Parasitology

cambridge.org/par

Research Article

Cite this article: Hasan MM, Roy BC, Biswas H, Rahman M, Anisuzzaman A, Alam MZ, Talukder MH (2022). Efficacy of flukicides on *Fasciola gigantica*, a food-borne zoonotic helminth affecting livestock in Bangladesh. *Parasitology* **149**, 1339–1348. https://doi.org/10.1017/S0031182022000580

Received: 10 January 2022
Revised: 20 April 2022
Accepted: 20 April 2022
First published online: 10 May 2022

Key words:
Bangladesh; *Fasciola gigantica*; flukicides; food-borne trematode; *in vitro*; scanning electron microscopy

Author for correspondence:
Md. Hasanuzzaman Talukder,
E-mail: talukdermhasan@bau.edu.bd

Efficacy of flukicides on *Fasciola gigantica*, a food-borne zoonotic helminth affecting livestock in Bangladesh

Mohammad Manjurul Hasan[1,2], Babul Chandra Roy[1], Hiranmoy Biswas[1,2], Moizur Rahman[3], Anisuzzaman Anisuzzaman[1], Mohammad Zahangir Alam[1] and Md. Hasanuzzaman Talukder[1]

[1]Department of Parasitology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh; [2]Department of Livestock Services, Dhaka, Bangladesh and [3]Faculty of Veterinary and Animal Sciences, University of Rajshahi, Rajshahi 6205, Bangladesh

Abstract

Fasciola gigantica, the causative agent of tropical fasciolosis, is a food-borne zoonotic trematode that affects around 80% livestock of Bangladesh. Triclabendazole (TCBZ), nitroxynil (NTON) and oxyclozanide (OCZN) are frequently used against fascioliasis; however, the current status of potency of these flukicides was unknown. In this study, *in vitro* efficacy of TCBZ, NTON and OCZN at various concentrations on *F. gigantica* has been evaluated by relative motility (RM), morphological distortions of apical cone through an inverted microscope, architectural and ultra-structural changes through histopathological and scanning electron microscopy (SEM). It is observed that TCBZ, NTON and OCZN at higher concentrations significantly ($P < 0.05$) reduced RM of the flukes compared to untreated control. NTON at 150 $\mu g\, mL^{-1}$ was the most potent to reduce the motility within 4 h whereas TCBZ and OCZN were much delayed. Histopathological changes showed swollen, extensive cracking, numerous vacuoles and splitting of the tegument surrounding the spines; spine dislodged from its socket in treated flukes compared to untreated worms. Histopathological changes were more conspicuous at higher doses of TCBZ, NTON and OCZN. SEM has shown the disruption of the apical cone, apart from swelling of the tegument on the ventral surface corrugation and disruption of the ventral apical cone. All these changes indicate that NTON is the most potent in killing flukes *in vitro* among the tested flukicides and suggest the presence of TCBZ-resistant fluke populations in Bangladesh. It is imperative to explore the *in vivo* effects of these flukicides and subsequently their molecular mechanisms.

Introduction

Fascioliasis, caused by *Fasciola* spp., is a food- and water-borne, zoonotic and neglected tropical disease which affects both animals and humans throughout the globe but more commonly found in tropics and sub-tropics (Mehmood *et al.*, 2017; Fairweather *et al.*, 2020). It is reported as one of the most widely spread diseases over 50 countries of the world (Mas-Coma, 2003; Mas-Coma *et al.*, 2005; Toledo and Fried, 2014; Mehmood *et al.*, 2017). It is estimated that 2.4 million people over 60 countries are infected with fascioliasis and more than 180 million people are at risk in the world (WHO, 1995; Mas-Coma *et al.*, 1999; Zerna *et al.*, 2021). Fascioliasis is considered an important and devastating disease in the world including Bangladesh which causes severe economic losses due to morbidity and mortality, declined weight gain (up to 20%), decreased milk and meat production (3–15%), damage and condemnation of liver of infected animals in the livestock industry (Mohanta *et al.*, 2014; Khan *et al.*, 2017; Aghayan *et al.*, 2019; Opio *et al.*, 2021). The global economic losses are evaluated to be more than USD 3.2 billion annually due to fascioliasis (Mas-Coma *et al.*, 2005; Charlier *et al.*, 2007; Luo *et al.*, 2021). In Bangladesh, the financial losses caused by fascioliasis are estimated to USD 0.16/slaughtered goat and would be USD 115.44/1000 slaughtered goats due to liver condemnation and USD 2374.9 annually (Islam and Ripa, 2015).

Globally, the incidence of fascioliasis has increased over the past two decades, possibly due to change in farming practices, climate and development of anthelmintic resistance (Sabourin *et al.*, 2018). *Fasciola gigantica* is one of the most endemic and parasitic diseases of ruminants in Bangladesh (Amin and Samad, 1988; Islam and Samad, 1989; Rahman *et al.*, 2017). The prevalence of *F. gigantica* in live animals has been reported to vary from 21 to 53% in cattle, 10 to 32% in goats, 8.4 to 31% in sheep and 19 to 51% in buffaloes (Islam *et al.*, 2014; Rahman *et al.*, 2017) and its prevalence in slaughtered animals also vary from 15 to 66% in cattle, from 3.8 to 22% in goats, 81% in sheep and from 23 to 47% in buffaloes, respectively (Talukder *et al.*, 2010; Islam *et al.*, 2014). However, the actual burden of *F. gigantica* in domestic ruminants might be much higher than the mentioned values since fascioliasis is mainly the subclinical disease (Khatun *et al.*, 2015). A recent retrospective epidemiological study has been carried out in domestic ruminants where the hot spot, clusters and risk factors of fascioliasis are identified in Bangladesh (Rahman *et al.*, 2017).

Fascioliasis is mainly treated with anthelmintic drugs, due to the absence of commercially available vaccines (Davis *et al.*, 2020). The most commonly used anthelmintic drugs against fascioliasis in animals are triclabendazole (TCBZ), nitroxynil (NTON), oxyclozanide (OCZN), clorsulon (CLORS), closantel and albendazole (ALBZ) (Kelley *et al.*, 2016). TCBZ is considered the first choice of anthelmintic for the treatment of fascioliasis due to its effect against immature and mature flukes (Rolfe and Boray, 1987) and other anthelmintics are effective against mature flukes only (Fairweather and Boray, 1999). TCBZ inhibits the polymerization of the tubulin molecules into the cytoskeletal microtubule structures (Fairweather, 2005; Fairweather, 2009). NTON stops the oxidative phosphorylation in the cell mitochondria of flukes and disturbs the production of adenosine triphosphate, thus impairing the motility of the parasites, whereas OCZN acts by uncoupling the oxidative phosphorylation in flukes (Boray and Happich, 1968; Rapic *et al.*, 1988). Currently, various flukicidal drugs are recommended commercially but their efficacy in fascioliasis has not been established (Fairweather *et al.*, 2020) and thus making the control of fascioliasis difficult (Novobilský and Höglund, 2015). There are several previous *in vitro* studies on both Fasciola *hepatica* and *F. gigantica* where the efficacy of flukicides has been analysed to evaluate the changes in various parameters namely relative motility (RM) (Saowakon *et al.*, 2009; Jeyathilakan *et al.*, 2012; Tansatit *et al.*, 2012; Lorsuwannarat *et al.*, 2014; Shareef *et al.*, 2014; Chang and Flores, 2015), morphometric (Shafiei *et al.*, 2014; Ahasan *et al.*, 2016; Shalaby *et al.*, 2016) and morphology followed by histopathological changes of treated flukes (Saowakon *et al.*, 2009; Jeyathilakan *et al.*, 2012; Lorsuwannarat *et al.*, 2014; Hanna *et al.*, 2015; Shalaby *et al.*, 2016). Consequently, the tegument is one of the tissues that exposed most immediately to anthelmintics and is likely to represent a primary drug target region (McKinstry *et al.*, 2003). Scanning electron microscopy (SEM) has been proven to be a useful tool for evaluating the surface changes on the tegument, suckers and spines of flukes resulting in anthelmintic action (Stitt and Fairweather, 1993; Fairweather and Boray, 1999; Meaney *et al.*, 2002; Halferty *et al.*, 2008; Saowakon *et al.*, 2009; Shalaby *et al.*, 2009; Diab *et al.*, 2010; Lorsuwannarat *et al.*, 2014; Shareef *et al.*, 2014; Omran and Ahmad, 2015).

In Bangladesh, much less information is available regarding the effectiveness of anthelmintics where the efficacy of NTON has been reported as 92.57% followed by TCBZ (91.55%) and ALBZ (84.53%) (Aktaruzzaman *et al.*, 2015). Although animals are treated commonly with various flukicides, the pathological changes may occur in liver and bile ducts which indicate flukicidal resistance against flukes. It has been reported previously that around 200–500 liver flukes, *F. hepatica*, are detected in a single infected liver during the necropsy of sheep (Soulsby, 2012). Even though TCBZ, NTON, OCZN and ALBZ are used randomly in ruminants of Bangladesh, numerous (~120) liver flukes have recovered from a single infected liver of goat collected from slaughter house. The presence of numerous flukes suggests the possibility of development of anthelmintic resistance, which may be due to indiscriminate use of anthelmintics or inappropriate dose and timing of flukicidal drug administration in Bangladesh. Resistance against anthelmintics has already been reported against nematodes in this country (Hoque *et al.*, 2003; Dey *et al.*, 2020). However, the current status of flukicides against *F. gigantica* has not yet been evaluated in Bangladesh. Since there is scarcity of information on the *in vitro* efficacy of flukicidal drugs on *F. gigantica* isolates in Bangladesh, the present study aimed to assess the potency of commonly used flukicidal drugs *in vitro* on *F. gigantica* by observing the fluke's motility, histopathology, morphometric and ultra-structural changes using SEM.

Table 1. Doses of flukicides used for *in vitro* experiment

Name of flukicides	Dosage (μg mL^{-1})		
TCBZ	10	20	40
NTON	50	100	150
OCZN	0.02	0.2	2

Materials and methods

Drugs and dosage

Pure form of drugs, TCBZ, NTON and OCZN was purchased directly from Germany (WITEGA Laboratorien Berlin-Adlershof GmbH, Berlin, Germany) and stored in the laboratory of the Department of Parasitology, Bangladesh Agricultural University, Mymensingh. Then the doses of these drugs were determined according to the calculation of previously published data (Table 1) (McKinstry *et al.*, 2007; Shalaby *et al.*, 2009; Fairweather *et al.*, 2012; Tansatit *et al.*, 2012; Lorsuwannarat *et al.*, 2014; Arafa *et al.*, 2015).

Collection and isolation of live flukes from the liver of slaughtered goats

Livers were collected immediately after slaughtering of goats from the local abattoir and brought to the laboratory of the Department of Parasitology, Bangladesh Agricultural University, Mymensingh. Then flukes were recovered from livers following the standard procedure described previously in the laboratory (Shalaby *et al.*, 2009). Flukes were cleaned from blood and debris using phosphate-buffered saline.

Culture of flukes

After washing, the alive flukes were cultured in a Petri dish containing RPMI-1640 medium incorporated with 50% (v/v) heat-denatured rabbit serum, 2% (v/v) rabbit red blood cells and antibiotics (penicillin, 50 IU mL^{-1}; streptomycin, 50 mg mL^{-1}) as per recommendation (Ibarra and Jenkins, 1984) at 37°C in the presence of 5% CO_2.

After an hour the dead or slow-moving flukes were removed from the culture plate. Then the media was changed with the help of pipette and the culture plate was placed in the incubator for subsequent *in vitro* experiments with flukicides.

Treatment of flukes with flukicidal drugs with selected doses

After half an hour of incubation, flukes were treated with flukicides when the motility was very live and spontaneous. Five flukes were cultured in each well of the culture plate containing RPMI-1640 media as per recommendation and treated with three selected drugs such as TCBZ, NTON and OCZN at different concentrations as described in Table 1 and subsequently repeated thrice independently.

Observation on the motility of flukes

The motility of the flukes was observed with a stereo zoom microscope and recorded hourly until death of the flukes. The motility of each parasite at each incubation period was scored using the criteria (Table 2) described previously (Kiuchi *et al.*, 1987).

https://doi.org/10.1017/S0031182022000580 Published online by Cambridge University Press

Table 2. Measurement criteria of motility and movement of flukes

Sl. no.	Score	Criteria	Calculation	Remarks
1	3	Movement of the whole body	RM value = (MI test × 100)/MI control Motility index = $\sum nN_n/\sum N_n$ n = score, N_n = number of flukes with the score of n (Kiuchi *et al*., 1987)	The control group where all the parasites scored 3 had the RM value of 100, and the smaller RM values indicated stronger drug activity
2	2	Movement of only some parts of the body		
3	1	Immobile but not dead		
4	0	Immobile and dead		

Morphometric changes in apical cone, suckers, spines and tegument of treated flukes

The morphometric measurements of the apical cone of control and treated flukes were performed using a computer image analysis system (ELICA QWin 500, Cambridge, England) following the manual and keys described previously (Valero *et al*., 1996; Shafiei *et al*., 2014; Ahasan *et al*., 2016; Diyana *et al*., 2020). The measurements include the lineal biometric characters such as length and width of apical cone, maximum diameter of oral and ventral suckers, length and width of spine as well as area of tegumental swelling around the ventral sucker (Shalaby *et al*., 2009).

Histopathological changes of flukes treated with flukicides

Five dead flukes from each treated and control groups were taken for paraffin embedding. Then the flukes were fixed in 10% formaldehyde for 24 h, dehydrated with an ascending series of ethanol and cleaned with xylene. They were then embedded in paraffin, sectioned at a thickness of 5 μm using a rotary microtome and stained with haematoxylin and eosin. Then the specimens were examined under an inverted microscope to check for the abnormalities and were photographed (Jeyathilakan *et al*., 2010; Hanna *et al*., 2015).

Scanning electron microscopy

Following incubation, the oral cone (including ventral sucker) of all flukes was fixed intact for 12 h in a 3:1 mixture of 4% (w/v) glutaraldehyde in 0.12 M Millonig's buffer (pH 7.4) and 1% aqueous osmium tetroxide. The specimens were washed repeatedly in double-distilled water, dehydrated through a graded series of ethanol (10, 20, 30, 40, 50, 70, 80, 90, 95 and 100%) for 10 min in each step, then dried with hexamethyldiacetylazene, fixed to aluminium stubs and coated with gold–palladium. The gold-coated specimens were examined under the Joel SEM (Jeol Corp., Mitaka, Japan) operated at 10 kV at the Centre for Advanced Research in Sciences in the University of Dhaka, Bangladesh.

Statistical analyses

One-way analysis of variance (ANOVA) with the *post hoc* Schaffer multiple comparison test was employed to identify the group having a statistically significant difference from the other groups in comparison with the control group. Differences between means at $P < 0.05$ were considered as the level of significance.

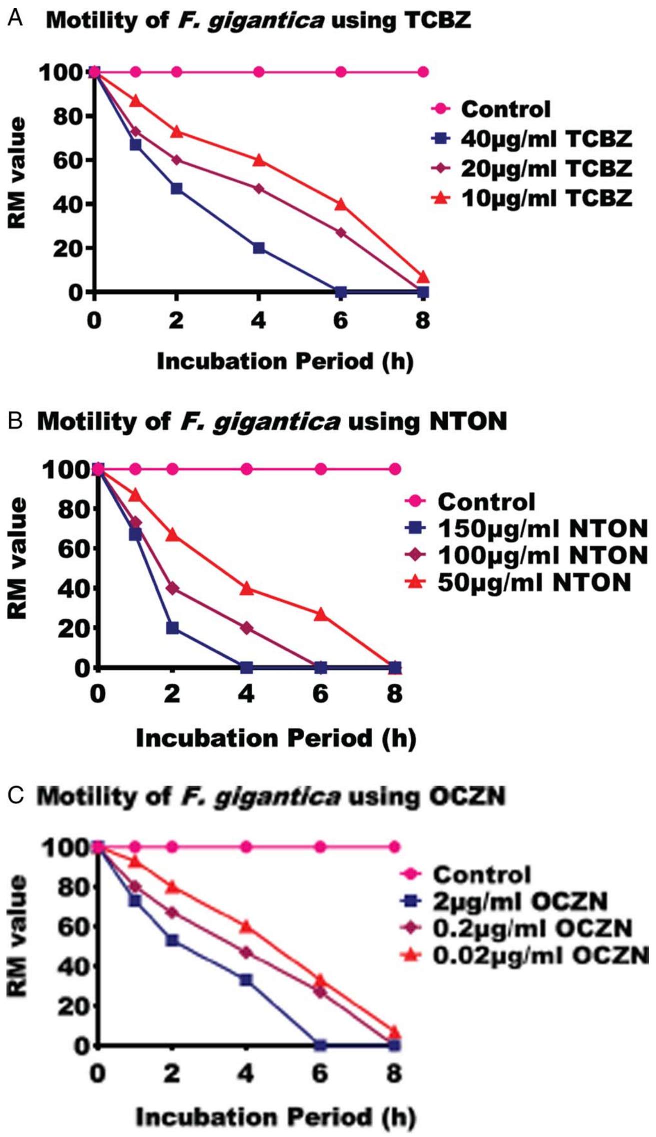

Fig. 1. RM rate of *Fasciola gigantica* treated with various concentrations of TCBZ (A), NTON (B) and OCZN (C) *in vitro* method.

Results

NTON efficiently kills F. gigantica

To determine the efficacy of commercially available anthelmintics, *F. gigantica* was incubated with TCBZ, NTON and OCZN at different concentrations and time frames as described in the 'Materials and methods' section. It was observed that NTON at 150 $\mu g\ mL^{-1}$ drastically reduced the RM (=0) within 4 h of incubation whereas OCZN at 2 $\mu g\ mL^{-1}$ and TCBZ at 40 $\mu g\ mL^{-1}$ performed it by 6 h. The initial reduction of RM (decreased RM value) was found at 1 h of incubation with NTON at the concentration of 50 $\mu g\ mL^{-1}$ and the RM value declined gradually until 8 h of incubation. Flukes incubated with NTON at the concentration of 100 $\mu g\ mL^{-1}$ exhibited reduced motility at a more rapid rate than the previous dose, as the RM value dropped rapidly from 1 h and the parasites became completely immobile and dead at 6 h (RM = 0) whereas drastic reduction of RM value was observed at 4 h with NTON at 150 $\mu g\ mL^{-1}$ (RM = 0) (Fig. 1B). In case of flukes incubated with TCBZ at various concentrations (10, 20 and 40 $\mu g\ mL^{-1}$), the fluke's motility and RM value were decreased throughout the experimental period. The flukes treated with TCBZ at 10 and 20 $\mu g\ mL^{-1}$ showed a gradual reduction of RM value at 1–8 h, and sharply decreased and finally

https://doi.org/10.1017/S0031182022000580 Published online by Cambridge University Press

Table 3. Data showing the visual observations on the motility of adult flukes of *Fasciola gigantica* recovered from untreated control and TCBZ-, NTON-, OCZN-treated

Treatment (h)	Motility at 10 μg mL^{-1} TCBZ	Motility at 20 μg mL^{-1} TCBZ	Motility at 40 μg mL^{-1} TCBZ	Motility at 50 μg mL^{-1} NTON	Motility at 100 μg mL^{-1} NTON	Motility at 150 μg mL^{-1} NTON	Motility at 0.02 μg mL^{-1} OCZN	Motility at 0.2 μg mL^{-1} OCZN	Motility at 2 μg mL^{-1} OCZN
1	++++	+++	++	+++	++	+	++++	+++	++
2	+++	++	+	++	+	–	+++	++	+
4	++	+	–	+	–	Dead	++	+	–
6	+	–	Dead	–	Dead	Dead	+	–	Dead
8	–	Dead	Dead	Dead	Dead	Dead	–	Dead	Dead

Highly active (+++), moderately active (++) and immotile (–).

dead from 6 to 8 h (RM = 0). However, the flukes became completely immobile and dead at 6 h (RM = 0) during treatment with TCBZ at 40 μg mL^{-1} (Fig. 1A). The flukes were incubated with OCZN at 0.02, 0.2 and 2 μg mL^{-1} at 1–8 h period showed rapid reduction of RM values which was similar to that of TCBZ (Fig. 1C). In contrast, flukes in the control group showed active movement throughout the duration of the experiment. The results reveal that flukes treated with TCBZ, NTON and OCZN exhibited reduced motility in a concentration and time-dependent manner. Data showing the visual observations on the motility of *F. gigantica* recovered from the control and treated groups are provided in Table 3.

Anthelmintic treatment causes morphological distortion of apical cone of the flukes

Tegumental disruption in the apical cone region was more pronounced and the ventral surface was severely affected than the dorsal of flukes treated with high concentration of flukicides (TCBZ at 40 μg mL^{-1}; NTON at 150 μg mL^{-1} and OCZN at 2 μg mL^{-1}) at 8 h of incubation compared to that of the low concentration treated and control group flukes. Both oral and ventral suckers were distorted with NTON (150 μg mL^{-1}) and OCZN (2 μg mL^{-1}). Losses of spine were observed in 20 and 40 μg mL^{-1} of TCBZ; 100 and 150 μg mL^{-1} of NTON and 0.2 and 2 μg mL^{-1} of OCZN. The tegumental swelling around ventral sucker was more pronounced in 40 μg mL^{-1} TCBZ; 150 μg mL^{-1} NTON and 2 μg mL^{-1} OCZN treated flukes, whereas no such damages were detected in flukes of the control group (Table 4).

Architectural changes in the flukes treated with anthelmintics

To reveal the architectural changes, histopathology of flukes from all groups was conducted. This study revealed that treated flukes at 8 h of incubation showed more prominent architectural changes. Flukes treated with TCBZ at 10 μg mL^{-1}, NTON at 50 μg mL^{-1} and OCZN 0.02 μg mL^{-1} concentrations at 8 h of incubation showed the formation of small blebs on the tegument surface i.e. spine embedded in the slightly damaged tegument and muscle lying underneath the basement membrane. Flukes treated with TCBZ at 20 μg mL^{-1}, NTON at 100 μg mL^{-1} and OCZN 0.2 μg mL^{-1} concentrations at 8 h of incubation showed mild separation of tegument between the spines and underlying tissue and dislodged of spines. Flukes treated with TCBZ at 40 μg mL^{-1}, NTON at 150 μg mL^{-1} and OCZN 2 μg mL^{-1} concentrations at 8 h of incubation revealed the formation of small vacuoles, small blebs and disrupted blebs on the tegument, while spine, muscle and other structures underneath the basement membrane showed normal but dilate. However, none of these changes were detected in flukes of the control group (Fig. 2).

Ultra-structural changes of flukes treated with anthelmintics

Ultra-structural changes of flukes were analysed by SEM. The most remarkable changes were found with TCBZ (40 μg mL^{-1}) showing severe tegumental distortion (arrow) and sloughing of ventral sucker and NTON (150 μg mL^{-1}) showing ridged tegumental distortion with losses of numerous spines and marked distortion at the tips of the spine (Figs 3–6).

SEM of apical cone surface of treated *F. gigantica* following 8 h of incubation in 40 μg mL^{-1} of TCBZ showed disruption apart from swelling of the tegument of the ventral surface (Fig. 3). SEM of the ventral sucker at higher magnification (500×) showed tegumental distortion and sloughing in all TCBZ-treated flukes whereas these changes were more visible with TCBZ at 40 μg mL^{-1} (Fig. 4). SEM of the tegument showed extensive lesions in some areas of ventral apical cone with some distorted spine at 40 μg mL^{-1} of TCBZ-treated flukes (Fig. 5). SEM of the tegument at higher magnification (5000×) showed breaking of large tegument with losses of numerous spines and crumbled up (arrow) at their tips and corrugated appearance at 40 μg mL^{-1} of TCBZ-treated flukes (Fig. 6).

SEM of the apical cone surface of treated *F. gigantica* following 8 h of incubation at 150 μg mL^{-1} of NTON showed relatively little disruption and tegumental swelling with regional variations in severity surrounding the oral and ventral sucker's swollen rim, apart from swelling of the tegument on the ventral surface (Fig. 3). SEM of ventral sucker at higher magnification (500×) showed severe tegumental distortion and sloughing in all NTON-treated flukes (Fig. 4). SEM of the tegument showed extensive lesions in some areas of ventral apical cone in NTON-treated flukes (Fig. 5). SEM of the tegument at higher magnification (5000×) showed ridged tegumental distortion with losses of numerous spines and extensive breaking of tips of spine in NTON-treated flukes but more pronounced in 150 μg mL^{-1} NTON-treated flukes (Fig. 6).

SEM of the apical cone surface at 2 μg mL^{-1} of OCZN showed relatively little disruption, apart from swelling of the tegument on the ventral surface (Fig. 3). SEM of the ventral sucker shows swollen rim, blebbing, interior severe erosion of the muscular rim at higher magnification (500×) of OCZN-treated flukes (Fig. 4). SEM of tegument showed swollen tegument covering the spines (S) and extensive damaging of lesion on the tegument between the spines in all OCZN-treated flukes (Fig. 5). SEM of the tegument at higher magnification (5000×) showed ridged tegumental distortion with losses of numerous spines and marked distortion at the tips of the spine at 2 μg mL^{-1} of OCZN-treated flukes (Fig. 6). However, SEM of apical cone surface of control fluke (*F. gigantica*) showed smooth ventral sucker with thick rims covered with transverse folds and spines. The anterior part of the ventral surface of flukes showed the spines are small and closely

Table 4. Morphometric data of apical cone of control flukes and the three groups of treated flukes

	Fluke									
		Treated with TCBZ			Treated with NTON			Treated with OCZN		
Measurements	Control fluke	10 mg mL^{-1}	20 mg mL^{-1}**	40 mg mL^{-1}**	50 μg mL^{-1}*	100 μg mL^{-1}**	150 μg mL^{-1}**	0.02 μg mL^{-1}	0.2 μg mL^{-1}**	2 μg mL^{-1}**
Apical cone length (mm)	1.82 ± 0.07	1.58 ± 0.06	1.18 ± 0.09	0.82 ± 0.03	1.39 ± 0.06	1.0 ± 0.09	0.61 ± 0.03	1.46 ± 0.05	1.08 ± 0.08	0.72 ± 0.03
Apical cone width (mm)	2.04 ± 0.06	1.59 ± 0.12	1.36 ± 0.14	0.92 ± 0.02	1.41 ± 0.11	1.16 ± 0.14	0.72 ± 0.03	1.46 ± 0.11	1.25 ± 0.49	0.82 ± 0.02
Maximum diameter of oral sucker (mm)	0.71 ± 0.06	0.62 ± 0.05	0.44 ± 0.03	0.31 ± 0.03	0.43 ± 0.05	0.24 ± 0.03	0.13 ± 0.02	0.52 ± 0.06	0.33 ± 0.04	0.22 ± 0.03
Maximum diameter of ventral sucker (mm)	1.02 ± 0.13	0.92 ± 0.13	0.64 ± 0.09	0.45 ± 0.06	0.71 ± 0.14	0.44 ± 0.09	0.26 ± 0.06	0.82 ± 0.13	0.53 ± 0.09*	0.36 ± 0.06
Spine length (mm)	44.01 ± 2.81	50.71 ± 3.24	Loss of spine	Loss of spine	40.85 ± 3.11	Loss of spine	Loss of spine	45.62 ± 3.19	Loss of spine**	Loss of spine
Spine width (mm)	30.87 ± 2.58	33.68 ± 2.1	Loss of spine	Loss of spine	23.29 ± 2.11	Loss of spine	Loss of spine	28.72 ± 2.23	Loss of spine**	Loss of spine
Area of tegumental swelling around ventral sucker (mm^2)	0.00	0.60 ± 0.05	1.41 ± 0.14	1.62 ± 0.05	0.75 ± 0.04	1.62 ± 0.05	1.84 ± 0.12	0.65 ± 0.06	1.25 ± 0.7	1.6 ± 0.103

*$P < 0.05$; **$P < 0.01$ (according to one-way ANOVA with *post hoc* Duncan test).

spaced (Fig. 3). Wall of the ventral suckers showed smooth and flourish at higher magnification (Fig. 4). SEM of tegument surface appeared rough due to the presence of numerous normal spines and surface folding (Fig. 5) and spine showed finger-like protrusions at their tips (Fig. 6).

Discussion

Fasciola gigantica is a food- and water-borne devastating parasite, which induces cirrhosis, calcification and eventually causes severe damage and condemnation of liver leading to high economic losses in the livestock industry. This study evaluated the comparative efficacy of TCBZ, NTON and OCZN on *F. gigantica in vitro* technique by observing the RM value, morphological distortion of apical cone of the flukes as well as architectural changes by microscopy and ultra-structural changes of the flukes by SEM for the first time in Bangladesh.

In this study, flukes treated with TCBZ, NTON and OCZN revealed reduced motility in a concentration and time-dependent manner of flukicides. Among the tested flukicides, NTON was the most potent flukicides against *F. gigantica* which showed greater and faster reduction of fluke's motility. The present findings are in agreement with the findings reported previously where the RM values of the flukes exposed with TCBZ in crude extracts of *Artocarpus lakoocha* and artesunate were observed to decrease within 1 h (Fairweather *et al.*, 1984; Bennett and Köhler, 1987; Saowakon *et al.*, 2009; Tansatit *et al.*, 2012). Decreased contraction and motility were first observed after 3 h of incubation with TCBZ at the concentrations 80 and 175 μg mL^{-1}. TCBZ markedly reduced the parasite's motility at the concentration of 175 μg mL^{-1} at 6 h, and killed the worms after 12 h exposure (Saowakon *et al.*, 2009). The RM values of TCBZ-treated flukes decreased significantly from 6 to 24 h for 20, 40 and 80 μg mL^{-1} dosages (Tansatit *et al.*, 2012). Tegumental disruption in the apical cone region, severely affected the ventral surface, distorted oral and ventral suckers seen as morphological changes; formation of some vacuoles, small blebs and disrupted blebs on the tegument surface, separation of tegument between the spines and underlying tissues and dislodged spines as histopathological changes were observed. Severe tegumental distortion and sloughing of the ventral sucker, extensive lesions of some areas of ventral apical cone with some distorted spine, ridged tegumental distortion with losses of numerous spines and crumbled up at their tips and corrugated appearance were determined by SEM in this study. The present findings from this study are consistent with the findings reported previously where sequential changes in the tegument including swelling, followed by blebbings that later ruptured, leading to the erosion and desquamation of the tegument syncytium were observed (Saowakon *et al.*, 2009). Tegumental disruption in the apical cone region became more pronounced and the ventral surface was more severely affected than the dorsal one. The tegumental swelling which was seen in the previous concentration was more pronounced and both oral and ventral suckers were distorted (Shalaby *et al.*, 2009). The morphological changes after treatments with drugs, comprising swelling of tegumental ridges, followed by blebbing and later rupturing of the blebs, leading to erosion and lesion, and disruption of the tegument were observed (Tansatit *et al.*, 2012).

Architectural changes were detected through histopathological examination revealing that TCBZ, NTON and OCZN resulted in the disintegration of the tegument, vacuolization, blebbing formation, disruption of blebs and disrupting the fluke's surface which included detachment of spines. The findings of this study are in concurrence with the previous studies performed *in vitro* on *Fischoederius cobboldi* treated with ethanol extract of *Terminalia catappa* where vacuolization, blebbings and partial disruption of

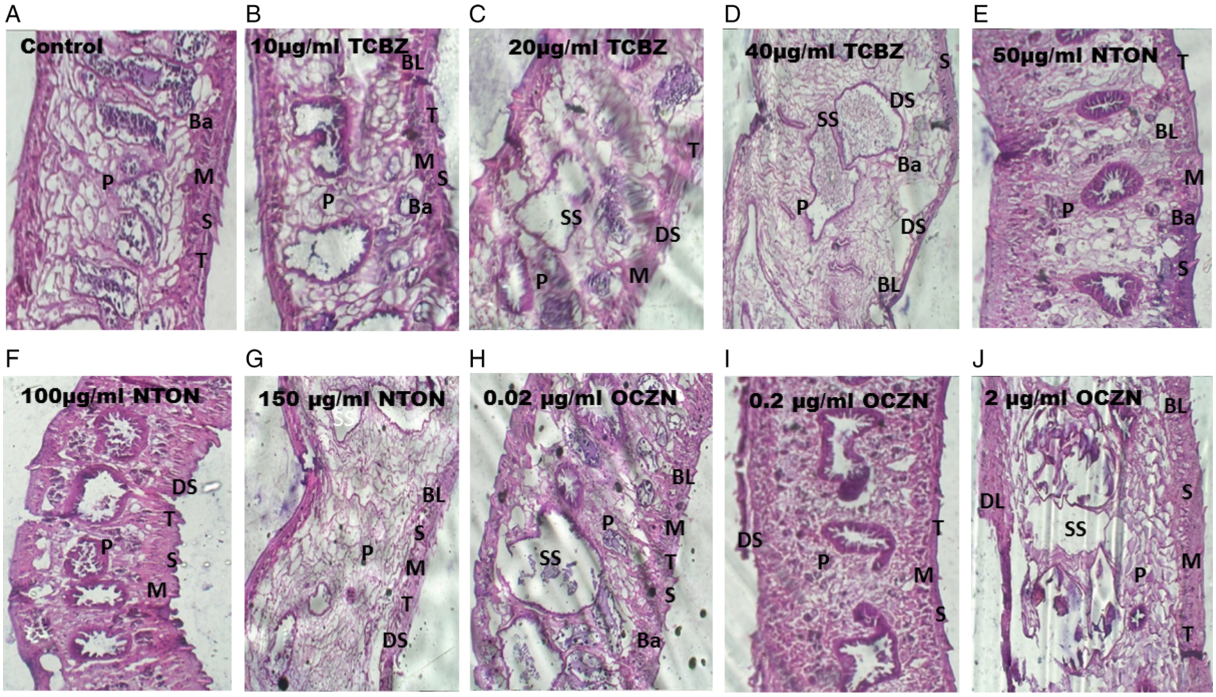

Fig. 2. Stereomicroscopic figures showing the histopathology of the tegument of *F. gigantica*. (a) Control *F. gigantica* incubated in RPMI-1640 medium for 8 h, showing the tegument with normal appearances of parenchyma (P), spines (S) embedded in the intact tegument (T) and muscle layers (M) lying underneath the basement membrane (Ba). Flukes are treated with TCBZ at 10 μg mL^{-1}; NTON at 50 μg mL^{-1} and OCZN at 0.02 μg mL^{-1} for 8 h of incubation, showing the formation of small blebs (BL) in the surface of tegument (T), mild separation of tegument between the spines (S) and underlying tissue (T) and dislodged spines (DS) in the micrograph of (b), (e) and (h). Flukes are treated with TCBZ at 20 μg mL^{-1}; NTON at 100 μg mL^{-1} and OCZN at 0.2 μg mL^{-1} for 8 h of incubation, showing the small blebs (BL), disruption of blebs (DL) on the tegument and formation of small vacuoles (SS) in the cytoplasm, while spine (S), muscle (M) lying the underneath the basement membrane (Ba) in the micrographs of (c), (f) and (i). At high concentration of TCBZ at 40 μg mL^{-1}, NTON at 150 μg mL^{-1} and OCZN at 2 μg mL^{-1}, showing the formation of more outward small vacuoles (SS), small blebs (BL), degeneration and sloughing of tegument and disruption of blebs (DL) in the micrograph of (d), (g) and (j), respectively.

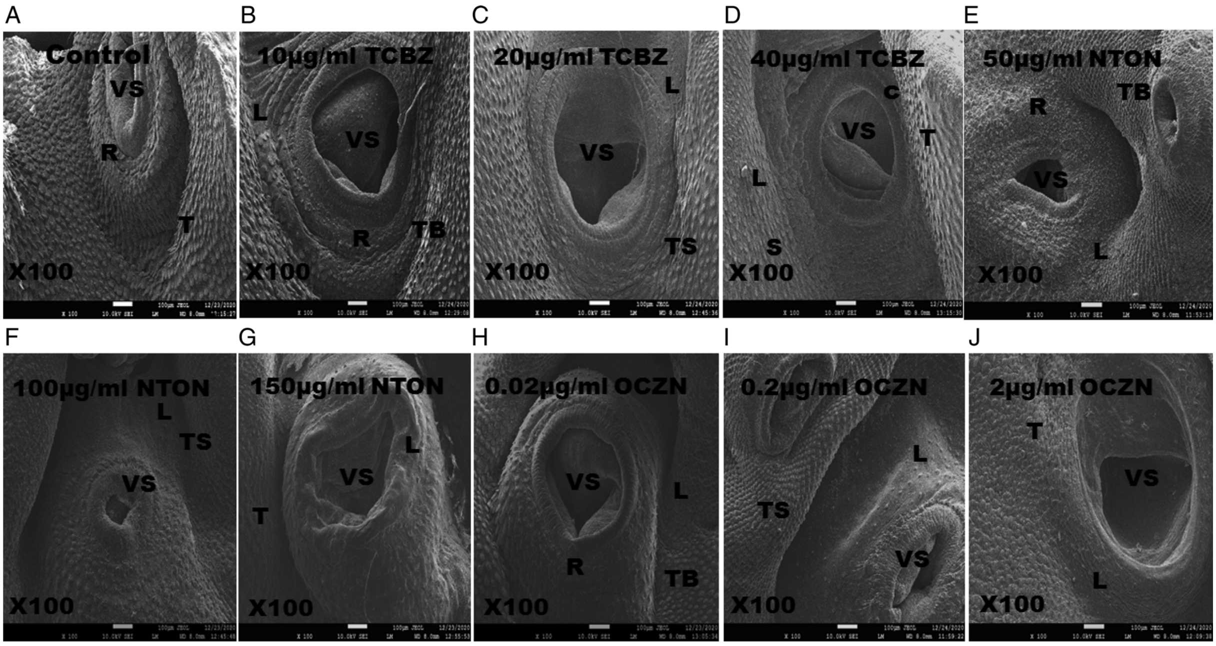

Fig. 3. SEM images of the control and treated flukes. (A) SEM of apical cone surface of control flukes showing smooth ventral sucker (VS) with thick rims (R) covered with transverse folds (T) and appear spineless. SEM of the ventral sucker (B), (E) and (H) showing tegumental blebbing (TB), little disruption (L) apart from the swelling of tegument (T) on the ventral surface at a concentration of TCBZ at 10 μg mL^{-1}; NTON at 50 μg mL^{-1} and OCZN at 0.02 μg mL^{-1} for 8 h of incubation. Pronounced tegumental swelling (TS) and disruption (L) and distortion of the ventral suckers (VS) showed in the SEMs (C), (F) and (I) during flukes were treated with CBZ at 20 μg mL^{-1}; NTON at 100 μg mL^{-1} and OCZN at 0.2 μg mL^{-1} concentrations. SEM of flukes treated with TCBZ at 40 μg mL^{-1}, NTON at 150 μg mL^{-1} and OCZN at 2 μg mL^{-1} concentrations showing relatively little disruption (L) on the ventral surface of flukes, apart from the swelling of the tegument (T) and swollen rim (R) of ventral sucker.

parasites tegument were reported (Anuracpreeda *et al.*, 2016) and treated flukes with 20 μg mL^{-1} TCBZ (Ebeid *et al.*, 2011). Flukes treated with higher doses of TCBZ, NTON and OCZN showed some vacuoles, the formation of small blebs and disrupted blebs on the tegument, while spine, muscle and other structures underneath the basement membrane showed normal but dilated that

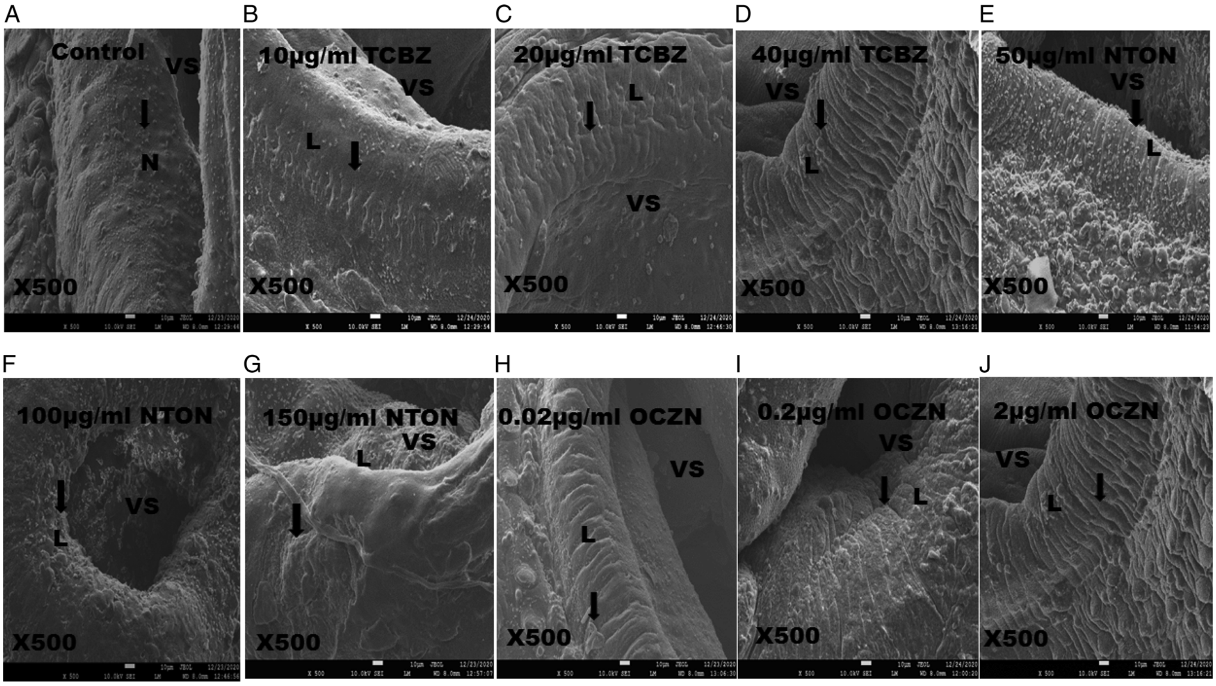

Fig. 4. (A) SEM showing smooth and flourish wall of the ventral sucker (VS) of control fluke at higher magnification (500×). (B), (C), (E), (F), (H) and (I) SEM of the ventral sucker of flukes treated with TCBZ at 10 μg mL^{-1}, TCBZ at 20 μg mL^{-1}, NTON at 50 μg mL^{-1}, NTON at 100 μg mL^{-1}, OCZN at 0.2 μg mL^{-1} and OCZN at 0.2 μg mL^{-1} showing severe tegumental distortion (arrow marks) and sloughing (L) but all these lesions were more pronounced at the concentrations of TCBZ at 40 μg mL^{-1}, NTON at 150 μg mL^{-1} and OCZN at 2 μg mL^{-1} in the SEM (D), (G) and (J).

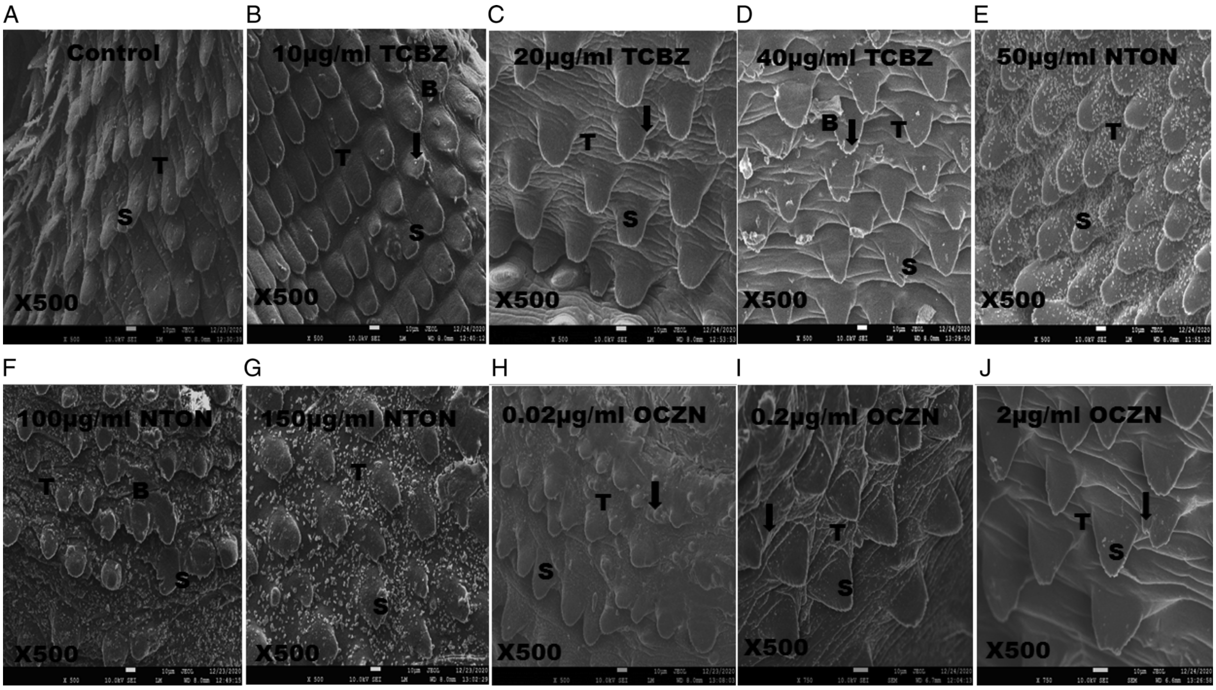

Fig. 5. (A) SEM micrograph of tegument (T) surface in control flukes shows normal appearance. (B–D) SEM of tegument of treated flukes with various concentrations of TCBZ showing gradual distortion of spines. (E–G) SEM of treated flukes with various concentrations of NTON showing gradual distortion of spines and blebbing of tegument. (H–J) SEM of treated flukes with various concentrations of OCZN showing severe damaged (arrow marks) of tegument and gradual distortion of spines (S).

supports the previous finding where authors found the vacuole formation in the longitudinal section of fluke treated with OCZN (Jeyathilakan *et al.*, 2012). Histopathologically, the flukes showed marked separation of tegument from the cuticle and corrugated at 5% concentration *Areca catechu*. The longitudinal smooth muscle showed a massive contraction that leads to vacuolation of parenchyma (Jeyathilakan *et al.*, 2010). Hence, these histopathological changes indicate the potency of flukicides against *F. gigantica*.

However, architectural changes through histopathological changes were used to investigate a limited area of the surface change, whereas more changes in the tegument of the parasites

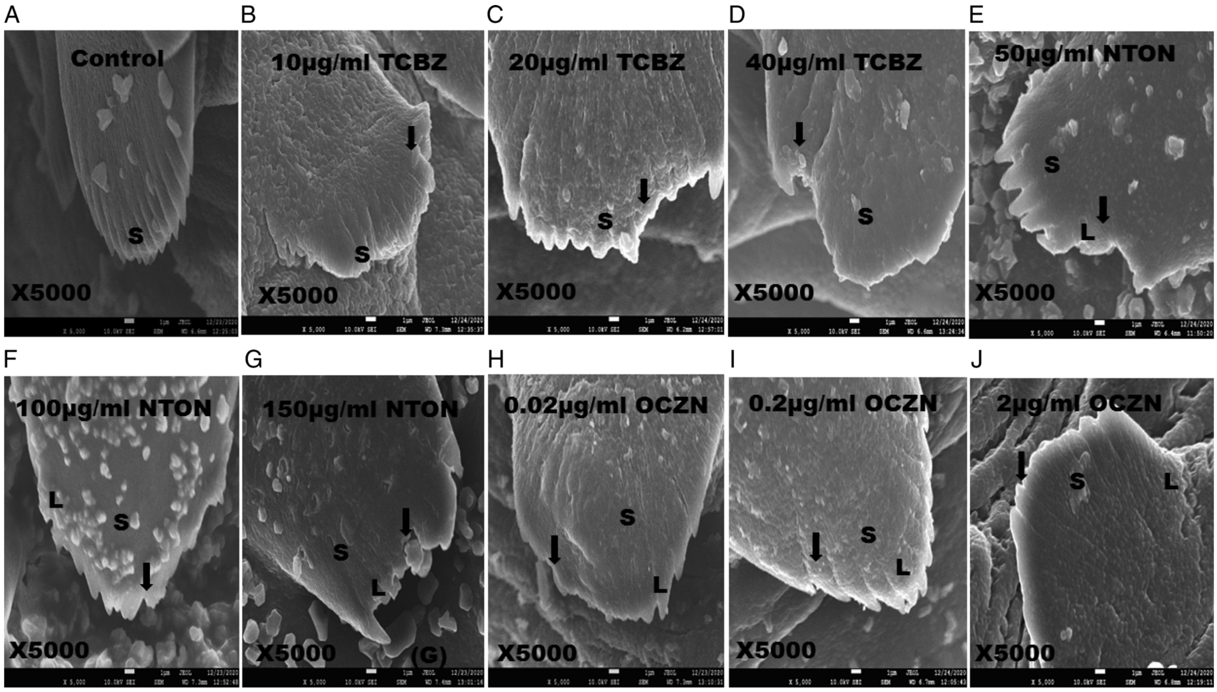

Fig. 6. (A) SEM image of spines (S) of control fluke shows finger-like protrusion at their tips. (B–J) SEM micrographs of treated flukes with various concentrations of TCBZ, NTON and OCZN showing extensive distortion (arrow marks) of the tegument (T) with losses of numerous spines (S). Severe tegumental distortion was found in flukes treated with higher concentration (150 μg mL^{-1}) of NTON (G).

could be observed by SEM. In response to TCBZ, NTON and OCZN on *F. gigantica* changes were noticed by SEM, depending on the used concentration of drugs and exposure time.

Regarding the ultra-structural changes through SEM of the apical cone surface of *F. gigantica* following 8 h of incubation in TCBZ showing relatively less disruption, apart from swelling of the tegument on the ventral surface, especially after the longer incubation periods in this study. The surface alterations observed in the present study resemble the earlier study where SEM of tegumental swelling followed by blebbing and rupturing of the blebs and disruption of tegument has been reported (Meaney *et al.*, 2002; Tansatit *et al.*, 2012). The present study is also in agreement with the previous study where similar surface changes were observed in *F. hepatica* after treatment with CLORS, NTON and artemether (McKinstry *et al.*, 2003; Meaney *et al.*, 2003; Keiser and Morson, 2008; Anuracpreeda *et al.*, 2016). In contrast, extensive damage to the tegument has been reported in TCBZ-susceptible *F. hepatica*, whereas only localized and minor disruption of the tegument covering the spines is recorded in TCBZ-resistant flukes (Robinson *et al.*, 2002). The ultra-structural changes mentioned above might be plausibly due to response to the various anthelmintics, dependence on the thickness, variation in the anatomy and physiology, routes of drug uptake and metabolism of drug in different areas of the tegument.

In the present study, the ventral surface of treated flukes showed more severe disruption than the dorsal surface, and the anterior part of the fluke has also been affected in response to drug action. The findings of this study are in accordance with the previous *in vitro* studies of NTON where more disruption on the dorsal surface has been reported than the ventral surface and the anterior region of the fluke was more disrupted compared to the posterior region (McKinstry *et al.*, 2003). In contrast, the present study is inconsistent with earlier study where the dorsal surface was found more severely affected than the ventral surface (Dawes, 1966; Anderson and Fairweather, 1988). However, the dorso-ventral changes have also been reported in juvenile flukes (Stitt and Fairweather, 1993).

The current study revealed that NTON causes swelling and severe disruption of the tegumental surface as well as swelling of the basal in-folds in the tegumental syncytium in treated flukes. OCZN was found to be also effective against *in vitro F. gigantica* that caused severe surface damage. The findings of this study are consistent with the previous studies where extensive swelling and blebbing of the tegument on both surfaces had reported in NTON-treated flukes (McKinstry *et al.*, 2003). The results of the present study are also in accordance with previous studies where similar changes have been described for other fasciolicides (Fairweather *et al.*, 1986, 1987; Anderson and Fairweather, 1988, 1995; Skuce and Fairweather, 1990; Stitt and Fairweather, 1993, 1994; Meaney *et al.*, 2002, 2003; Robinson *et al.*, 2002; Buchanan *et al.*, 2003). Severe body surface damage has also been reported *in vitro* OCZN-treated *Gigantocotyle explanatum* where abrasion of surface papillae, formation of lesions and peeling of the tegumental syncytium are recorded.

In this study, enlarged, swollen and sloughing off numerous spines and submersion of spines by externally swollen tegument was detected in the treated flukes. The present findings are in accordance with the previous *in vitro* studies on NTON where widespread swelling and extensive furrowing of the tegument had been reported and the spines had submerged with the swollen tegument surrounding them in the mid-body region (Fairweather *et al.*, 1984; McKinstry *et al.*, 2003). The cause of swollen and sloughing off spines may plausibly be due to flukicidal action in the thin tegument covering the spines because a hole may be formed in the syncytium in the tegument which allow more access of the flukicidal drugs to internal tissues (McKinstry *et al.*, 2003).

The severity of tegumental changes in NTON-, TCBZ- and OCZN-treated flukes was increased at higher concentration and longer exposure times which caused the immobility and death of the parasites. However, NTON causes faster and greater death of flukes at 4 h compared to TCBZ and OCZN which cause slower (6–8 h) death of parasites. NTON may be considered as a safe substitute for TCBZ/OCZN treatment for fascioliasis in rural animals.

https://doi.org/10.1017/S0031182022000580 Published online by Cambridge University Press

Therefore, it is concluded that TCBZ, NTON and OCZN are still effective against *F. gigantica in vitro* testing particularly in goats from Bangladesh. The delayed reduction of RM of TCBZ-treated flukes suggests the presence of TCBZ-resistant fluke populations in Bangladesh. Further study is imperative to explore the effects of the flukicidal drugs *in vivo* and their molecular mechanisms.

Acknowledgements. The authors acknowledge the support from the Department of Livestock Services, Ministry of Fisheries and Livestock, Bangladesh, National Agricultural Technology Programme-2 (NATP-2), BARC; Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh and Centre for Advanced Studies and Research for Scanning Electron Microscopy, University of Dhaka, Bangladesh. The abstract (P-3006) was presented in the conference of WAAVP2021, Dublin, Ireland, 18–22 July 2021.

Author contributions. Mohammad Manjurul Hasan: Conceptualization, methodology, formal analysis, resources, visualization, writing – original draft preparation, investigation, writing – review and editing. Hiranmoy Biswas: Methodology, investigation, formal analysis. Dr Babul Chandra Roy: Methodology, formal analysis, resources, validation, software, writing – review and editing. Dr Moizur Rahman: Methodology, formal analysis, writing – review. Dr Anisuzzaman: Formal analysis, resources, investigation, data curation, writing and editing. Dr Mohammad Zahangir Alam: Resources, co-supervision, writing-review & editing. Dr Md. Hasanuzzaman Talukder: Conceptualization, methodology, project administration, supervision, validation, visualization, investigation, resources, writing – review and editing.

Financial support. The authors acknowledge National Agricultural Technology Programme-2 (NATP-2), BARC, Bangladesh for providing financial support (Grant no. NATP-2/PIU-BARC-44/2017-6330, dated: 25/04/2019) to conduct this research work.

Conflict of interest. The authors declare there are no conflicts of interests.

Ethical standards. The study was approved by the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University [AWEEC/BAU/2019(1)]. The authors maintained the highest possible ethical standards in their works as per guidelines.

References

Aghayan S, Gevorgian H, Ebi D, Atoyan HA, Addy F, Mackenstedt U, Romig T and Wassermann M (2019) *Fasciola* spp. in Armenia: genetic diversity in a global context. *Veterinary Parasitology* **268**, 21–31.

Ahasan SA, Valero MA, Chowdhury EH, Islam MT, Islam MR, Mondal MMH, Peixoto RV, Berinde L, Panova M and Mas-Coma S (2016) CIAS detection of *Fasciola hepatica*/*F. gigantica* intermediate forms in bovines from Bangladesh. *Acta Parasitologica* **61**, 267–277.

Aktaruzzaman M, Mohamed Z, Naim-Ul-Alam M, Siddiqul Islam M and Howlader MR (2015) Evaluation of anthelmintic efficacy of triclabendazole, nitroxynil and albendazole against naturally acquired fascioliasis in cattle of Bangladesh with special reference to its residual effect. *International Journal of Pharmacology and Toxicology* **3**, 1–4.

Amin MR and Samad MA (1988) Clinico-therapeutic studies on bovine fascioliasis. *Bangladesh Veterinarian* **5**, 20–22.

Anderson HR and Fairweather I (1988) *Fasciola hepatica*: scanning electron microscopic observations of juvenile flukes following treatment *in vitro* with the deacetylated (amine) metabolite of diamphenethide (DAMD). *International Journal for Parasitology* **18**, 827–837.

Anderson HR and Fairweather I (1995) *Fasciola hepatica*: ultrastructural changes to the tegument of juvenile flukes following incubation *in vitro* with the deacetylated (amine) metabolite of diamphencthide. *International Journal for Parasitology* **25**, 319–333.

Anuracpreeda P, Chankaew K, Puttarak P, Koedrith P, Chawengkirttikul R, Panyarachun B, Ngamniyom A, Chanchai S and Sobhon P (2016) The anthelmintic effects of the ethanol extract of *Terminalia catappa* L. leaves against the ruminant gut parasite, *Fischoederius cobboldi*. *Parasitology* **143**, 421.

Arafa WM, Shokeir KM and Khateib AM (2015) Comparing an *in vivo* egg reduction test and *in vitro* egg hatching assay for different anthelmintics against *Fasciola* species, in cattle. *Veterinary Parasitology* **214**, 152–158.

Bennett JL and Köhler P (1987) *Fasciola hepatica*: action *in vitro* of triclabendazole on immature and adult stages. *Experimental Parasitology* **63**, 49–57.

Boray JC and Happich FA (1968) Standardised chemotherapeutical tests for immature and mature *Fasciola hepática* infections in sheep. *Australian Veterinary Journal* **44**, 72–78.

Buchanan J, Fairweather I, Brennan G, Trudgett A and Hoey E (2003) *Fasciola hepatica*: surface and internal tegumental changes induced by treatment *in vitro* with the sulphoxide metabolite of albendazole ('Valbazen'). *Parasitology* **126**, 141.

Chang ACG and Flores MJC (2015) Morphology and viability of adult *Fasciola gigantica* (giant liver flukes) from Philippine carabaos (*Bubalus bubalis*) upon *in vitro* exposure to lead. *Asian Pacific Journal of Tropical Biomedicine* **5**, 493–496.

Charlier J, Duchateau L, Claerebout E, Williams D and Vercruysse J (2007) Associations between anti-*Fasciola hepatica* antibody levels in bulk-tank milk samples and production parameters in dairy herds. *Preventive Veterinary Medicine* **78**, 57–66.

Davis CN, Winters A, Milic I, Devitt A, Cookson A, Brophy PM and Morphew RM (2020) Evidence of sequestration of triclabendazole and associated metabolites by extracellular vesicles of *Fasciola hepatica*. *Scientific Reports* **10**, 13445.

Dawes B (1966) Experimental fascioliasis: some effects on *Fasciola hepatica* of treatment of rat hosts with Bithionol ('Actamer'). *Helminthologia* **7**, 297–307.

Dey AR, Begum N, Anisuzzaman, Alim MA and Alam MZ (2020) Multiple anthelmintic resistance in gastrointestinal nematodes of small ruminants in Bangladesh. *Parasitology International* **77**, 102105.

Diab TM, Mansour HH and Mahmoud SS (2010) *Fasciola gigantica*: parasitological and scanning electron microscopy study of the *in vitro* effects of ivermectin and/or artemether. *Experimental Parasitology* **124**, 279–284.

Diyana JNA, Mahiza MIN, Latiffah H, Fazila SHN, Lokman IH, Hazfalinda HN, Chandrawathani P, Ibitoye EB and Juriah K (2020) Occurrence, morphometric, and molecular investigation of cattle and buffalo liver adult fluke in peninsular Malaysia main abattoirs. *Journal of Parasitology Research* **2020**, 5436846.

Ebeid MH, Moustafa AM, Arnaout FK, Degheidy NS, Omer EA, Shalaby HA and Abd El-Hamed AF (2011) *In vitro* evaluation of anthelmentic efficacy of *Balanites egyptiaca* on *Fasciola gigantica*. *Benha Veterinary Medical Journal* **22**, 56–67.

Fairweather I (2005) Triclabendazole: new skills to unravel an old (ish) enigma. *Journal of Helminthology* **79**, 227–234.

Fairweather I (2009) Triclabendazole progress report, 2005–2009: an advancement of learning? *Journal of Helminthology* **83**, 139–150.

Fairweather I and Boray JC (1999) Fasciolicides: efficacy, actions, resistance and its management. *The Veterinary Journal* **158**, 81–112.

Fairweather I, Holmes S and Threadgold L (1984) *Fasciola hepatica*: motility response to fasciolicides *in vitro*. *Experimental Parasitology* **57**, 209–224.

Fairweather I, Anderson H and Baldwin T (1987) *Fasciola hepatica*: tegumental surface alterations following treatment *in vitro* with the deacetylated (amine) metabolite of diamphenethide. *Parasitology Research* **73**, 99–106.

Fairweather I, Anderson H and Threadgold L (1986) *Fasciola hepatica*: tegumental changes induced *in vitro* by the deacetylated (amine) metabolite of diamphenethide. *Experimental Parasitology* **62**, 336–348.

Fairweather I, Mcshane D, Shaw L, Ellison S, O'Hagan N, York E, Trudgett A and Brennan G (2012) Development of an egg hatch assay for the diagnosis of triclabendazole resistance in *Fasciola hepatica*: proof of concept. *Veterinary Parasitology* **183**, 249–259.

Fairweather I, Brennan GP, Hanna REB, Robinson MW and Skuce PJ (2020) Drug resistance in liver flukes. *International Journal for Parasitology: Drugs and Drug Resistance* **12**, 39–59.

Halferty L, Brennan GP, Hanna REB, Edgar HW, Meaney MM, McConville M, Trudgett A, Hoey L and Fairweather I (2008) Tegumental surface changes in juvenile *Fasciola hepatica* in response to treatment *in vivo* with triclabendazole. *Veterinary Parasitology* **155**, 49–58.

Hanna REB, Mcmahon C, Ellison S, Edgar HW, Kajugu PE, Gordon A, Irwin D, Barley JP, Malone FE, Brennan GP and Fairweather I (2015) *Fasciola hepatica*: a comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland sheep farms in Northern Ireland using faecal egg counting, coproantigen ELISA testing and fluke histology. *Veterinary Parasitology* **207**, 34–43.

Hoque MN, Begum N and Nooruddin M (2003) Albendazole resistance in gastrointestinal nematode parasites of cattle in Bangladesh. *Tropical Animal Health and Production* **35**, 219–222.

Ibarra OF and Jenkins DC (1984) An *in vitro* screen for new fasciolicidal agents. *Parasitology Research* **70**, 655–661.

Islam M and Ripa RN (2015) Prevalence of fascioliasis in slaughtered goat in Bengal meat abattoir house and its economic impact on business. *Journal of Chemical, Biological and Physical Sciences (JCBPS)* **5**, 2684.

Islam MA and Samad MA (1989) Efficacy of commercial fasciolicides against mixed infection of fascioliasis and amphistomiasis in cattle. *Bangladesh Veterinarian* **6**, 27–32.

Islam KM, Rahman M, Islam MS, Adhikary GN and Rauf SMA (2014) Epidemiological studies of fascioliasis (*Fasciola gigantica*) in black Bengal goats. *Eurasian Journal of Veterinary Science* **30**, 152–156.

Jeyathilakan N, Murali K, Anandaraj A and Abdul Basith S (2012) *In vitro* evaluation of anthelmintic property of ethno-veterinary plant extracts against the liver fluke *Fasciola gigantica*. *Journal of Parasitic Diseases* **36**, 26–30.

Jeyathilakan N, Murali K, Anandaraj A and Abdul Basith S (2010) *In vitro* evaluation of anthelmintic property of herbal plants against *Fasciola gigantica*. *Indian Journal of Animal Sciences* **80**, 1070.

Keiser J and Morson G (2008) *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. *Experimental Parasitology* **118**, 228–237.

Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P and Spithill TW (2016) Current threat of triclabendazole resistance in *Fasciola hepatica*. *Trends in Parasitology* **32**, 458–469.

Khan M, Anisur Rahman AKM, Ahsan S, Ehsan A, Dhand N and Ward MP (2017) Bovine fascioliasis risk factors and space-time clusters in Mymensingh, Bangladesh. *Veterinary Parasitology: Regional Studies and Reports* **9**, 104–109.

Khatun M, Asaduzzaman M, Pallab M and Chakrabartty P (2015) Risk factor analysis of fascioliasis in two geo-climatic regions of Bangladesh. *International Journal of Science and Research* **4**, 41–43.

Kiuchi F, Miyashita N, Tsuda Y, Kondo K and Yoshimura H (1987) Studies on crude drugs effective on visceral larva migrans. I. Identification of larvicidal principles in betel nuts. *Chemical and Pharmaceutical Bulletin* **35**, 2880–2886.

Lorsuwannarat N, Piedrafita D, Chantree P, Sansri V, Songkoomkrong S, Bantuchai S, Sangpairot K, Kueakhai P, Changklungmoa N, Chaichanasak P, Chansela P and Sobhon P (2014) The *in vitro* anthelmintic effects of plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica*. *Experimental Parasitology* **136**, 5–13.

Luo X, Cui K, Wang Z, Li Z, Wu Z, Huang W, Zhu X-Q, Ruan J, Zhang W and Liu Q (2021) High-quality reference genome of *Fasciola gigantica*: insights into the genomic signatures of transposon-mediated evolution and specific parasitic adaption in tropical regions. *PLoS Neglected Tropical Diseases* **15**, e0009750.

Mas-Coma S (2003) Adaptation capacities of *Fasciola hepatica* and their relationships with human fascioliasis: from below sea level up to the very high altitude. *Taxonomy, Ecology and Evolution of Metazoan Parasites* **2**, 81–123.

Mas-Coma MS, Esteban JG and Bargues MD (1999) Epidemiology of human fascioliasis: a review and proposed new classification. *Bulletin of the World Health Organization* **77**, 340–346.

Mas-Coma S, Bargues MD and Valero MA (2005) Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology* **35**, 1255–1278.

Mckinstry B, Fairweather I, Brennan GP and Forbes AB (2003) *Fasciola hepatica*: tegumental surface alterations following treatment *in vivo* and *in vitro* with nitroxynil (Trodax). *Parasitology Research* **91**, 251–263.

Mckinstry B, Brennan GP, Halferty L, Forbes AB and Fairweather I (2007) Ultrastructural changes induced in the tegument and gut of *Fasciola hepatica* following *in vivo* and *in vitro* drug treatment with nitroxynil (Trodax). *Parasitology Research* **101**, 929–941.

Meaney M, Fairweather I, Brennan G, Ramasamy P and Subramanian P (2002) *Fasciola gigantica*: tegumental surface alterations following treatment *in vitro* with the sulphoxide metabolite of triclabendazole. *Parasitology Research* **88**, 315–325.

Meaney M, Fairweather I, Bernnan GP, Mcdowell LSL and Forbes AB (2003) *Fasciola hepatica*: effects of the fasciolicide clorsulon *in vitro* and *in vivo* on the tegument surface, and a comparison of the effects on young- and old-mature flukes. *Parasitology Research* **91**, 238–250.

Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, Saleem MH, Ur Rehman M, Iqbal MK, Wang Y, Ahmad HI, Abbas T, Hussain R, Ghori MT, Ali S, Khan AU and Li J (2017) A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. *Microbial Pathogenesis* **109**, 253–262.

Mohanta UK, Ichikawa-Seki M, Shoriki T, Katakura K and Itagaki T (2014) Characteristics and molecular phylogeny of *Fasciola* flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. *Parasitology Research* **113**, 2493–2501.

Novobilský A and Höglund J (2015) First report of closantel treatment failure against *Fasciola hepatica* in cattle. *International Journal for Parasitology: Drugs and Drug Resistance* **5**, 172–177.

Omran E and Ahmad N (2015) Effect of nitroxynil (fasciolid) on adult *Fasciola gigantica* and *Fasciola hepatica* in infected cows. *Parasitologists United Journal* **8**, 107–114.

Opio LG, Abdelfattah EM, Terry J, Odongo S and Okello E (2021) Prevalence of fascioliasis and associated economic losses in cattle slaughtered at lira municipality abattoir in northern Uganda. *Animals* **11**, 681.

Rahman AKMA, Islam SKS, Talukder MH, Hassan MK, Dhand NK and Ward MP (2017) Fascioliasis risk factors and space-time clusters in domestic ruminants in Bangladesh. *Parasites & Vectors* **10**, 228.

Rapic D, Dzakula N, Sakar D and Richards RJ (1988) Comparative efficacy of triclabendazole, nitroxynil and rafoxanide against immature and mature *Fasciola hepatica* in naturally infected cattle. *The Veterinary Record* **122**, 59–62.

Robinson MW, Trudgett A, Hoey EM and Fairweather I (2002) Triclabendazole-resistant *Fasciola hepatica*:[beta]-tubulin and response to *in vitro* treatment with triclabendazole. *Parasitology* **124**, 325.

Rolfe PF and Boray JC (1987) Chemotherapy of paramphistomosis in cattle. *Australian Veterinary Journal* **64**, 328–332.

Sabourin E, Alda P, Vázquez A, Hurtrez-Boussès S and Vittecoq M (2018) Impact of human activities on fasciolosis transmission. *Trends in Parasitology* **34**, 891–903.

Saowakon N, Tansatit T, Wanichanon C, Chanakul W, Reutrakul V and Sobhon P (2009) *Fasciola gigantica*: anthelmintic effect of the aqueous extract of *Artocarpus lakoocha*. *Experimental Parasitology* **122**, 289–298.

Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR and Moshfe A (2014) Molecular and morphological characterization of *Fasciola* spp. isolated from different host species in a newly emerging focus of human fascioliasis in Iran. *Veterinary Medicine International* **2014**, 10–20.

Shalaby HA, El Namaky AH and Kamel ROA (2009) *In vitro* effect of artemether and triclabendazole on adult *Fasciola gigantica*. *Veterinary Parasitology* **160**, 76–82.

Shalaby HA, El Namaky AH and Kamel ROA (2016) *In vitro* tegumental alterations on adult *Fasciola gigantica* caused by mefloquine. *Journal of Parasitic Diseases* **40**, 145–151.

Shareef PAA, Brennan GP, Mcveigh P, Khan MAH, Morphew RM, Mousley A, Marks NJ, Saifullah MK, Brophy PM, Maule AG and Abidi SMA (2014) Time-dependent tegumental surface changes in juvenile *Fasciola gigantica* in response to triclabendazole treatment in goat. *Acta Tropica* **136**, 108–117.

Skuce P and Fairweather I (1990) The effect of the hydrogen ionophore closantel upon the pharmacology and ultrastructure of the adult liver fluke *Fasciola hepatica*. *Parasitology Research* **76**, 241–250.

Soulsby EJL (2012) *Helminths Arthropods and Protozoa of Domesticated Animals*. Eastbourne, UK: Baillière, Tindall & Cassell.

Stitt AW and Fairweather I (1993) *Fasciola hepatica:* tegumental surface changes in adult and juvenile flukes following treatment *in vitro* with the sulphoxide metabolite of triclabendazole (Fasinex). *Parasitology Research* **79**, 529–536.

Stitt A and Fairweather I (1994) The effect of the sulphoxide metabolite of triclabendazole ('Fasinex') on the tegument of mature and immature stages of the liver fluke, *Fasciola hepatica*. *Parasitology* **108**, 555–567.

Talukder S, Bhuiyan M, Hossain M, Uddin M, Paul S and Howlader M (2010) Pathological investigation of liver fluke infection of slaughtered black Bengal goat in a selected area of Bangladesh. *Bangladesh Journal of Veterinary Medicine* **8**, 35–40.

Tansatit T, Sahaphong S, Riengrojpitak S, Viyanant V and Sobhon P (2012) *Fasciola gigantica*: the *in vitro* effects of artesunate as compared to triclabendazole on the 3-weeks-old juvenile. *Experimental Parasitology* **131**, 8–19.

Toledo R and Fried B (2014) *Digenetic Trematodes*. New York, NY: Springer.

Valero MA, Marcos MD and Mas-Coma S (1996) A mathematical model for the ontogeny of *Fasciola hepatica* in the definitive host. *Research and Reviews in Parasitology* **56**, 13–20.

WHO (1995) *Control of Food Borne Trematode Infections*. Geneva, Switzerland: WHO.

Zerna G, Spithill TW and Beddoe T (2021) Current status for controlling the overlooked caprine fasciolosis. *Animals* **11**, 1819.