severely effect on the ventral than the dorsal tegument.
In conclusion, the present study demonstrated the fasciociial properties of ATS which caused reduction of the motility and disruption to the tegument of the 3-week-old juvenile *F. gigantica* after in vitro incubation. ATS might act by generating the carbon-centered free radicals which, in turn, caused lipid peroxidation and protein alkylation within the parasites tegumental surface membrane and mitochondria. Furthermore, ATS resulted in the disruption of cytoskeleton and vacuolization of the tegument.

Acknowledgments

This work was financially supported by the Mahidol University (to Prasert Sobhon) and by a Ph.D. scholarship from the Commiss. on Higher Education, and Faculty of Graduate Studies, Mahidol University (to Tawewan Tansatit). The authors thank the Novartis Foundation on Higher Education. The authors thank the Novartis Foundation on Higher Education, and Faculty of Graduate Studies, Mahidol University (to Prasert Sobhon) and by a Ph.D. scholarship from the Commission on Higher Education, and Faculty of Graduate Studies, Mahidol University (to Tawewan Tansatit). The authors thank the Novartis Foundation on Higher Education, and Faculty of Graduate Studies, Mahidol University (to Tawewan Tansatit). The authors thank the Novartis Foundation on Higher Education, and Faculty of Graduate Studies, Mahidol University (to Tawewan Tansatit).

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