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In Vitro Efficacy Of Antimalarial Drug Primaquine Against Adult Cestode And Trematode Helminthes Of Sheep

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Abstract: *The fact that the efficiency of anthelmintic drugs may diminishthrough tenyears of use depending on virousissues, one of these issues includes producers' over-dependence on such chemicals for parasites control, has dragged the concern of many researchers to useothordrugs . Both scanning electron and light microscopic studieswere used ,for the first time, to determine the effect of antimalarial drug primaquine on some gastrointestinal helminthes of sheep following 24 h in vitro incubation. These results were compared with those observed on adult worms exposed to albendazole (reference drug). Two species of helminthes, Monieziaexpansa and Paramphistomummicrobothrium; cestode and trematode representatives were subjected to 10, 20 and 30µg/ml ofprimaquine.Thehelminthes'tegument was affected and altered by primaquine. Furthermore, the response to primaquine action was more obvious in P. microbothrium than M. expansa adult worms. These effects involved destruction, alterations, and deformationof the tegument of P. microbothrium, whilelimited effect was observed on the tegument ofM. expansa.This study suggestedthat primaquine is not only a potent antimalarial drug, but also effective trematocidal drug causing significant damage to the fluke's tegument.*

Keywords:*Primaquine,Antimalarial,Albendazole,Monieziaexpansa, Paramphistomummicrobothrium,In vitro effect*

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I. Introduction

Primaquine has been used since the early 1950s and is the most widespread 8-aminoquinoline antimalarial drug (WHO 2015). *In vitro*, it displayed antischistosomalactivities against both juvenile and adult worms of *Schistosoma mansoni*, that caused the body of parasite to be deformed in a prominent manner (KamelandBayaumy2017).According to literatures primaquinewas found to affectthe schistosomula lysosomal acidic vesicles which responsible for endocytosis and detoxification (Holtfreter et al., 2011). Besides, it reduced the survival of both male and female worms and inhibiteddaily egg output (Mitsui and Aoki 2010).Recently, there were no published studies concerning using primaquine as anti-trematodcidal or anti-cestodaldrug. On the other hand, gastrointestinal helminthes infection is the most common parasitic infection of ruminants worldwide, more particularly in third-world countries because it infects livestock to a large extent and is now well recognized as the highest disease cost to small ruminant industry(Bedada et al. 2018).Two species of helminthes have the most economic significance;the cestode*Monieziaexpansa*and the trematode *Paramphistomummicrobothrium*. *M.expansa* is a very common parasite that infects sheep, as well as goat populations and could be considered as an important problem in sheep breeding(Maziadand El-Nemr 2002).The vast majority species of paramphistomes in Egypt is*P.microbothrium*(HiekalandHilali 1993). It causes economic loss greater than those caused by many other parasites (Hanna et al. 1988). As denoted by Khani et al. (2008)the immature paramphistomes caused very high death, representing 80-90% in domesticated ruminants. The sheep industry still relies heavily on the use of anthelmintics to alleviate the infections of gastrointestinal helminthes, in spite of these drugs are expensive and usually do not block reinfection (Fikru et al. 2006). Moreover, Shalaby(2013) reported that the efficacy of anthelmintics might decreasethrough about ten years of use based on producers' over-dependence on such chemicalsin treatment as well as poor administration practices such as under-dosing. So,the need for new anthelmintics from a different chemical group in veterinary medicine is insistent. Asmentioned by McKinstry (2007) that helminthes' tegument is vital for the absorptive and protective functions, the current work was undertaken to assess whether the primaquine had any effect on the tegument of both adult *M. expansa* and *P.microbothrium* following incubation *in vitro*. The results were matchedwith those detected in the helminthes' tegument after exposure to albendazole, as it was one of the most effective of the broad-spectrum anthelmintic agents.

II. Materials And Methods

Drugs

Primaquine bisphosphate was obtained from Sigma-Alderich Chemical Co. (St. Louis, MO, USA).10 mg/ml primaquine was prepared as a stock solution with 3ml double distilled water(Holtfreter2011).Albendazole (Vermizole®) was purchased from Amoun Pharmaceutical Company (El-Obour City, Cairo, Egypt).

Parasites

P. microbothrium and *M. expansa*adult worms were collected from the rumens and intestines, of naturally infected sheep slaughtered in Cairo abattoir .Worms were washed in different changes of sterilized culture medium-RPMI 1640.

Anthelmintic effects of primaquine

The adult worms,after recovery, were conveyed to fresh RPMI containing 50 % (v/v) heat denatured rabbit serum, 2 % (v/v) rabbit red blood cells; as recommended by Ibarra and Jenkins(1984), and primaquine at concentrations of 10, 20 and 30 µg/ml. Then the worms were incubated at 37 °C for 24 h in an atmosphere of 5 % CO_2. A reference drug group was prepared by incubating the adult worms in RPMI 1640 culture medium containing 10 µg/ml albendazole sulfoxide, (ABZ-SO) active form, for 24 h. This concentration is close to the ultimate blood levels of the sulfoxide metabolite *in vivo*(Fetterer1982).A stock solution of albendazole was prepared in DMSO.Thenit added to RPMI medium to give a solvent concentration of 0.1% (v/v). Anormal control group was prepared by incubating worms in RPMI medium without adding drugs. Six worms from eachgroup were examined.

Light microscopy

After incubation, 10% buffered formol saline was used to fix the adult worms.The samples were dehydrated with a graded ethanol series then embedded in paraffin. Longitudinal sections of *M. expansa* gravid segments and cross sections of *P. microbothrium* were cut with a microtome. The sections (4-6 µm thick) were stained with hematoxyline and eosin according to the method of Bancroft et al.(1996).The tegument of adult worms was studied and photographed using an Olympus CX41 microscope.

Scanning electron microscopy (SEM)

After incubation for 24 h in 30µg/ml primaquine, the anterior end of adult *M. expansa* and intact adult *P. microbothrium* were fixed 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. After washing with distilled water, dehydration of the specimens occurredby increasing concentrations of ethanol (from 50 to 100%), and then dried in a HCP-2 critical point drying apparatus (Hitachi, Japan) using liquid carbon dioxide as a transitional medium for 15 min. The specimens were mounted on aluminum stubs and coated with gold in an ion-sputtering apparatus for 4 min. The specimens were examinedusing aJeol scanning electron microscope (Jeol Corp., Mitaka, Japan) operated at 15 kV.

III. Results

The control untreated adult worms exhibited no loss of motility during the 24 h, the whole period of incubation. While, the adult *M. expansa*worms treated with 30 µg/ml primaquine responded less sensitive to changes in the surrounding conditions than the control worms, at a time, the adult *P. microbothrium* showed complete loss of motility. All the adult worms incubated at both 10 and 20 µg/ml primaquine exhibited active movement throughout the incubation period of 24 h. On the other hand, those exposed to 10 µg/ml ABZ-SO (reference drug) showed paralysis or death of the adult worms. To determine the mechanisms by which primaquine, at 30 µg/ml concentration, affected the adult worm activity, the possible tissue damage induced after treatment was evaluated analyzing histological sections of the tegument of the adult worm and the structures of the tegmental surface.

Light microscopic observations

M. expansa

The tegument ofthe control worms' gravid segments showed features similar to that of the fresh normal specimen. Briefly, it showed an intensively stained syncytial layer; lay on a thick basement membrane of amorphous material containing granular inclusions. The basement membrane appeared to be continuous with the general filling material which lay between parenchymal cells of the interior of the proglottis. Beneath the outer tegumentary layer was a sheath of muscle fibers; an outer layer of circular muscle and an internallayer of longitudinal muscle. Those layers were followed by a subtegumentary layer of branching parenchymal cells filled the whole space around the uterine branches (Figs.1a - c). In the primaquine treated worms, the tegument

appeared to be more swollen than normal, while the underlying structures still appeared normal (Figs.1d - f). This swelling became more pronounced in ABZ-SO treated worms. In those specimens, the tegument lost its normal aspect and appeared to be extremely corrugated, swollen and pale, accompanied by the appearance of prominent wrinkles on its outer layer. In some areas, disruption of the muscle bundles was observed (Figs.1g - i).

P. microbothrium

No significant differences in the tegumental features were noticed between normal and control worms incubated for 24 h in media free from drug. The tegument showed even and intensely stained cytoplasmic syncytial layer, which rested on basal lamina. The latter linked the tegument to the underlying two muscular layers which sent their processes outwardly to join up with the tegument. The tegumental cells located underneath the muscular layers (Figs.2a - c). The primaquine treated flukes revealed severe tegumental disruption and sloughing of patches of the outer tegumental layer exposing the basal lamina. The muscle bundles and the parenchymal tissues showed severe degenerative changes (Figs.2d - f). Degenerative changes of the outer tegumental layer were also apparent following treatment with ABZ-SO, but the muscles underlying the tegument still exhibited a normal appearance (Figs.2g - i).

Scanning electron microscopic observations

M. expansa

A globular scolex provided with four oval suckers radially located at its peripheral margin were observed at the anterior end of the control adult *M. expansa*. The tegument behind the suckers had no infoldings (Fig.3a).The scolex appeared to be more swollen than the control with narrowing of the sucker's openingfollowing treatment with primaquine. The tegument lost its normal aspect showing corrugated tegumental surface (Fig.3b). Following ABZ-SO treatment, the adult cestodeexhibited swollen scolex with severely folded tegument around the suckers sothat the contraction of their openings were obvious (Fig.3c).

P. microbothrium

In the control, the adult fluke had a pear-shaped body with a narrow anterior end and broader posterior end (Figs.4a, b). The oral sucker located near the anterior tip, was transversely elongated oval, whereas the acetabulum was positioned close to the posterior tip. The tegument covering the body was composed of transverse folds alternating with grooves (Fig.4c). Aggregation of dome-shaped papillae arranged in rows was clearly visible in these anterior folds. In almost all specimens examined ofthe primaquine treated flukes, distortion of both oral sucker and acetabulum was extreme to such extent that little distinguishable structure remained (Figs.4d, e). Apparently, patches of thickened tegument were seen throughout the posterior region around the acetabulum. At high magnification, the tegumental thickening was observed due to a layer of syncytial secretion overlying the surface of the tegument. Also, this syncytial secretion was extended to the mid-body region of the fluke so that the tegumental transverse folds could not be recognized (Fig.4f). At high magnification, neatand round holes were noticeable at the surface (Fig.4f, inset). In ABZ-SO treated flukes, the tegumental alterations appeared similar to that of the primaquine treated flukes. Yet, a number of pits caused by rupture of papillae were observed at higher magnification of the tegumental surface at the anterior end of the fluke (Fig.4g, inset). Acetabulum showed considerable damage; the texture of the tegument was distorted and the papillae were lost (Fig.4h). The tegumental surface at the mid-body region exhibited severe blebbing (Fig.4i, inset).

In all experiments of scanning electron microscopic studies, no significant differences in the tegumental features were seen between control and normal fresh worms incubated for 24 h in media free from drug.

IV. Discussion

The current study demonstrated the comparative morphological effects of primaquine and ABZ-SO (reference drug) against *M. expansa* and *P. microbothrium* adult worms; cestode and trematode representatives. This is the first study demonstrating the *in vitro* effects of primaquine on some gastrointestinal helminthes of sheep. Remarkably, primaquine is the fourth antimalarial drug showing anthelmintic properties after artemisinins (artemether and artesunate), trioxolanes and mefloquine(Keiser et al. 2006; Keiser and Morson 2008; Shalaby et al. 2009; Shalaby et al. 2016).In this study, the response to primaquine action was more potent in *P. microbothrium* than *M. expansa* adult worms. During the whole period of the *in vitro* experiments, the 30µg/ml primaquine treated *M. expansa* showed a slower rate of activity than the controls and none of the treated cestodes died. While, 30µg/ml primaquine treated *P. microbothrium* and ABZ-SO treated worms showed complete loss of motility. These observations might refer to the superiority of primaquine in killing the trematodes that might be appropriate *in vivo* to drive the worms out from the host's gastrointestinal tract, as had been illustrated for albendazole(Tinar et al. 1988). Indeed, in the present study,the data of the tegument

histological observations of *P. microbothrium* after *in vitro* administration of primaquine were similar to that induced by ABZ-SO, and more severe than that were observed in the primaquine treated *M. expansa*. However, the assessment of drug-derived effects was essentially depended upon electron micrographs, rather than light micrographs. Since, electron micrographs elucidated the detailed morphology and different changes of the worm's tegument permitting the interpretation of its functionality. In this aspect, the tegumental distortion of *P. microbothrium* including the oral sucker and the acetabulum, as well as the tegumentalthichening throughout the mid-body region of the fluke; that was marked with neat round holes, were observed during the *in vitro* action of primaquine. The surface changes were more severe than those observed in the primaquine treated *M. expansa*, in which limited tegumental swelling had occurred in their scolices.The tegumental distortion has been seen in the adult flukes treated with ABZ-SO. Previous studies had shown that the albendazole possessed a broad spectrum activity against all classes of parasitic helminthes. This drug had been recorded to induce tegumental disruption and muscular degeneration by binding specifically to β-tubulins, thereby inhibiting polymerization and functioning of the cellular motor proteins(Lacey 1988;Ahmed and Nizami 1991).In the current study, ABZ-SO showed a potential *in vitro* effect against *P. microbothrium* and *M. expansa*, where the tegument of the adult worms was severely distorted and tegumental blebs appeared especially at the mid-body region of the flukes. Similar findings were reported for, biologically related trematodes, *Cotylophoroncotylophorum*(Radwan et al. 2012) and*Fasciola hepatica* (Buchanan et al. 2003)treated *in vitro* with 10 µg/ml of albendazole for 12 hours. The thickening of the tegument without bleb formation was observed in the primaquine treated flukes, but the surface was marked with neat, round holes that might be resulted from rupture of blebs. These tegumental alterations were also reported for *P. microbothrium*(Shalaby et al. 2010)and *F. gigantica*(Shalaby et al. 2009)treated *in vitro* with, an antimalarial drug, artemether. The tegumental alterations; including swelling, blebbing that was later disrupted causing erosion, were common features of drug-treated trematodes and cestodes after exposure to anthelmintics(Meansy et al. 2002). Additionally, the tegumental thickening was probable to represent part of a stress response on the part of the fluke and had been observed in another anthelmintic studies on *F. hepatica* during the early stages of drug action (Stitt and Fairweather 1993, Anderson and Fairweather 1995, McKinstryet al. 2007). In an effort to maintain the safety of the apical membrane,the layer was formed by the accelerated release of tegumental secretions at the surface.

Our observations on primaquine treated worms pointing to the tegument as the main interface for drug uptake. Much of the literature suggested trans-tegumental uptake might play a more significant role in drug entrance into trematode and cestode parasites(Toner et al. 2009). However, in this study, the *P. microbothrium*tegument was severely affected than that of *M. expansa* following their exposure to primaquine. Traditionally, the complex trematode and cestode tegument was believed to act as absorptive surface (Bashtar et al. 2011).*P. microbothrium* exhibited a high degree of corrugation comprising of alternating grooves and folds increasing the surface area of absorption by the tegument. The higher absorptive ability in the tegument of *P. microbothrium* reflected on the higher level of disruption to the flukes when exposed to primaquine. A recent published study(Kamel and Bayaumy 2017) showed that 24 h after incubation in primaquine at the concentration of 20 µg/ml resulted in extensive damage of, a biologically related trematode, *Schistosoma mansoni* including degeneration of both the tegumental and subtegumental layers. Also, Holtfreter et al.(2011) suggested a strong impact of the primaquine on the tegument of *S. mansoni*.

In conclusion, the present study has suggested that primaquine is not only potent antimalarial drug, but also effective trematocidal drug causing significant damage of the fluke's tegument. It means that the parasite's first and main line of defense is destroyedallowing the drug potential access to other, internal tissues of the fluke, which will lead to more widespread damage. However, several questions remain to be answered, for example the mechanism by which primaquine exerts its effect on the parasite's tegument, and why the intensity of primaquine-induced damage in *P. microbothrium* is greater than that in *M. expansa*.

Authors' contributions

HAS, AHE and HMA designed, conceivedand performed the experiments; FEM,RAK and TKF collected worms from abattoir and revised the manuscript;HAS,AHE wroteand preparedthemanuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figures captions

Fig. 1 Light micrographs of the tegument longitudinal section of adult *M. expansa* gravid segments. (a, b, c,) Normal control worms, (d, e, f) Following 24 h incubation with 30 µg/ml primaquine. Note slightly swollen tegument. (g, h, i) Following 24 h incubation with 10 µg/ml albendazole sulphoxide. The tegument is extremely corrugated, swollen and pale as compared with the controls. The outer tegumental layer shows prominent wrinkles (*arrows*) with patches of tegumental sloughing. Some areas exhibit disruption of the muscle bundles.

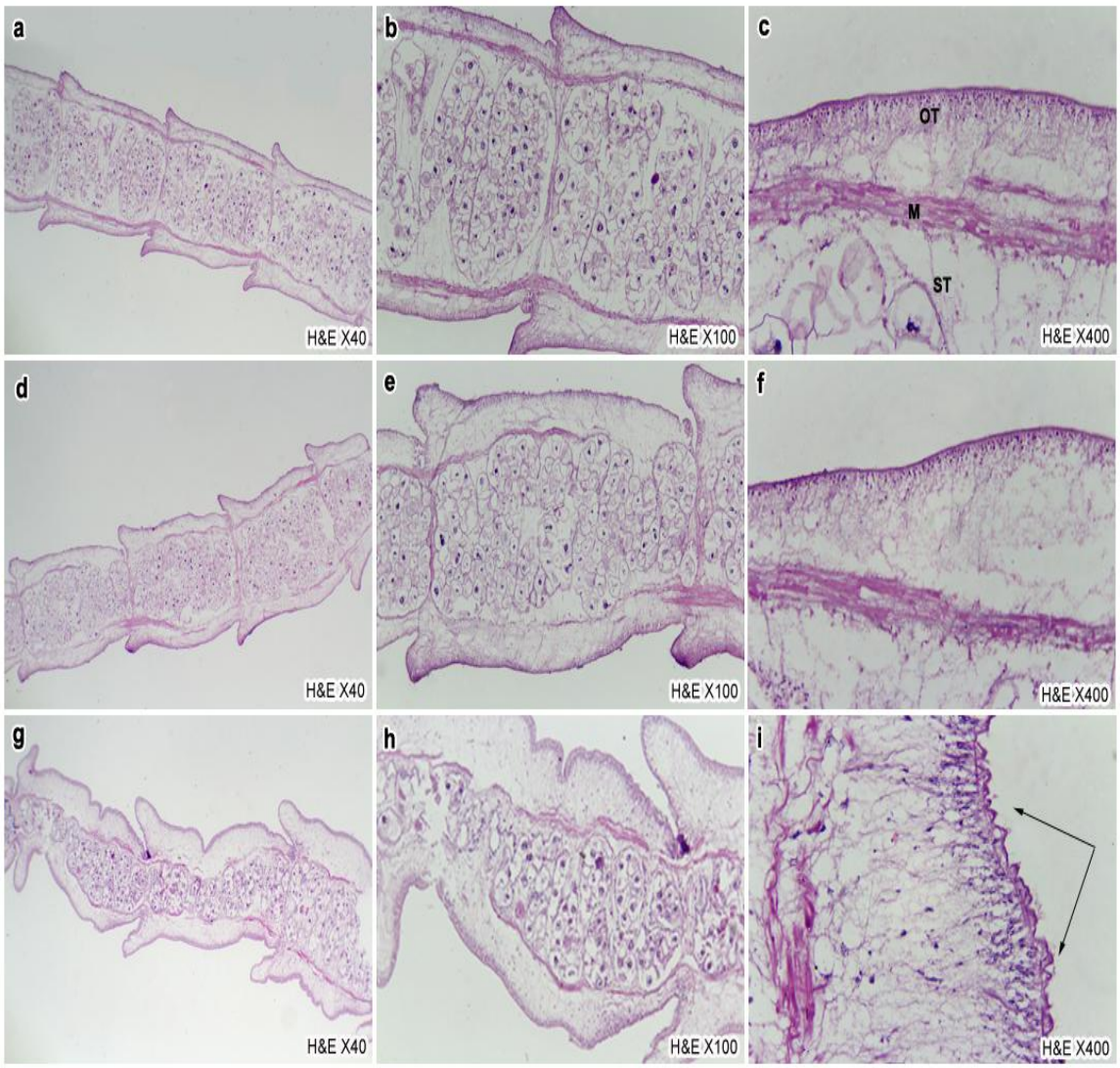
Fig. 2 Light micrographs of the tegument cross section of adult *P. microbothrium*.
(a, b, c) Normal control fluke. (d, e, f) Following 24 h incubation with 30 µg/ml primaquine. Note severe tegumental disruption and sloughing of patches of the outer tegumental layer exposing the basal lamina. The muscle bundles and the parenchymal tissues show severe degenerative changes. (g, h, i) Following 24 h incubation with 10 µg/ml albendazole sulphoxide. Note degenerative changes of the outer tegumental layer. *T* tegument, *BL* basal lamina, *M* muscular layer, *TC* tegumental cell, *P* parenchyma

Fig.3 Scanning electron micrographs of the anterior end of adult *M. expansa*
(a) Normal control worm showing a globular scolex provided with four oval suckers and smooth tegument. (b) Following 24 h incubation with 30 µg/ml primaquine.The scolex becomes more swollen than the control with narrowing of the sucker's opening and corrugation of the tegumental surface. (c) Following 24 h incubation with

10 µg/ml albendazole sulphoxide. The scolex is swollen with severely folded tegument around the suckers and contraction of their openings.

Fig.4 Scanning electron micrographs (SEMs) of the adult *P. microbothrium*.
(a, b, c) Normal control fluke. (a) SEM of the anterior end showing transversely elongated oral sucker. (b) SEM of the posterior end showing the acetabulum. (c) SEM of the mid-body region of the fluke's body exhibiting smooth transverse folds alternating with grooves. (d, e, f) Following 24 h incubation with 30 µg/ml primaquine.(d, e) SEMs of the anterior and posterior ends reveal distortion of both oral sucker and acetabulum as well as a layer of syncytail secretion overlying the surface of the tegument throughout the posterior region around the acetabulum. (f) SEM of the mid-body region showing neat rounded holes (*inset)*. (g, h, i) Following 24 h incubation with 10 µg/ml albendazole sulphoxide. (g) SEM of the anterior end showing a number of pits caused by rupture of papillae (*inset*). (h) SEM of the acetabulum exhibiting distortion of the tegument texture. (i) SEM of the mid-body region exhibiting severe blebbing (*inset*).

Figure 1



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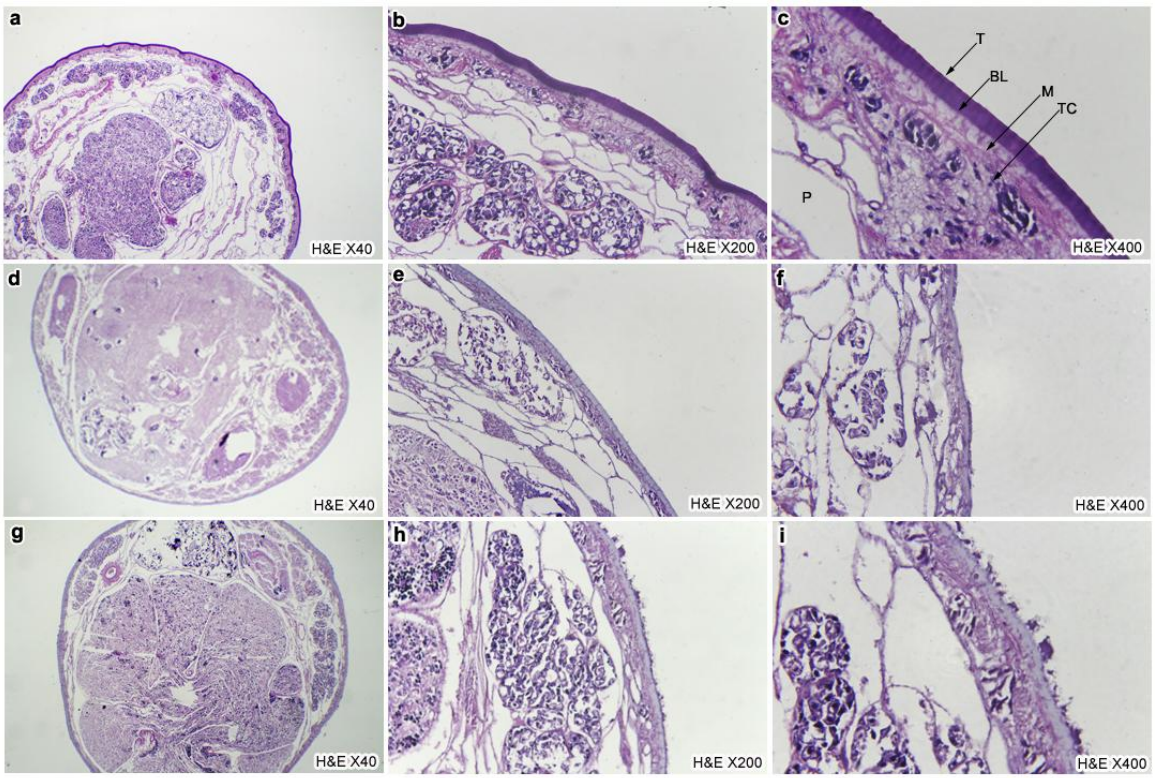


Figure 3

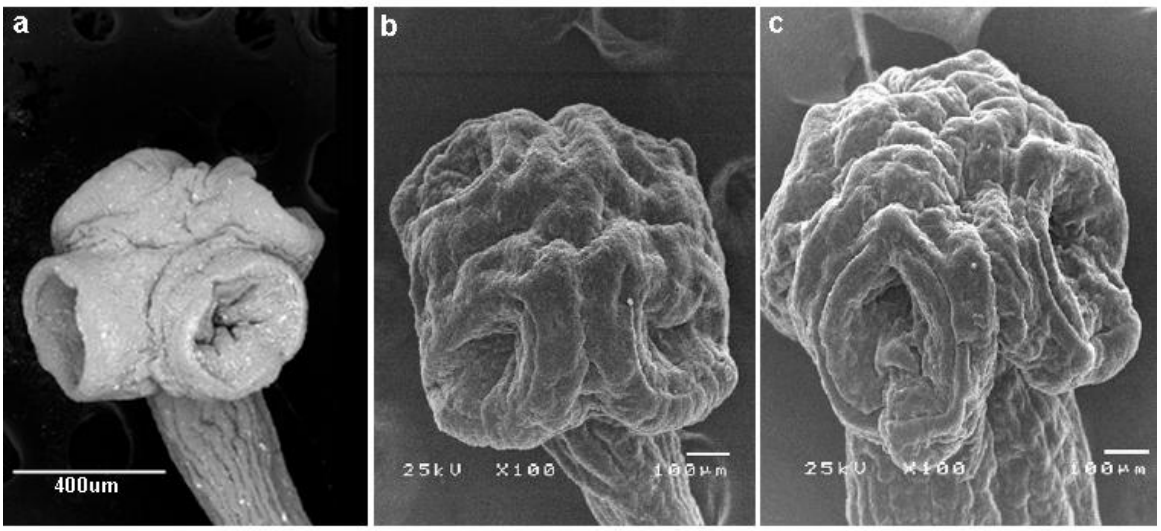
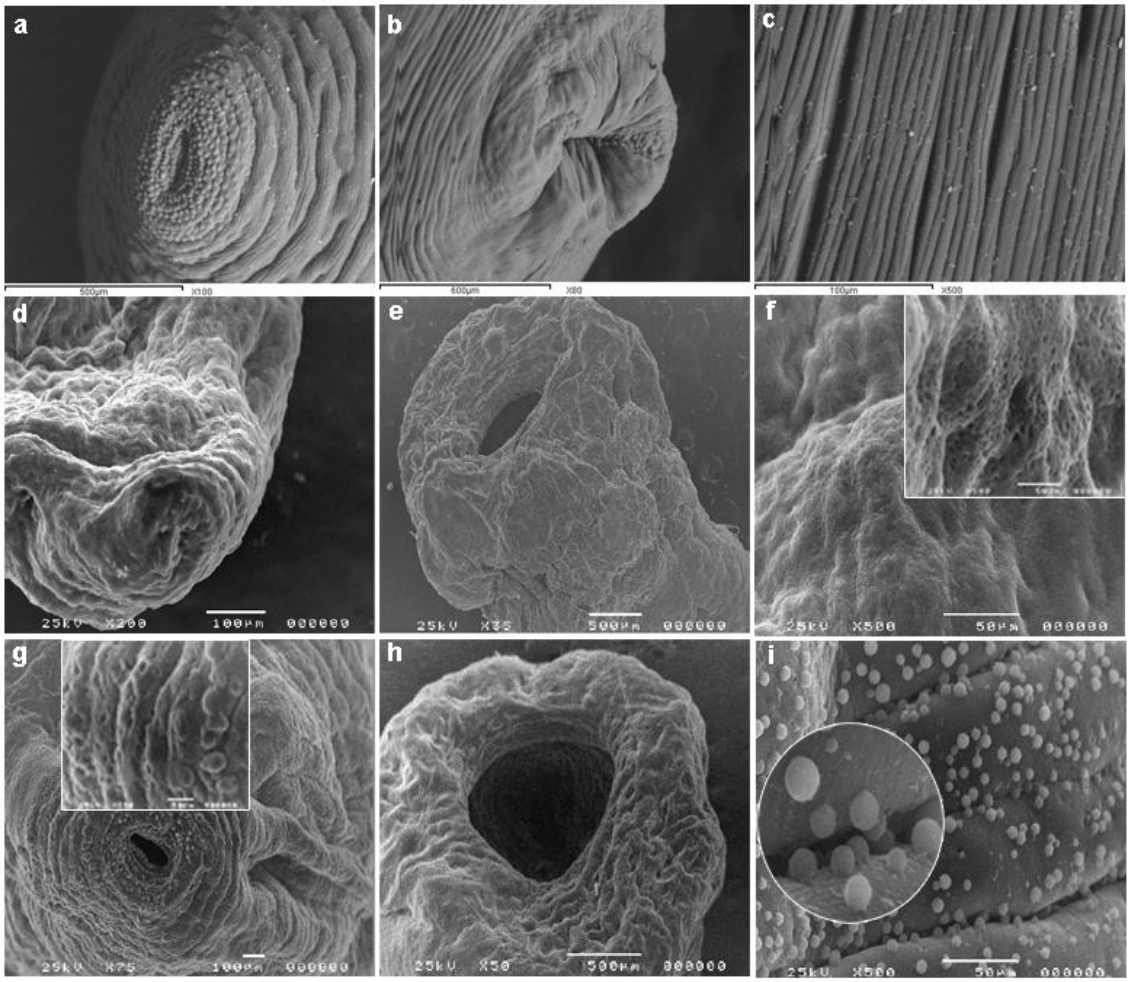


Figure 4



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