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

Tawewan Tansatit [a], Somphong Sahaphong [b], Suda Riengrojpitak [b], Vithoon Viyanant [c], Prasert Sobhon [d,*]

[a] Departments of Preclinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakhonpathom 73170, Thailand
[b] Department of Pathobiology, Faculty of Science, Mahidol University, RamaVI Rd., Bangkok 10400, Thailand
[c] Faculty of Allied Health Sciences, Thammasat University, Phahonyothin Rd., Klongluang, Pathumthani 12120, Thailand
[d] Department of Anatomy, Faculty of Science, Mahidol University, RamaVI Rd., Bangkok 10400, Thailand

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ABSTRACT

The *in vitro* effect of artesunate (ATS) on the 3-week-old juveniles of *Fasciola gigantica* was compared with triclabendazole (TCZ) by incubating the parasites in M-199 medium containing the drugs at concentrations of 20, 40, and 80 μg/ml for 1, 3, 6, 12, and 24 h. The anthelmintic activities of these drugs were evaluated based on the relative motility value (RM) and the alterations of the tegument as observed by scanning (SEM) and transmission (TEM) electron microscopy. The RM values of TCZ-treated flukes decreased significantly from 6 to 24 h for all dosages. For ATS-treated flukes, RM value decreased markedly from 12 to 24 h, but the rates of decline were less than TCZ at the same doses. When observed by SEM, the tegument showed similar sequence of morphological changes after treatments with both drugs, comprising of swelling of tegumental ridges, followed by blebbing and later rupturing of the blebs, leading to erosion and lesion, and disruption of the tegument. When examined by TEM, ultrastructural changes in the tegument and associated structures after treatments with TCZ and ATS were similar which comprised of swelling, blebbing of the tegument, dilation of basal infoldings, and depolymerization of the microtrabecular network. After a longer incubation time, the tegument was completely sloughed off and the tegument cell bodies became necrotic. Additionally, in ATS-treated flukes, mitochondria showed severe swelling, rupturing of outer membrane, and their interior filled with flocculent materials.

1. Introduction

Fasciola gigantica remains one of the most important helminth parasites of livestocks 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, and lower in the southern provinces (Sukhapesna et al., 1990; Suksaithaichana et al., 1993; Tantasuvan and Kitikoon, 1996). Fasciolosis is characterized by severe anemia and extensive hepatic tissue damage, with intense inflammatory reactions in response to the migration of immature parasites and the feeding of mature flukes (Behm and Sangster, 1999). At present there is no vaccine available for the prevention of fasciolosis (McManus and Dalton, 2006), and hence anthelmintic drugs remain the most effective mean for control. Triclabendazole, the most potent flukicide, is highly effective against both adult and juvenile stages of *Fasciola spp*. (Boray et al., 1983; Rapic et al., 1988), and has been used worldwide. However, the flukes' resistance to this drug is increasing since 1995 (Fairweather and Boray, 1999; Moll et al., 2000; Overend and Bowen, 1995). Thus, new drugs that can kill immature as well as mature flukes are urgently needed. Qinghao (*Artemesia annua*), a famous sweet wormwood from China, has been used in Traditional Chinese Medicine as a remedy for hemorrhoid and fever for more than 2000 years (Hien and White, 1993; Klayman, 1985). The active substance is artemisinin, a unique sesquiterpene lactone endoperoxide, extracted from the leaves and the flowers of Qinghao (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 *Schistosoma mansoni* (Utzinger et al., 2002), *Schistosoma. mekongi* (Jiraungkoorskul et al., 2005, 2006), *Opisthorchis viverrini*, and *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 fasciocidal activities. A randomized controlled study showed a high

* Corresponding author. Fax: +66 2 354 7168.
E-mail address: scpso@mahidol.ac.th (P. Sobhon).

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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 on 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 cause 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 ollula*, infected with miracidia hatched from eggs of adult *F. gigantic*a collected from the bile ducts and gall bladders of the cattle and buffaloes at a slaughter house in Pathum Thanee 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 purchased from Tropical Medicine Hospital, Faculty of Tropical Medicine, Mahidol University. Artesunate (ATS) was 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% (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% CO_2. 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 3 = movement of the whole body
score 2 = movement of only some parts of the body
score 1 = immobile but not dead (unstained with 1% methylene blue)
score 0 = 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:

$$\text{RM value} = \frac{\text{MI test}}{\text{MI control}} \times 100$$

$$\text{Motility index(MI)} = \frac{\sum nN_n}{\sum N_n}$$

n = score, N_n = 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 (OsO_4) 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.