ULTRASTRUCTURAL EFFECTS OF ALBENDAZOLE ON THE BODY WALL OF GNATHOSTOMA SPINIGERUM THIRD STAGE LARVAE

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Abstract. This study investigated the effects of albendazole (ABZ) on the ultrastructure of Gnathostoma spinigerum advanced third-stage larvae. Two groups of experimentally infected mice received 60 or 90 mg/kg ABZ orally once a day for 21 consecutive days. Both groups had damage to the body walls of the worms, especially to the non-contractile part of the muscular layer. The severity of the damage was dose related, the higher the dose, the greater the damage. The body wall of the ABZ treated larvae demonstrated a decrease in the number of mitochondria in the non-contractile muscular part, especially in the internal surface of the sarcolemma. Some mitochondria developed large vacuoles, and became distorted and degenerated. The nuclei degenerated and had irregular shapes and the number of glycogen granules decreased. The present study demonstrates the structural damage induced by the toxic effects of ABZ and increases our knowledge of the mechanism of action of ABZ against G. spinigerum.

INTRODUCTION

Gnathostoma spinigerum is a pathogenic nematode causing gnathostomiasis in humans and certain animals, prevalent mainly in Asia (Miyazaki, 1960). Recently, human gnathostomiasis has become an emerging disease in western countries imported by travellers who visited endemic areas (Moore et al, 2003). Humans are accidental hosts. Infection routes include eating raw or semi-cooked foods containing infective larvae, parasite penetration through the skin after direct contact with contaminated meat or from mother to child during pregnancy (Daengsvang, 1981). The infected larvae do not develop to mature worms in humans but can survive for a long time in the human body. The worm usually penetrates into subcutaneous tissue, causing intermittent migratory swelling (Daengsvang, 1981). Sometimes it migrates to the central nervous system, producing various signs and symptoms, at times becoming life threatening (Boongird et al, 1977; Jaroonvesama, 1988; Schmutzhard et al, 1988).

Albendazole (ABZ) has been reported to be effective treatment against various helminthic infections in humans (Horton, 2000) including G. spinigerum infection in mice (Maleewong et al, 1992) and humans (Kraivichian et al, 1992). When G. spinigerum infected mice were treated with various sub-
lethal ABZ regimens (30, 60 or 90 mg/kg once a day) for 21 consecutive days, there was a significant reduction in the number of infectious larvae (Maleewong et al., 1992). The changes in *G. spinigerum* advanced third-stage larvae (aL3) after exposure to the metabolized ABZ, albendazole sulphoxide, *in vitro* were demonstrated by scanning electron microscope (SEM) (Sukontason et al., 2000). Changes were observed in the surface regions of the neck and body of the worm. The cuticular surface on the neck was swollen and overlaid with fuzzy material and the spines on the posterior part of the body were removed. However, transmission electron microscope (TEM) documentation of the ultrastructure of *G. spinigerum* after *in vivo* treatment with ABZ was still lacking. The present work analysed the ultrastructure of *G. spinigerum* aL3 in experimentally infected mice by TEM after treatment with ABZ, documenting the direct mode of action of this anthelmintic drug on disruption of parasite metabolism.

**MATERIALS AND METHODS**

Outbred male Swiss albino mice, weighing 25-30 g, were obtained and maintained at the animal unit of the Faculty of Medicine, Khon Kaen University. Four mice were randomly placed into different cages (27.5 x 18.7 x 12.5 cm³) containing wood shavings. The rodent chow and water were given ad libitum. All animal experiments were performed according the Guidelines for Animal Experimentation of the National Research Council of Thailand and the study was approved by the Animal Ethics Committee of Khon Kaen University. The experimental mice were orally infected with 5 *G. spinigerum* aL3 each using a blunt 18-gauge needle fitted to a 1-cc syringe. The aL3 were prepared according to procedures described previously (Maleewong et al., 1992).

Treatment with albendazole (Research Institute, Smith Kline & French Laboratories, Welwyn Garden City, Herts, UK) was begun 28 days after inoculation with aL3. The infected mice were randomly separated into two treated and one untreated (control) groups; each group consisted of 12 animals. The treated groups were given powdered ABZ suspended in 0.3 ml of distilled water orally. In the first treated group each mouse received 60 mg/kg ABZ once daily (at 09:00 AM) for 21 consecutive days. Each mouse in the second treated group received 90 mg/kg ABZ once daily for the same period of time. The untreated control mice received the vehicle substance only.

Only one mouse from control group, which died before the end of the experimental period, was excluded. All surviving mice were killed by ether inhalation 28 days after commencement of treatment, then dissected. All the organs were inspected under a light stereomicroscope (15-45X) for *Gnathostoma* larvae by the compression method. Five and 4 aL3 were recovered from the mice in the groups medicated with 60 and 90 mg/kg, respectively.

The worms recovered from the treated groups and those recovered from the untreated control group (mean ± SD = 4.5 ± 0.5) were fixed in modified Karnovsky fixative solution [2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) pH 7.2] and subsequently processed for SEM and TEM.

For TEM, the fixed worms were gently washed twice in the same buffer then post-fixed in a solution containing 1% osmium tetroxide in 0.1 M PBS pH 7.2, dehydrated in graded series of ethanol concentrations and embedded in Epon 812 resin (Fluka, Buchs Switzerland). Polymerization was performed at 60°C for 3 days. Ultrathin sections were collected on copper grids, counter
stained with uranyl acetate and lead acetate, and examined with a Hitachi-H600 transmission electron microscope. In the untreated control group, the surface morphology of the G. spinigerum L3 was processed by SEM as previously described (Maleewong et al., 1988) and is shown in Fig 1A.

The effect of albendazole was evaluated by examining the severity of larval damage. The structures evaluated were (1) the number of normal mitochondria and (2) the number of glycogen granules in the area of the non-contractile part of the muscular layer. Differences between the two groups were analyzed by Student’s t-test or Mann-Whitney rank sum test as appropriate.

RESULTS

Examination of untreated G. spinigerum L3 showed the body wall was composed of 3 layers, a cuticle layer, a hypodermis which forms the middle layer of the body wall, and a muscular layer (Fig 1B). The body wall and the organelles of the untreated control specimens were typical. The outermost cuticle was a non-cellular substance and was separated from the hypodermis by a thin basal lamina (Fig 1C). The middle hypodermis was thin and syncytial, containing a lot of organelles, mitochondria rough endoplasmic reticula and evenly dispersed glycogen granules (Fig 1C). The innermost muscular layer was enclosed by the sarcolemma (Fig 1C) and consisted of 2 parts: the upper contractile and the lower non-contractile parts (Fig 1C). The former part was composed of muscle fibers which were oriented parallel to the body axis and arranged in groups or fasciculi (Fig 1D). In the lower non-contractile part, no muscle fibers were found; instead the internal surface of the sarcolemma, the central region and the area beneath the contractile parts (Fig 1B and 1C) were occupied by mitochondria with round or cylindrical shapes (Fig 1D). A number of evenly dispersed glycogen granules (Fig 1D) and large round nuclei were also observed in this area.

Transmission electron micrographs of both ABZ treated groups revealed a damaged body wall, especially in the non-contractile part of the muscular layer (Fig 2A). Comparing the 60 mg/kg ABZ and the 90 mg/kg ABZ treated mice, it became obvious the severity of damage was dose-related (Table 1; Figs 2 and 3). The amount of damage increased with the dose. The body walls

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of mitochondria /10 μm²</th>
<th>No. of glycogen granules /1 μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.0 ± 2.3 (5)</td>
<td>63.8 ± 24.4 (5)</td>
</tr>
<tr>
<td>60</td>
<td>16.8 ± 3.8 (5)b</td>
<td>20.4 ± 2.3 (5)b</td>
</tr>
<tr>
<td>90</td>
<td>1.2 ± 0.5 (4)b</td>
<td>5.8 ± 1.7 (4)b</td>
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</tbody>
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Mnc = the non-contractile part of the muscular layer; n = number of larvae examined
a number of mitochondria with complete features
b Significant difference (p<0.05) from the untreated group
Fig 1–Scanning electron micrograph showing a *Gnathostoma spinigerum* advanced third-stage larva (A) and 3 transmission electron micrographs showing a transverse section of the body wall of *G. spinigerum* (B-D) from the untreated control group. The following structures are marked: cuticle (Cu), muscle fasciculus (F), hypodermis (H), muscle (M), the contractile part of the muscular layer (Mc), the non-contractile part of the muscular layer (Mnc), mitochondria (Mt), basal lamina (white asterisk), glycogen granules (arrow) and the sarcolemma (white triangle).
Fig 2—Transverse section of a transmission electron micrograph showing the body wall of *Gnathostoma spinigerum* advanced third-stage larvae from mice treated with 60 mg/kg albendazole (A-E). The following structures are marked: cuticle (Cu), hypodermis (H), muscle fasciculus (F), the contractile part of the muscular layer (Mc), the non-contractile part of the muscular layer (Mnc), mitochondria (Mt), nucleus (Nu), vacuole in mitochondria (V), glycogen granules (arrow) and the sarcolemma (white triangle).
Fig 3—Transverse section of a transmission electron micrograph showing the body wall of *Gnathostoma spinigerum* advanced third-stage larvae from mice treated with 90 mg/kg albendazole (A-E). The following structures are marked: cuticle (Cu), muscle fasciculus (F), hypodermis (H), the contractile part of the muscular layer (Mc), the non-contractile part of the muscular layer (Mnc), mitochondria (Mt), nucleus (Nu), vacuole in mitochondria (V), glycogen granules (arrow) and the sarcolemma (white triangle).
Fig 4–Transmission electron micrograph showing white blood cells accumulation in the outer surface of *Gnathostoma spinigerum* advanced third-stage larvae from mice treated with 90 mg/kg albendazole (A–B). The following structures are marked: cuticle (Cu) and the contractile part of the muscular layer (Mc). Ne, neutrophils; Mo, Mononuclear cell.

of the ABZ treated larvae demonstrated a statistically significant decrease in the number of mitochondria in the non-contractile muscular part, especially at the internal surface of the sarcolemma (Table 1; Figs 2B and 3B). Some mitochondria had large vacuoles, became degenerated and distorted (Figs 2D and 3D). The nuclei also degenerated and developed irregular shapes (Figs 2E and 3E). A significant decrease in the number of glycogen granules was seen (Table 1; Figs 2D and 3D). An additional observation was the accumulation of neutrophils and mononuclear cells at the outer surface of the larvae of the mice treated with 90 mg/kg ABZ (Fig 4).

**DISCUSSION**

Albendazole is a benzimidazole derivative and one of the most important drugs presently used to treat parasitic infections. It is extensively used in human and veterinary medicine against helminths (Horton, 2000; Reuter *et al.*, 2000; Buchanan *et al.*, 2003; van den Enden, 2009) and protozoa (Katiyar *et al.*, 1994; Farthing, 2006). Moreover, it is the drug of choice for the treatment of gnathostomiasis (Kraivichian *et al.*, 1992). Its mechanism of action, derived from experimental evidence by exposing intestinal helminths of several different species to ABZ, seems to be that it disrupts the parasite metabolism at several sites, most of which are involved in energy production: inhibition of mitochondrial fumarate reductase and reduction of glucose transport (Lacey, 1988) resulting in an inhibition of glucose uptake. In addition, for benzimidazoles, it is believed the final common pathway of metabolic disruption is inhibition of beta tubulin polymerase causing the disruption of cytoplasmic microtubule formation (Lacey, 1988) and production of cellular modifications (Osman *et al.*, 1994). It has been suggested that ABZ, together with other classical benzimidazoles, is capable of uncoupling oxidative phosphorylation in isolated mammalian mitochondria (McCracken and Stillwell, 1991; Carr *et al.*, 1993).

A new feature reported in the present study was the *in vivo* effects of ABZ on the ultrastructure of *G. spinigerum* al3 documented by TEM. We demonstrated treatment with ABZ led to body wall alterations in the worm particularly in the muscular layer. ABZ affected the number and morphology of mitochondria, degenerated nuclei and muscle fibers, and decreased the number of glycogen granules which possibly resulted in an inhibition of the glucose uptake (Lacey, 1988; Albonico *et al.*, 1999).
These data seem to indicate ABZ disrupts metabolism and hinders ATP formation required for survival and reproduction of the worm (Lacey, 1990). These changes reflect nutritional and energy deficiency and loss of cell function controls which possibly cause larval damage as revealed by a previous study (Sukontason et al, 2000). This damage may lead to exposure of antigenic determinants to the host’s immune system and trigger immunological attacks as revealed by white blood cell accumulation in the outer worm surface of ABZ treated mice (Fig 4).

A similar outcome was observed in Dictyocaulus viviparus exposed to sub-lethal doses of ABZ (Osman et al, 1994). In male worms, severe ultrastructural damage of the spermatogonia, spermatocytes and spermatozoa was seen, and in female worms abnormalities were seen in the cytoplasm of the oogonia with a decrease in nuclei and in the uteri and ovijector, which contained only undifferentiated ova (Osman et al, 1994). The TEM analysis of hydatid cysts from infected mice treated with ABZ showed a completely damaged germinal layer with vacuolated areas, many lipid droplets and residual lamellar bodies (Ceballos et al, 2008). The toxicity of ABZ was dose-related and damaged the mitochondria and smooth endoplasmic reticulum in the intestinal epithelium of the earthworm, Eisenia fetida. Some mitochondria had damage of the inner membrane with vacuolization (Gao et al, 2007).

While the doses of albendazole used in the present study were higher than recommended doses (400-800 mg/day for 21 consecutive days) for treatment of human gnathostomiasis (Kraiwichian et al, 1992), the revealed pathological changes at the ultrastructure level increase knowledge regarding the toxic effects of ABZ on round worms and its mechanism of action as an anthelmintic drug.

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