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Caenorhabditis elegans as a model for the screening of anthelminthic compounds: Ultrastructural study of the effects of albendazole

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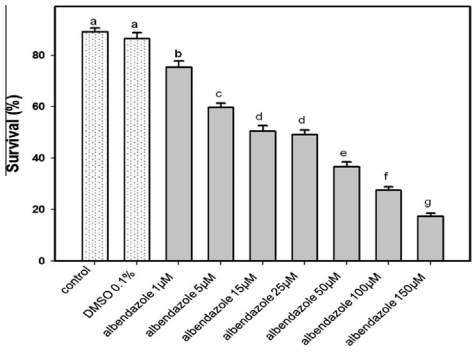
HIGHLIGHTS

- *C. elegans* is excellent for the screening of compounds with anthelmintic activity.
- Albendazole has potent anthelmintic activity against different stages of *C. elegans*.
- Degeneration of mitochondria was the main damage in albendazole-treated *C. elegans*.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the effects of albendazole on the viability, morphology and ultrastructure of different life stages of *Caenorhabditis elegans*. The albendazole EC_{50} value after seven days of treatment was 18.43 μM. This concentration was very efficient against all the stages. Light and electron microscopy analysis showed damage to the body wall of the adults and larvae. An intense desquamation of the cuticle of larvae and of the surface of the eggs was observed, preventing their hatching and development. The main ultrastructural damage detected was the degeneration of the mitochondria in the noncontractile muscle of the body wall, which appeared as large vacuoles. This study reaffirmed the use of *C. elegans* as a screening system for compounds with potential anthelmintic activity and showed the effects of albendazole on the different life stages of these worms.

1. Introduction

Helminthiasis is a serious problem worldwide resulting in high human morbidity and enormous economic losses in livestock (Waller, 1999), especially in tropical and sub-tropical countries.

Anthelmintic drugs are used for the control of parasitic infections caused by helminths. The demand for new and effective anthelmintics is immense, as the chemical drugs currently employed in the control of helminths are expensive, and most of them lose their efficacy in 20 years due to the problem of resistance (Mehlhorn et al., 2011).

Albendazole is a benzimidazole, which has broad spectrum activity, both in vitro and in vivo, against the common helminths

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of humans, such as *Ancylostoma duodenale*, *Necator americanus*, *Trichuris trichiura*, *Ascaris lumbricoides*, *Enterobius vermicularis* and *Strongyloides stercoralis* (Horton, 2000). It has been shown that the benzimidazoles exert their effect by binding selectively and have a high affinity to the β-tubulin molecules. The disappearance of cytoplasmic microtubules suggests that benzimidazoles act by inhibiting the microtubule-mediated transport of secretory vesicles in the helminth absorptive tissues and the consequent release of digestive enzymes causing tissue damage (Köhler, 2001). Borges and Nollin (1975) first demonstrated the disintegration of the normal microtubules matrix in intestinal cells of *Ascaris suum* treated with mebendazole. Subsequent studies (Borgers et al., 1975) confirmed this observation in other benzimidazole-susceptible species of helminths.

Caenorhabditis elegans is a free-living nematode naturally found in soils of temperate climate, which has became a model organism for parasitic nematode research and an excellent system for the screening of compounds with potential anthelmintic activity, because it is inexpensive, readily available, and easy to work with (Simpkin and Coles, 1981). In addition, the use of *C. elegans* in assays to investigate nematode behavior, locomotion, reproduction and death is uncomplicated and reliable (Thompson et al., 1996).

The use of different techniques of microscopy has enabled advances in the studies of chemotherapy identifying potential target structures of different parasites. In this study, *C. elegans* was used as an experimental model for evaluating anthelmintic activity using albendazole as an anthelmintic reference.

2. Materials and methods

2.1. Maintenance of C. elegans

Wild-type *C. elegans* Bristol strain N2, obtained from the Caenorhabditis Genetics Center was grown on NGM (Nematode Growth Medium: 2.5 g peptone from casein, 3 g NaCl, 17 g agar, 0.5% cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KH_2PO_4/K_2HPO_4 in 1 l of water) seeded with *Escherichia coli* strain OP50, at 25 °C as described by Brenner (1974).

2.2. Drug

Albendazole was purchased from Sigma Chemical Company (St. Louis, MO, USA). For the experiments, it was dissolved in dimethyl sulfoxide (DMSO) from Merck. The maximal concentration of DMSO was 0.1% in all assays.

2.3. Anthelmintic activity of albendazole

2.3.1. Assays using adults

The Bristol N2 wild-type *C. elegans* strain was grown on nematode growth medium (NGM) in plates containing the bacterial lawn (*E. coli* OP50) as the food source (Brenner, 1974). The nematodes were collected by washing the bottom of the plates with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 0.25 g $MgSO_4{\cdot}7H_2O$ in 1 l water) and centrifugation at 800*g* for 5 min. They were collected and 30 nematodes were added to each well of a 96-well plate and incubated at 20 °C in S medium, supplemented with *E. coli*. Different concentrations of albendazole were added and the development and the survival of the nematodes were observed after three and seven days of incubation. Three independent experiments were performed.

2.3.2. Egg hatch assay

The adults were collected from the NGM plates, centrifuged at 800*g* for five minutes. The supernatant was discharged and the pellet was resuspended with a lysing solution (5 M NaOH and 1% hypochloride). The period of incubation with the lysing solution did not exceed five minutes.

The eggs were separated and centrifuged at 1000*g* for 10 min; the pellet was suspended in M9 buffer, and the number of eggs counted. The number of eggs was adjusted to approximately 30 in 50 μl and then they were incubated for 15 h at 20 °C in S medium in a 96-well plate, supplemented with *E. coli* and with different concentrations of albendazole for 15 h at 20 °C. At the end of incubation, the unhatched eggs and L1 larvae were counted and the percent of hatched eggs at different concentrations of the treatment was calculated. Three independent experiments were performed.

2.3.3. Larval development assay

Eggs were obtained as described above and subsequently placed in microtubes containing S medium and *E. coli* under gentle stirring for 15 h at 20°. Larvae of L1–L2 stage (20 larvae in 50 μl) were collected and incubated for 24 h at 20 °C in 96 well plates containing S medium, supplemented with *E. coli* and with different concentrations of albendazole at 20 °C. After 34 h only the L3–L4 larvae were collected. These larvae were incubated in the same way as the L1–L2 larvae. Three independent experiments were performed.

2.4. EC_{50} calculation and statistical analysis

The number of dead and living adult nematodes was evaluated. Nematodes that had straight or curved bodies as well as those immotile were considered as dead. The percentage of mortality was expressed as the number of dead worms in relation to the whole group of worms.

The EC_{50} (efficient concentration of the drug required to kill 50% of worms) values were determined using Sigma Plot 8.0 (Systat Software Inc., Chicago, IL, EUA). Results were expressed as mean values ± standard error of the mean (SEM). Statistical comparisons were performed by analysis of variance (One Way ANOVA) using the program Prime (MacGraw Hill version 1.0) and the significance $p > 0.05$.

2.5. Morphology and ultrastructural analysis of different stages of C. elegans treated with albendazole

2.5.1. Light microscopy (LM)

For LM studies, approximately 90 nematodes of each stage, obtained from three independent experiments, were mounted on glass slides and observed under an Olympus B×51 light microscope using bright field or differential interference contrast (DIC). The images were captured with an Olympus DP12 digital camera.

2.5.2. Scanning electron microscopy (SEM)

For SEM, approximately 90 nematodes of each stage, obtained from three independent experiments, were fixed in 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M cacodylate buffer, rinsed in 0.1 M cacodylate buffer, pH 7.2, post-fixed in a solution containing 1% osmium tetroxide and 1.25% potassium ferrocyanide, pH 7.2, for 1 h, dehydrated in a graded series of ethanol solutions (30–100%), critical point-dried in CO_2 using a Balzers apparatus and sputter-coated with gold (Balzers) and observed using a FEI Quanta 200 scanning electron microscope.

2.5.3. Transmission electron microscopy (TEM)

For TEM, approximately 90 nematodes, obtained from three independent experiments were fixed in 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2. The worms were washed in 0.1 M cacodylate buffer, pH 7.2, post-fixed for 1 h in a solution containing 1% osmium tetroxide and 1.25% potassium fer-

rocyanide, pH 7.2. They were then dehydrated in a graded series of acetone (30–100%) and embedded in epoxy resin. Thin sections were collected on copper grids, counterstained with uranyl acetate and lead citrate, and observed in a Jeol 1200 TEM.

3. Results

3.1. Activity of albendazole on different life stages of C. elegans

The action of a single dose of albendazole on adult nematodes was plotted in curves that showed the percent of survival in function of the concentration of the compound (Fig. 1). The effect was clearly dependent on the concentration of albendazole used. After seven days of treatment, the EC_{50} value was 18.43 μM. At 150 μM less than 20% of the worms remained viable.

The treatment of *C. elegans* with albendazole on the hatching of eggs showed that at 1 μM approximately 20% of the eggs did not hatch (Fig. 2). Above 25 μM, albendazole was very efficient in preventing the hatching of eggs. Again, the effect was dependent of the concentration of albendazole used.

The inhibitory activity of albendazole against L1–L2 and L3–L4 larvae is shown in Fig. 3. The L1–L2 larvae were more susceptible than L3–L4 larvae. At a concentration of 25 μM survival of about 50% and 25% of L1–L2 and L3–L4 was observed, respectively. The difference was more accentuated when 150 μM albendazole was used.

3.2. Effects of albendazole on the development and the morphology of different stages of C. elegans

Treatment with albendazole after three days induced a phenomenon called *Endotokia matricida*, whereby the adult worm retains the eggs, which hatch internally. This resulted in rupture of adult nematodes, due to the movements of the internal larvae (Figs. 4B and 5B).

Scanning electron microscopy analysis was carried out in the treated and untreated adult helminths. The untreated helminths presented the typical cephalic end with six lips and the cuticular surface transversally striated along the body (Fig. 5A). SEM revealed that the surface of nematodes treated with 25 μM albendazole for seven days had a modified appearance of the cuticular surface. The classical cuticular pattern was not seen, and the cuticle was intensely desquamated (Fig. 5C). At least, two-thirds of helminthes analyzed presented these modifications. SEM of the untreated 15 h-old eggs showed typical morphology of the surface (Fig. 5D), which indicated the normal development of larvae (Fig. 4C) The eggs treated with 25 μM albendazole for 15 h had the appearance of a granular, fibrillar and disorganized mass of cells (Fig. 4D). By SEM it was possible to verify severe damage on the surface of the eggs treated with albendazole (Fig. 5E). The ultrastructural damages were present in nearly 100% of the eggs analyzed.

The treated L1–L2 larvae showed morphological changes when compared to control organisms. Light microscopy samples showed disorganization of the internal structures of the larvae (Fig. 4F). By SEM it was possible to verify areas of the larvae surface with lesions (Fig. 5G). The L1–L2 larvae treated with albendazole had the typical dimensions and did not resemble the Dauer stage. After 24 h, untreated L3–L4 larvae presented typical adult morphology, with eggs in the uterus, indicating their complete development (Fig. 4G). The larvae treated with 25 μM albendazole for 24 h showed deeply injured regions of the body (Fig. 4H) and the cuticular surface was severely desquamated (Fig. 5I and J). We observed theses morphological damages in nearly 100% of the samples analyzed.

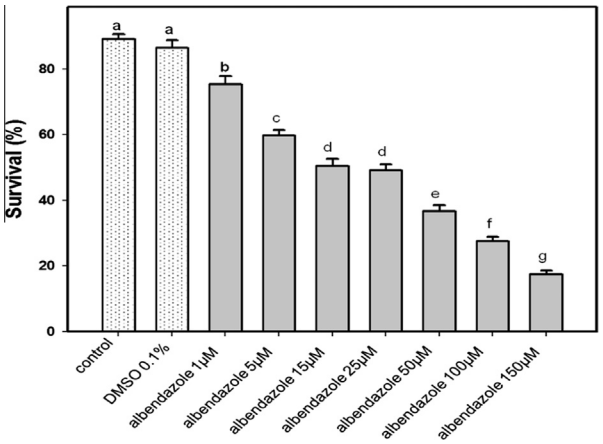

Fig. 1. The effect of albendazole on adult *C. elegans*. The values are the mean and standard error of the mean of three independent experiments. The letters represent the statistical analysis where different letters correspond to significant differences between the groups ($P > 0.05$; One Way ANOVA, Bonferroni post test.

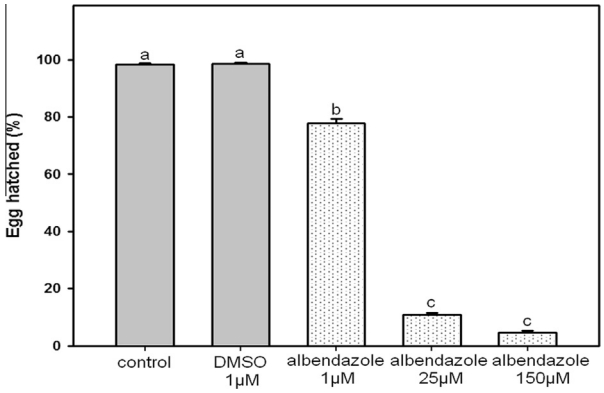

Fig. 2. The effect of albendazole on the egg hatching of *C. elegans* in vitro. The values are the mean and the standard error of the mean of three independent experiments. The letters represent the statistical analysis where different letters correspond to significant differences between the groups ($P > 0.05$; One Way ANOVA, Bonferroni post test.

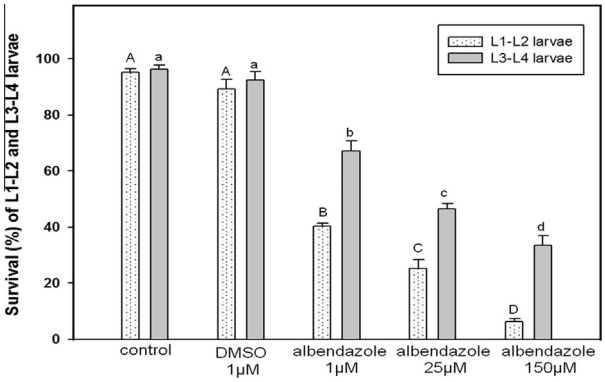

Fig. 3. The effect of albendazole on larvae L1–L2 and L3–L4. The values are the mean and the standard error of the mean of three independent experiments. The letters represent the statistical analysis where different letters correspond to significant differences between the groups ($P > 0.05$; One Way ANOVA, Bonferroni post test.

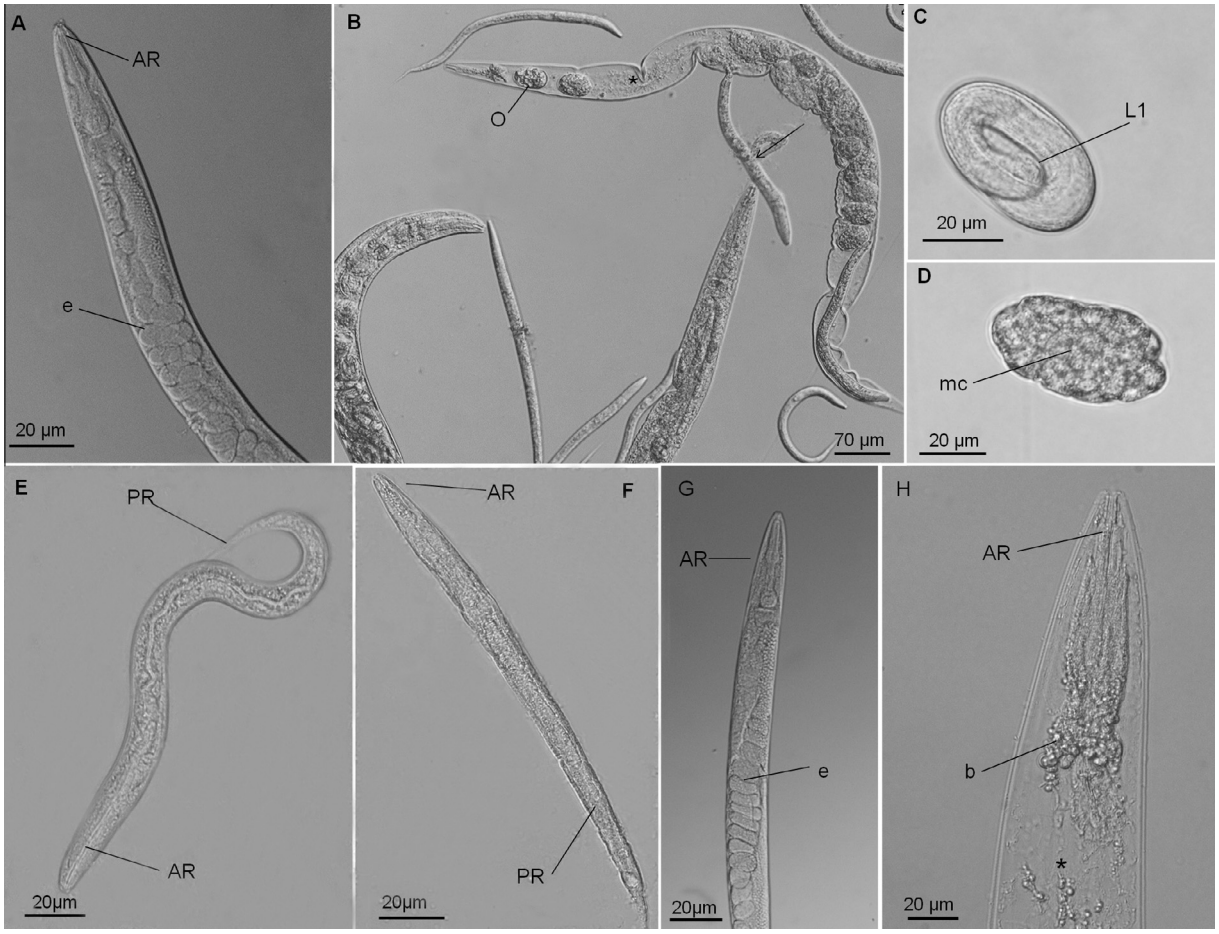

Fig. 4. Morphology of different life stages of *C. elegans* by light microscopy. A – Untreated adults show the anterior region (AR) with the uterus and some eggs (e). B – Adults treated with 25 µM albendzole, showing the *Endotokia matricida* in some adults. The eggs (e) that did not hatch are refringent and did not present any developing larva inside. Some larvae (arrows) were observed. C – Untreated egg where the larva of the first stage can be seen inside (L1). D – Egg treated in vitro with 25 µM albendazole. A granular refractile and disorganized mass is seen instead of the formation of L1 larva. E – Untreated L1 larva shows an intact body, the anterior region (AR) and the posterior region (PR). F – L1 larva treated with 25 µM albendazole show the anterior region (AR) and the posterior region (PR). The body is so severely disorganized that it is not possible to identify the internal structures. G – Anterior region (AR) of adult obtained after 34 h result of the full development of the eggs. H – Larvae between the L3–L4 stages show severely destroyed internal structures and bulb located in the anterior region (AR).

3.3. Effects of albendazole on ultrastructure of adult C. elegans

By TEM it was possible to observe *C. elegans* adults with a body wall composed of cuticle, hypodermis and muscular layer. The cuticle showed transverse cuticular striation. The hypodermis presented a syncytial layer, located below the cuticle and many mitochondrial profiles, nucleus and endoplasmic reticulum profiles. The hypodermis was projected into the pseudocoelom between the muscle fibers, forming the hypodermal chords (Fig. 6A, C and D). The musculature comprised contractile and non-contractile regions. The contractile region is made of radially organized myofilaments in each fiber separated by dense bodies (Fig. 6A, C and D). In cross sections (Fig. 6A) it is possible to see dense bodies present in the contractile region, which are connected to the sarcolemma. Each dense body delimits a sarcomere. The non-contractile region is rich in clusters of glycogen particles.

The uterus of hermaphrodites, as seen in cross-sections, was evolved by a single layer of epithelial cells (Fig. 6D). The embryos had a thin cuticle and a hypodermis similar to that of the adult form. (Fig. 6E).

The ultrastructural analysis of the adult form of *C. elegans* after treatment with 25 µM albendazole showed the cuticle with an irregular pattern of cuticle striation in many regions of the body (Fig. 7C), contrary to other regions where the cuticle showed the typical transversally striated cuticular surface (Fig. 7A and B). An intense disorganization was observed in the body wall (Fig. 7C). The hypoderm and the non-contractile part of the muscular layer were the regions with the most damage. Vacuoles with internal membranous elements (Fig. 7A, B, C and D), resembling autophagic vacuoles and organelles surrounded by concentric membranes (Fig. 7C) were observed. The albendazole affected especially the mitochondria; these organelles appeared as large vacuoles, (Fig. 7A, B and C). In addition, the musculature adjacent to the esophagus presented mitochondrial profiles presenting myelin-like figures in the matrix and general altered morphology (Fig. 7D and D1). These ultrastructural alterations were observed in at least 50 fields of different ultrathin sections analyzed from 9 samples of three different experiments.

4. Discussion

Many studies using benzimidazoles for the treatment of different nematodes and cestodes reported that depolymerization of microtubules caused disorganization of the cytoskeleton, leading to the interruption of transport of secretory vesicles and the incapacity of intestinal cells in the capture of glucose, causing the

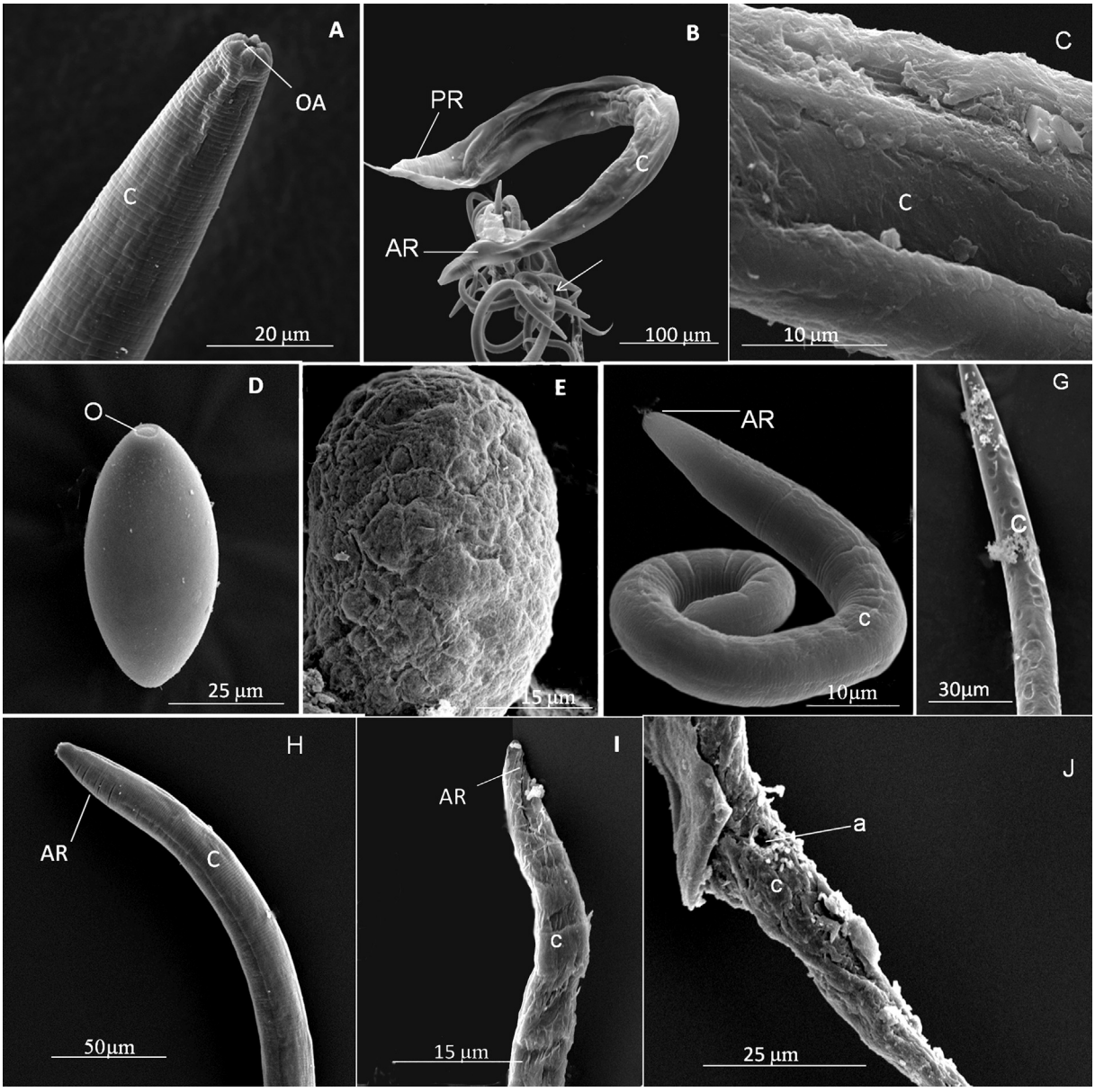

Fig. 5. Morphology of different life stages of *C. elegans* as seen by SEM. A – Anterior region showing the oral opening (AO) and the intact cuticle (C) with transversal striations. B – Adult nematode treated with 25 µM albendazole display an elongated body with the anterior (AR) and the posterior regions(PR); however, it was possible to verify damaged regions and larvae (arrows). C – Adult nematode treated with 25 µM albendazole show the middle region of the body with deep cuticular (C) desquamation. It is not possible to observe over the transverse striations. D – Untreated egg showing the operculum (OP). E – Egg treated with 25 µM albendazole with a fragile structure and without the development of larvae.

depletion of glycogen stores (Horton, 2000; Köhler, 2001; Lacey, 1990; Sanchez-Moreno et al., 1987).

Previous studies indicated that benzimidazoles showed activity against eggs, making them unviable and preventing the formation and the development of the larva (Lacey, 1988) as confirmed in this work.

During the life cycle of *C. elegans*, unfavorable conditions due to shortage of food or climate variations can lead the conversion of L1 larva into the Dauer stage. Treatment of the larvae between L1–L2 stages with albendazole did not lead to the conversion, since no morphological evidence of the Dauer stage, as the elongated and narrow body or a differentiated cuticle and the closing of the mouth, were observed (Cassada and Russell, 1975).

In this study, adult nematodes treated with the albendazole presented the process known as *E. matricida*, probably caused by the ability of this compound to induce starvation in the nematodes (Trent et al., 1983; Clutton-Brock, 1991). It is probable that the internal hatching of larvae could have been stimulated by prevention of the glucose uptake and impairment of the production of energy in the treated worms. This phenomenon is typical in species of the Rhabditidae family and it might increase survival as a response to stress (Chen and Caswell-Chen, 2004).

By SEM it was possible to verify damages caused by these compounds on the surface of eggs and cuticles of larvae and adults. Desquamation in the cuticle of L1–L2 larvae, L3–L4 and adults was often seen after different treatments. Oliveira-Menezes et al. (2007) observed by SEM that adults of *Wulchereria bancrofti* obtained from patients with filariosis treated with co-administration of diethylcarbamazine and albendazole, displayed damage in the cuticular surface and presented large ruffled-leaf cuticular

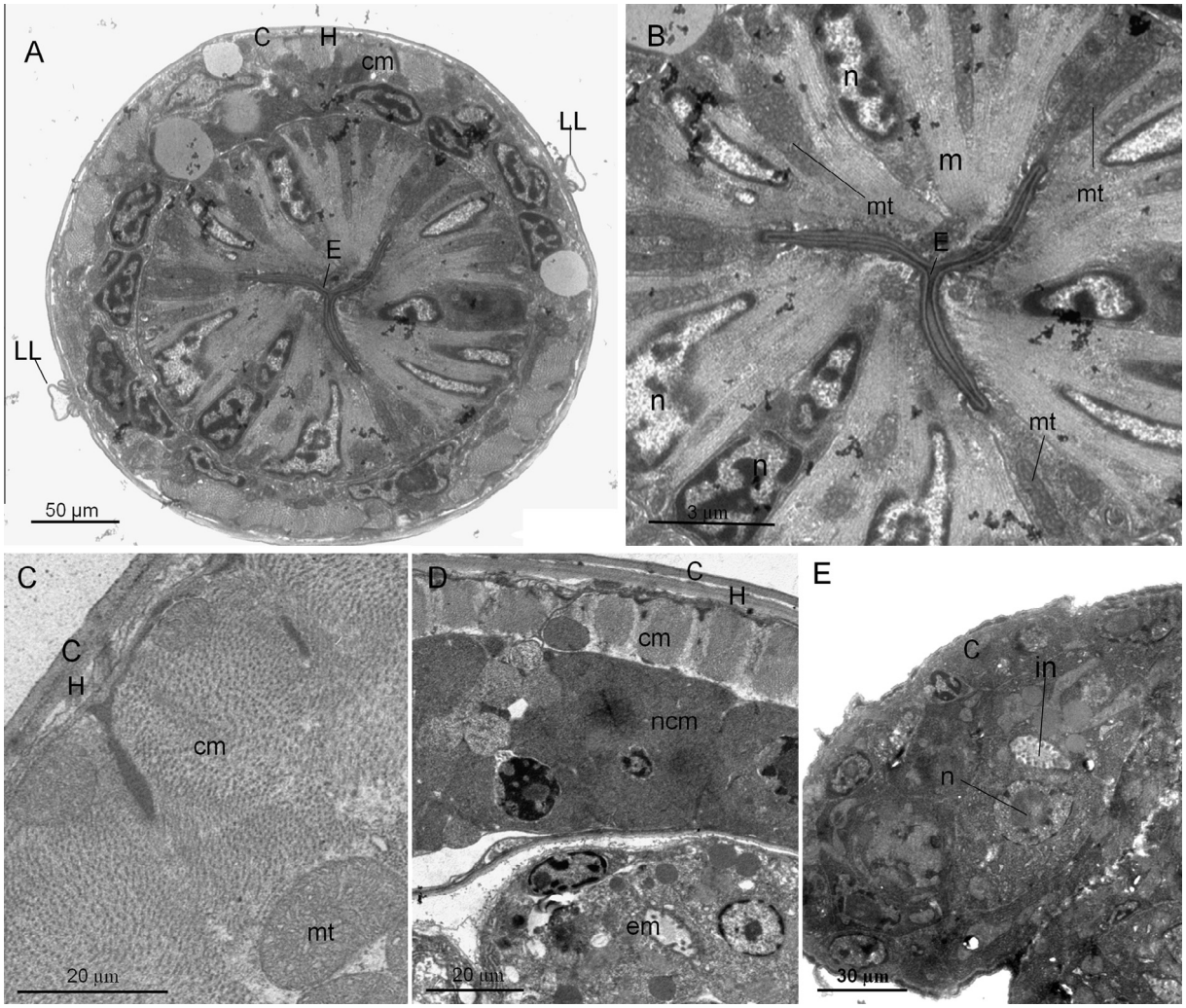

Fig. 6. Transmission electron microscopy of untreated *C. elegans*. A – Cross section of anterior region, showing the cuticle (C), the lateral line (LL), the musculature and the esophagus. B – Detail showing the musculature near the esophagus and several nucleus (N) and mitochondria (mt) profiles. C – Body wall formed by a cuticle (C), hypodermis (H), and a muscle cell composed of a contractile region (cm) and a non-contractile region with mitochondria (mt). D – Ultrathin section showing the body wall with the cuticle (C), hypodermis (H), and a muscle cell composed of a contractile region (cm) and a non-contractile region (ncm). The uterus delimited by an epithelial tissue and containing embryos (em) is also seen. E – Embryos with a thin cuticle (C), nucleus (n) and intestine (in).

projections. The nematodes from patients treated only with diethylcarbamazine did not show any damage.

The SEM of the larva L3 of nematode *Gnasthostoma spinigerum* treated with albendazole sulphoxide in vitro, showed severe damage in the cuticle of different regions of the worm body and the spines located in the posterior region were removed (Sukontason et al., 2000).

Previous studies have shown that benzimidazoles interfere with mitochondrial function, especially in the synthesis of ATP. Prichard (1970) suggested that tiabendazole inhibits the enzyme fumarate reductase in *Haemonchus contortus*. Van Den Bossche and Janssen (1967, 1969) showed that cabendazole, tiabendazole, levamizole and mebendazole inhibited the fumarate reductase and succinate dehydrogenase of *H. contortus*.

A feature reported in the present study was the effect of albendazole on the ultrastructure of *C. elegans* as seen by TEM. We showed that treatment with albendazole led to alterations of the nematode body wall and the morphology of mitochondria. Our results were similar to those observed by Arunyanart et al. (2009), who demonstrated that treatment of infected mice with *G. spinigerum* with albendazole affected mitochondria in the non-contractile musculature, especially in the internal surface of sarcolemma, where they appeared as large vacuoles. The authors also observed a decrease in quantity of these organelles in addition to a decrease in glycogen storage. The mitochondria observed as "empty" structures were interpreted as vacuoles. Several mitochondria were seen in an intermediate stage of degradation showing disrupted cristae and swelling of the matrix. It is likely that these changes can be linked to the action of benzimidazoles in mitochondrial metabolism. Cárdenas et al. (2010) observed the same ultrastructural damage when rodents infected with *Litomosoides chagasfilhoi* were treated in vivo with 40 mg albendazole. However the damage was restricted to the reproductive system of the nematode.

In conclusion our present observations show that albendazole has activity on eggs, larvae and adults of *C. elegans*, causing severe damage to the body wall. The intense degeneration of mitochondria observed in the treated nematodes suggests that this drug may have an important effect on this organelle and not only on microtubules. In addition, our observations support the view that *C. elegans* is a good model for the investigation of effects of drugs on helminths.

Conflict of interest statement

The authors declare no conflict of interest. Mention of trade names or commercial products in this publication is solely for

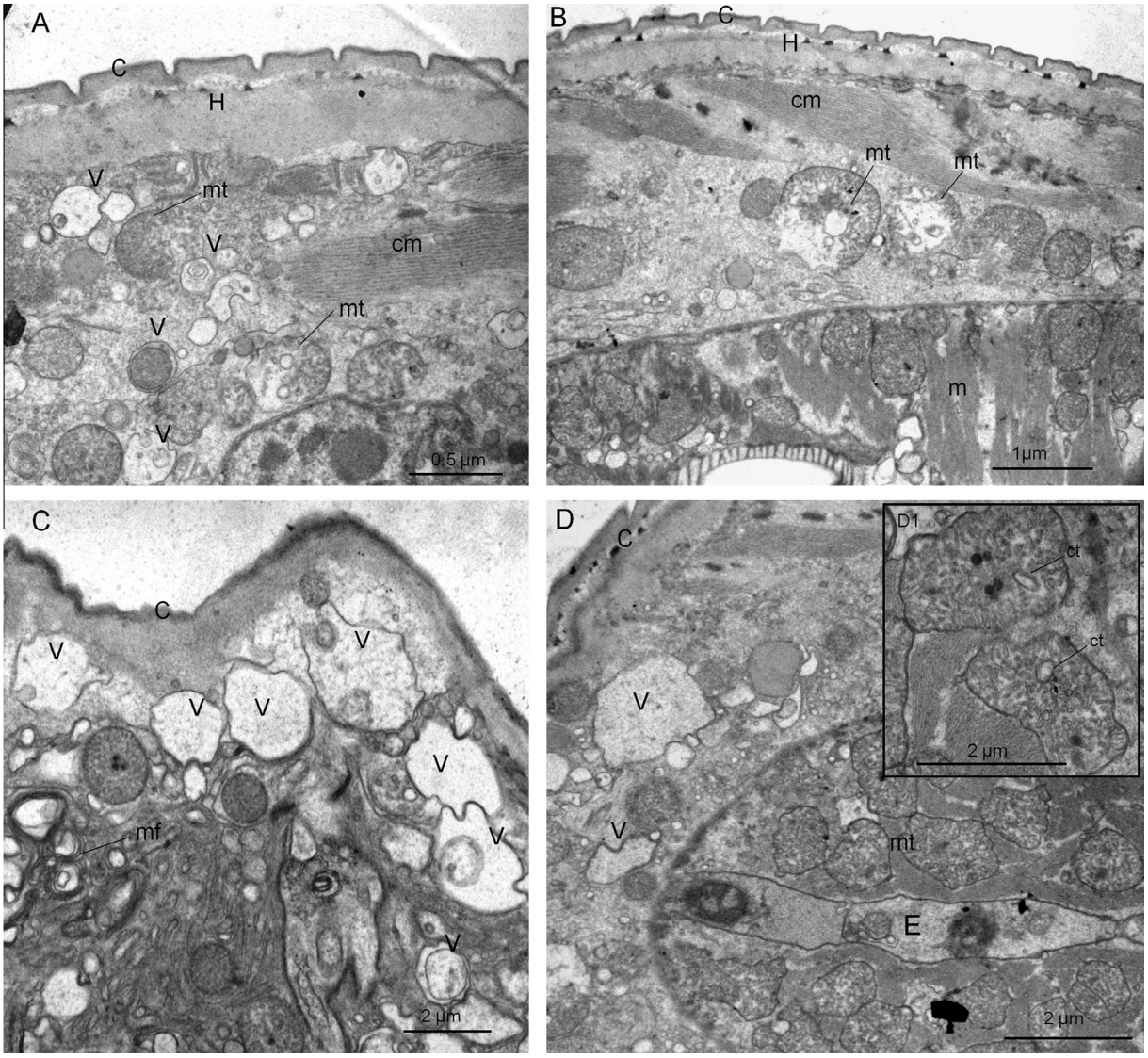

Fig. 7. Ultrastructural effects of albendazole. A – The cuticle (C) presents transversal striations, the hypoderm (H), the contractile region (cm) and a non-contractile region (ncm) with many degenerated mitochondria and vacuoles (V). B – Longitudinal section show the cuticle (C) with transversal striations, the hypoderm (H), the contractile region (cm) and a non-contractile region (ncm). The mitochondria (mt) were damaged, and it was possible to verify that the mitochondrial cristae and the matrix were extracted. C – Body region with intense disorganization, many vacuoles (V) originated from the degeneration of the mitochondria, and myelin-like figures (mf). D – Muscular region near the esophagus showing altered mitochondria (mt). D1 – Detail showing mitochondrial swollen cristae (ct).

the convenience of the reader. The final article has been approved by all authors.

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