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In vitro Effects of Praziquantel on *Fibricola seoulensis*

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= Abstract = *In vitro* effects of praziquantel on *Fibricola seoulensis* (Trematoda: Diplostomatidae) viability and tegumental and/or parenchymal layer changes were studied by light and scanning electron microscopy after exposing the worms to 0.01-100 μg/ml drug concentrations up to 24 hours. The metacercariae (diplostomula) were obtained from the snake, *Natrix tigrina lateralis*, and adult worms from albino rats 7-9 days after experimental infection.

From the view point of worm viability, the lowest effective lethal concentration of praziquantel was 0.1 μg/ml. In 1, 10 and 100 μg/ml concentrations rapid contraction and immediate death of worms were observed. Severe tegumental and subtegumental changes were recognizable by light and electron microscopy. These changes consisted of bleb formation followed by rupture and loss of surface integrity, and deformity of body configuration. The surface destruction was more pronounced at ventro-lateral margins of the forebody and dorsal junctional area between fore-and hindbody. Widening of the distance oral and ventral suckers was also a prominent feature. Tegumental spines and sensory papillae were least affected.

Key Words: ***Fibricola, Diplostomatidae, Praziquantel, In vitro effects, Bleb formation, SEM***

INTRODUCTION

Fibricola seoulensis is one of the intestinal flukes of man and animals in Korea. This fluke was first found in small intestines of house rats in Seoul and described as a new species (Seo *et al.* 1981). Frogs and snakes are the second intermediate hosts (Hong *et al.* 1982). Human infection was proven in 16 cases among snake eaters (Seo *et al.* 1982; Hong *et al.* 1984). Since further cases may occur study of chemotherapeutic agents is necessary.

Praziquantel, an acetylated isoquinolino-pyrazine, appears to be the most appropriate anthelmintic to use, since it is known to be highly effective in treatment of many kinds of trematode and cestode infections (Gönnert *et* Andrews 1977; Thomas *et* Gönnert, 1977; Rim *et al.* 1981a & b; Andrews *et al.* 1983; Lee 1984). Although the exact mechanism of worm death is not clearly understood, two striking phenomena have been observed in the worms exposed to praziquantel (Andrews *et al.* 1983), namely, the immediate tetanic contraction of worm musculature and rapid vacuolization of the syncytial tegument.

It has been reported that praziquantel treatment is effective in worm expulsion of *F. seoulensis* in humans (Hong *et al.* 1984), however, no study has been performed on *in vitro* effects. This study was undertaken in order to observe, by means of light and scanning electron microscopy, the *in vitro* effects of praziquantel on the viability and morphological features of *F. seoulensis* worms.

MATERIALS AND METHODS

1. Preparation of *F. seoulensis* Worms: Approximately a thousand of *F. seoulensis* adults, aged 7-9 days, were recovered from small intestine of two albino rats (Wistar strain) experimentally fed with 1,000 metacercariae (diplostomula) each. The metacercariae were obtained from the viscera of the snake, *Natrix tigrina lateralis,* purchased from snake collectors in Hoengseong-gun, Kangweon-do in June-July 1984. Procedure of metacercarial-isolation was the same as described by Hong *et al.* (1983).

2. Exposure of Worms to Praziquantel: The worms collected from experimental rats were

washed two times with phosphate-buffered saline and incubated at 37°C in small pertri dishes containing 7-10 ml of Tyrode solution and various concentrations (0.01, 0.1, 1, 10, 100 μg/ml) of praziquantel (Distocide®). The actively moving worms were incubated in each petri dish up to 24 hours (Table 1). Ten worms incubated in Tyrode solution not containing praziquantel were used for controls.

3. Observation of Drug Effects on Worms: The effects of praziquantel on *F. seoulensis* were observed by measuring the extent of worm activity after incubation with the drug, by light microscopy on the tegument and internal organs, and by scanning electron microscopy on the whole teguments.

Activity of worms in each petri dish containing praziquantel was checked at various intervals (5, 15, 30 minutes, 1, 2, 4, 6, 12 and 24 hours after exposure) and the worms were categorized as very active, moderately active, slightly active and dead (Table 1). The worms with no movement, widely distended their forebody in contrast to constricted hindbody, and with no reaction to pinching with a sharp pointed pin, were regarded as dead.

Light microscopic changes were observed 30 and 60 minutes after incubation on fresh and acetocarmine stained whole worms, as well as on their sectioned preparations. Fresh preparation was made by direct mounting of the worm form petri dish and worm sections made by routine procedure and stained with hematoxylin and eosin. Ultrastructural changes on the tegument of *F. seoulensis* were observed 5, 15, 30 and 60 minutes after incubation. The worms were washed three times with phosphate-buffered saline, fixed in cold 2.5% glutaraldehyde solution (pH 7.4) and postfixed in 1% osmium tetroxide. They were dehydrated, gold-coated, and observed with ISI SS-60 scanning electron microscope at 10 KV.

RESULTS

1. Viability of *F. seoulensis* after Exposure to Praziquantel: Praziquantel appeared to be very effective in killing adult worms of *F. seoulensis in vitro* especially with higher concentrations than 0.1 μg/ml (Table 1). In 1, 10 and 100 μg/ml solutions immediate death of worms occurred. After exposure to these concentrations, the worms, rapidly and strongly contracted their fore-and hindbody. This was followed by marked distension of the forebody. On death, the hindbody became narrower and nearly cylindrical in appearance, and the worms revealed no response to physical stimuli.

In 0.1 μg/ml concentration of praziquantel, the worms showed slightly active movements up to 15 minutes post incubation. Thereafter, however, they revealed no more movement and showed signs of death. By comparison, in 0.01 μg/ml solution, the worms appeared to be very active up to 30 minutes, but became less active after 1-6 hours, and showed only slight movements after 12-24 hours. With this concentration, worm death was hardly seen up to 24 hours of incubation. Control worms were consistently active until 24 hours of incubation. Control worms were consistently active until 24 hours in Tyrode solution, though some showed a little reduced movement after 12 hours. The lowest effective concentration of praziquantel to kill the worms *in vitro* is considered to be 0.1 μg/ml.

2. Light Microscopic Changes in *F. seoulensis* after Exposure to Praziquantel: The worms in control group (acetocarmine stained) showed their distinct bisegmented body (Fig. 1) and measured an average of 1.23 mm length and 0.66 mm width. The forebody was broad, tapering anteriorly and

Table 1. Activity of *F. seoulensis* after incubation with various concentrations of praziquantel

Drug concentr. (μg/ml)	Incubation time								
	5 min.	15 min.	30 min.	1 hr.	2 hr.	4 hr.	6 hr	12 hr.	24 hr.
100	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−
1	−	−	−	−	−	−	−	−	−
0.1	+	+	−	−	−	−	−	−	−
0.01	+++	+++	+++	++	++	++	++	+	+
control	+++	+++	+++	+++	+++	+++	+++	++	++

+++:very active ++:moderately active
+:slightly active −:dead

having oral sucker (OS), ventral sucker (VS) and tribocytic organ (TO). The hindbody was narrower than the forebody and had an ovary (OV), two butterfly-shaped testes (T) and uterus containing eggs. The texture of tegumental surface was grossly smooth but covered with fine, scale-like spines from anterior end to proximal one-third level of hindbody. The sectioned worm showed oral sucker, tribocytic organ, intestine, ovary, testes and other intact parenchymal portions.

In vitro incubated *F. seoulensis* in media containing 1-100 μg/ml of praziquantel for 30 and 60 minutes showed severe tegumental and subtegumental changes (Fig. 2-5). In the specimens incubated in 1 μg/ml concentration, numerous blebs, relatively small in size, were seen on the tegument under 200x magnification. In general, they were more abundant in forebody than in hindbody, and their number and size increased as incubation time was prolonged. In 10 or 100 μg/ml concentration, larger-sized blebs or vacuoles appeared on the tegument and within the subtegumental layers (Fig. 2, 3 and 4). These changes were more pronounced at lateral margins of forebody, where extensive tegumental deformity followed (Fig. 2). The deformity was characterized by marked surface elevation and loss of tegumental integrity. Tegumental changes in hindbody, chiefly bleb formation, were severe in the specimens incubated in 100 μg/ml praziquantel solution. The vacuoles, formed in subtegumental parenchymal portions, were demonstrated both in fresh-mounted (Fig. 3) and paraffin-sectioned preparations (Fig. 5).

The sectioned worms revealed poor stainability by hematoxylin and eosin, indicating a certain change in the property of internal organs. However, in fresh-mounted worms, no special change was grossly recognizable in internal organs including male and female genital organs. In all worms exposed to praziquantel severe widening of distance between oral and ventral suckers was noted.

3. Scanning Electron Microscopic Changes in *F. seoulensis* after Exposure to Praziquantel:

The scanning electron microscopic view of normal *F. seoulensis* revealed typical bisegmented body with smooth tegumental surface, sharply demarcated and ventrally curved lateral margins of forebody, oral sucker (OS), ventral sucker (VS) and tribocytic organ (TO) (Fig. 6: Seo *et al.*, 1984).

The tegumental change in *F. seoulensis* after exposure to praziquantel was characterized by formation of numerous blebs, variable in size, followed by rupture of them and alteration of tegumental integrity (Fig. 7-22). This was consistently observed in the worms exposed to drug concentrations higher than 0.1 μg/ml concentration. The tegumental blebs were formed as early as 5 minutes after exposure. At this time the blebs were most abundant in the dorsal surface of the junctional area between fore-and hindbody (Fig. 7 and 18). Though less in number, they were also found from the ventral and dorsal surfaces of both fore- and hindbody (Fig. 7, 12, 15 and 19). Oral and ventral suckers were intact, but the tribocytic organ showed several ruptured blebs on its surface (Fig. 15). Despite these changes at 5 minutes, the sensory papillae and tegumental spines were completely intact (Fig. 12 and 19) and general body configuration nearly unaffected (Fig. 7).

At 15 minutes after incubation of worms with praziquantel, the tegumental blebs on worms increased in number and size. Increase of bleb size and number was especially pronounced at ventral surface between oral and ventral suckers. Probably due to destruction of tegument and subtegument at this portion, the distance between oral and ventral suckers were widened and anterior end of body greatly flexed and folded ventralwards (Fig. 8). The lateral margins of forebody were also widened, becoming thinner, folding to the ventral side, and loosing their sharp and smooth tegumental texture (Fig. 8). The blebs in the dorsal junctional area between fore-and hindbody also increased in their number and size. Even at this time the sensory papillae and tegumental spines remained to be unaffected.

It was at 30 or 60 minutes after exposure when tegumental destruction due to rupture of blebs and crater formation became most extensive. However, unruptured blebs were also scatteredly seen around oral sucker (Fig. 11), ventral sucker (Fig. 14), and between the suckers (Fig. 13). Rupture of blebs and destruction of tegumental surface were found both in fore-(Fig. 16 and 17) and hindbody (Fig. 22). However, they were more frequently found along the ventro-lateral margins of forebody (Fig. 17). Oral and ventral suckers were seen to be grossly intact at this time, but in one worm, the intestinal content of the worm was found to have been regurgitated through oral sucker (Fig. 9). It was also observed that the inner part of the tribocytic organ protruded ventrally exposing its internal surface contour (Fig. 9). Generally the tegumental

areas bearing many spines were less affected by the drug in comparison to aspinous portions. The sensory papillae were also one of the least affected structures.

The worms exposed to 100 μg/ml praziquantel revealed a much distorted forebody (Fig. 10). The margins of forebody became thinner in appearance and severely folded ventralwards. However, it was notable that bleb formation was not marked in these worms (Fig. 10). With this durg concentration, the blebs were rather abundant at the dorsal portion of hindbody (Fig. 20).

DISCUSSION

The present study confirms that praziquantel is highly effective in killing adult worms of *F. seoulensis in vitro* with as low a concentration as 0.1 μg /ml. The flukes severely contracted their musculature and became paralyzed almost instantaneously after exposure to drug. Subsequently they relaxed their forebody which was already deformed and distorted. On other trematodes such as *Schistosoma mansoni, Clonorchis sinensis, Opisthorchis viverrini,* the drug has shown similar effects both *in vitro* and *in vivo* (Rim *et al.* 1982; Xiao *et al.* 1984; Sirisinha *et al.* 1984).

The effect of praziquantel on the morphological integrity of trematodes and cestodes has been known to be the formation of vacuoles or blebs on the tegument followed by rupture and crater formation (Becker *et al.* 1981; Mehlhorn *et al.* 1983; Sirisinha *et al.* 1984). This study generally agrees with the above reports. The origin of blebs is not clearly understood. Some workers believe that the blebs originated from near the basal lamina of tegumental cells (Becker *et al.* 1981; Mehlhorn *et al.* 1983) while others suggested the origin to be the nerve bulb cells beneath the sensory papillae (Rim *et al.* 1980; Kim *et al.* 1982). In the present study, the blebs were formed on the tegument and as well in the parenchymal layers. The tegumental blebs appeared to be independent of the sensory papillae. In *in vivo* experiment with *F. seoulensis* recovered from albino rats 1 hour after treatment with 10 mg/kg praziquantel (Cha 1985), the blebs were also found from the deeper layer of worms. It is suggested that rapidly after absorbing praziquantel through the tegument the cytoplams of tegumental cells become vacuolized and the vacuoles protrude onto the tegumental surface. The subtegumental and parenchymal cells also become vacuolized and destroyed by subsequent entrance of the durg.

Both the phenomena of muscle contraction and bleb formation in the worms exposed to praziquantel is known to be calcium dependent. This is especially true in schistosomes. Incubation of *S. mansoni* in calcium-depleted media blocked the action of praziquantel on worm musculature (Pax *et al.* 1978) and inhibited bleb formation on the tegument (Xiao *et al.* 1984). It was also demonstrated by Ruenwongsa *et al.* (1983) that praziquantel increased the permeability of *O. viverrini* tegument to calcium binding or transport across the membrane. However, it has not been elucidated whether increased uptake of calcium by parasites is directly related to their muscle contraction and vacuolization in the tegumental layer. On muscle contraction, Andrews *et al.* (1983) suggested that possibly the movements of calcium between compartments within parasites themselves could be responsible. Sirisinha *et al.* (1984) were of the opinion that the surface alteration due to bleb formation may be a non-specific, secondary change to other mechanism of action of praziquantel. They had noted that similar tegumental changes occurred when *O. viverrini* was exposed to fresh nomal human sera.

An interesting finding in this study was that regurgitated intestinal content was clearly visible in one worm exposed to 10 μg/ml concentration for 15 minutes. Considering this, it seems probable that a significant proportion of praziquantel is taken up orally by *F. seoulensis*. In this respect, *in vivo* study by Cha (1985) demonstrated the enlarged intestinal lumen and obliterated luminal surface. However, the effect of praziquantel on intestinal tract of trematodes should be further studied.

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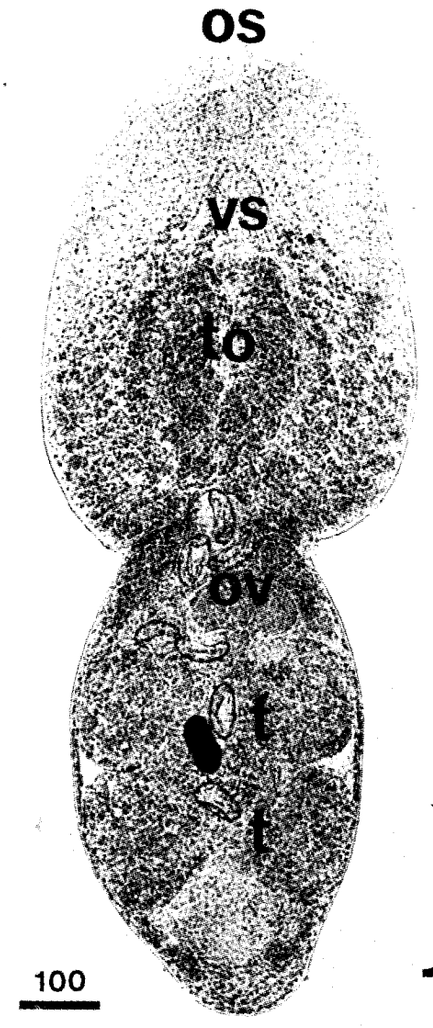
EXPLANATIONS FOR FIGURES

Fig. 1. *F. seoulensis,* an adult worm of normal control group showing oral sucker (os), ventral sucker (vs), tribocytic organ (to), ovary (ov), testes (t) and smooth tegumental surface. Acetocarmine stain.
Fig. 2. Anterior body of *F. seoulensis* incubated in 10 μg/ml praziquantel solution for 30 minutes. Note the destroyed tegumental surface (arrows) due to ruptured blebs. Fresh-mount.
Fig. 3. Magnification of Fig. 2, showing subtegumental blebs (arrows).
Fig. 4. Large blebs (arrows) protruded onto the tegumental surface of anterior body (100 μg/ml, 60 min.). Fresh-mount.
Fig. 5. A paraffin-sectioned worm (10 μg/ml, 30 min.). Note the vacuolized lateral margins (arrows) of anterior body as well as subtegumental and parenchymal layers. H-E stain.
Fig. 6. Scanning electron microscopic view of normal *F. seoulensis* showing oral sucker (OS), ventral sucker (VS), tribocytic organ (TO), its characteristic body configuration and smooth tegumental texture (See *et al.,* 1984).
Fig. 7. Dorsal view of *F. seoulensis* incubated in 10 μg/ml praziquantel solution for 5 minutes. Note the contracted fore-and hindbody, less smooth tegumental surface and numerous blebs (arrows) on junctional area between the body segments (Magnified in Fig. 18).
Fig. 8. Ventral view of forebody (10 μg/ml, 15 min.). Note widened distance between oral and ventral suckers, and ventral folding there. Irregularly wrinkled lateral margins are conspicuous.
Fig. 9. Another worm after prolonged exposure for 1 hour. Through oral sucker the intestinal content was regurgitated (arrow). Severe destruction and loss of tegumental integrity are prominent.
Fig. 10. A more severely deformed worm (100 μg/ml, 30 min.). Several blebs are seen around oral sucker.
Fig. 11. Tegumental blebs formed around oral sucker of *F. seoulensis* (10 μg/ml, 30 min.).
Fig. 12. *Ibid.* Note that the sites of blebs (arrow) are independent of sensory papillae, which are remained intact.
Fig. 13. Tegumental blebs on ventral surface between oral and ventral suckers (10 μg/ml, 30 min.).
Fig. 14. Grossly intact ventral sucker (10 μg/ml, 30 min.). But adjacent areas show blebs.
Fig. 15. Blebs formed around tribocytic organ, where normally there are very few sensory papillae (10 μg/ml, 5 min.).
Fig. 16. Two ruptured blebs near tribocytic organ (10 μg/ml, 30 min.).
Fig. 17. Crater formation on the tegument by ruptured blebs (10 μg/ml, 1 hour). Lateral margin of forebody.
Fig. 18. Blebs formed in dorsal junctional area between fore-and hindbody (Magnification of Fig. 7).
Fig. 19. Two blebs formed on a spinous area of mid-dorsal portion of forebody (10 μg/ml, 5 min).
Fig. 20. Numerous blebs, variable in size, formed on the dorsal junctional area between body segments (100 μg/ml, 30 min.).
Fig. 21. *Ibid,* on the dorsal proximal area of hindbody (10 μg/ml, 15 min.).
Fig. 22. Tegumental deterioration in the dorsal area of hindbody (10 μg/ml, 30 min.). Note a ruptured bleb and rough surface texture.

*All scales are given in microns.



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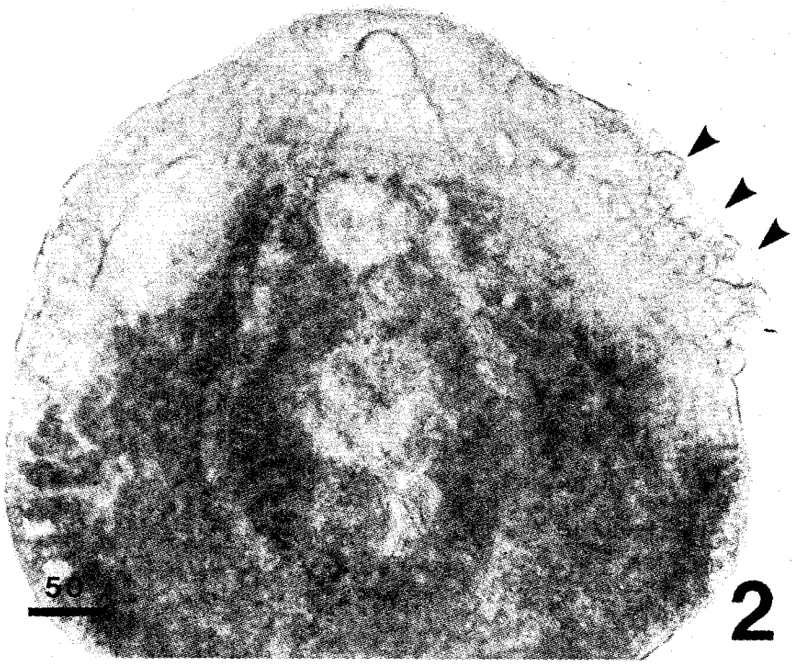

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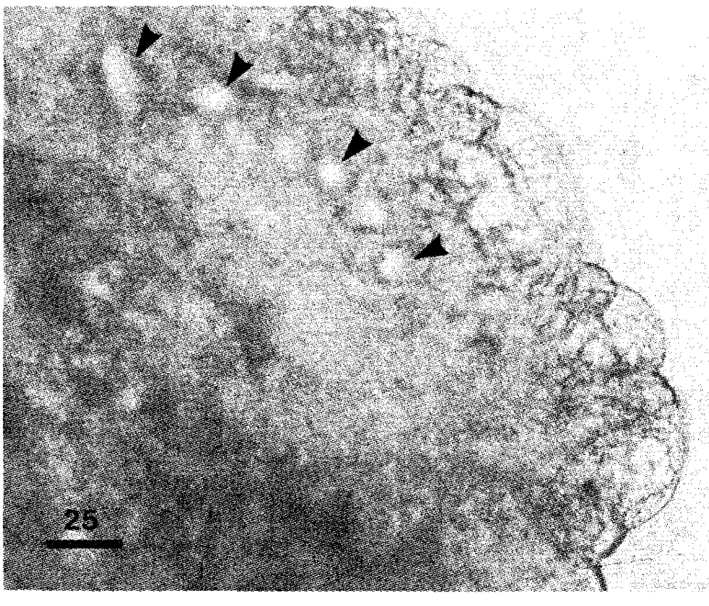

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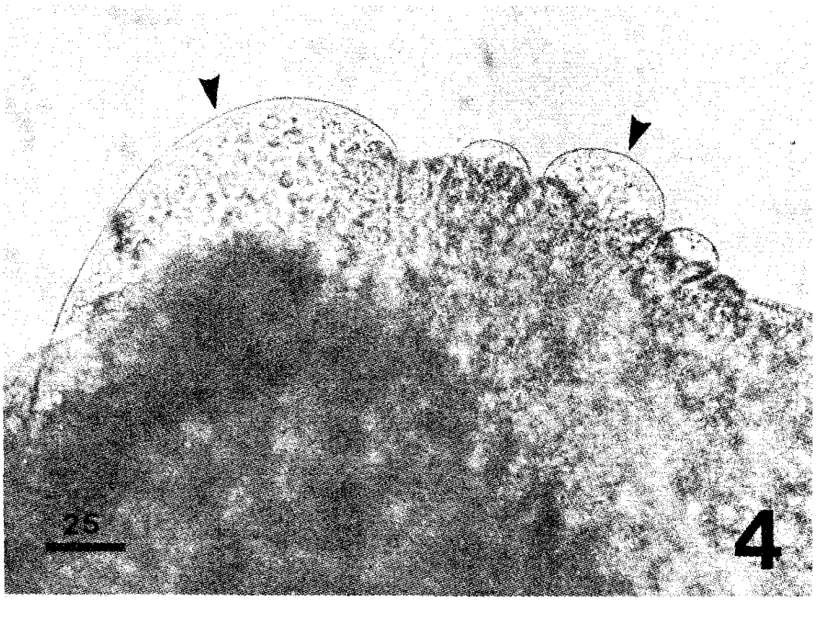

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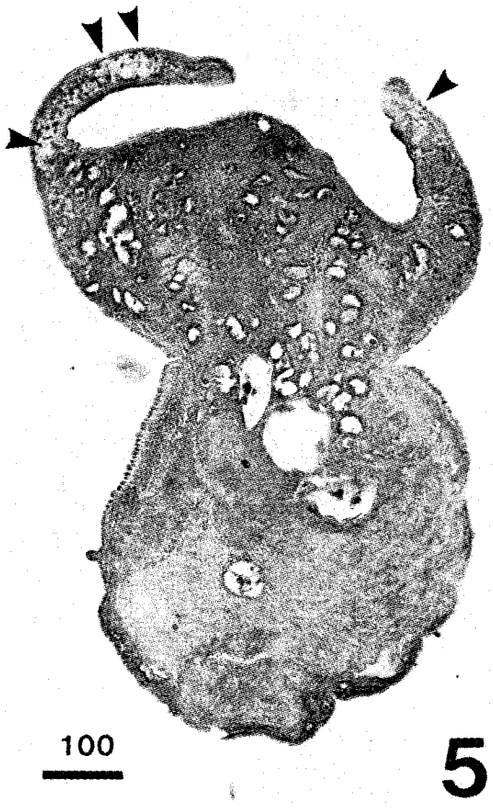

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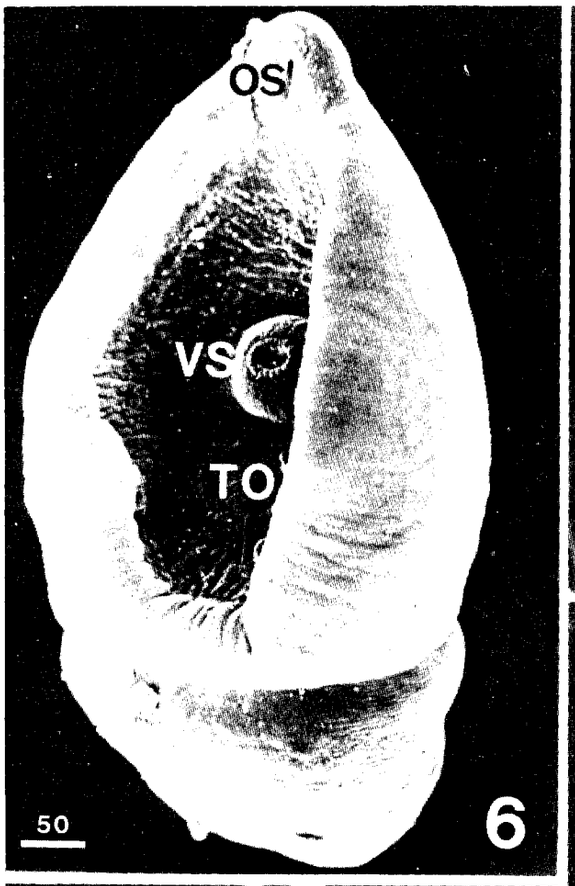
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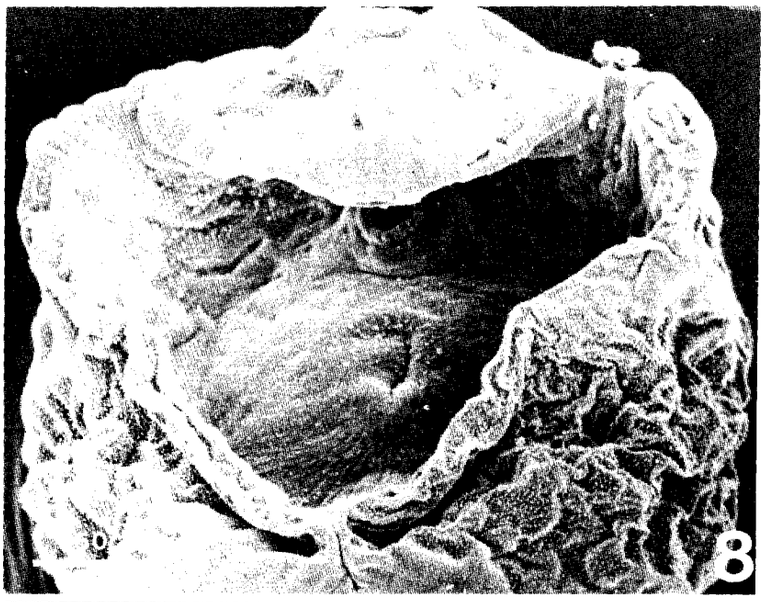
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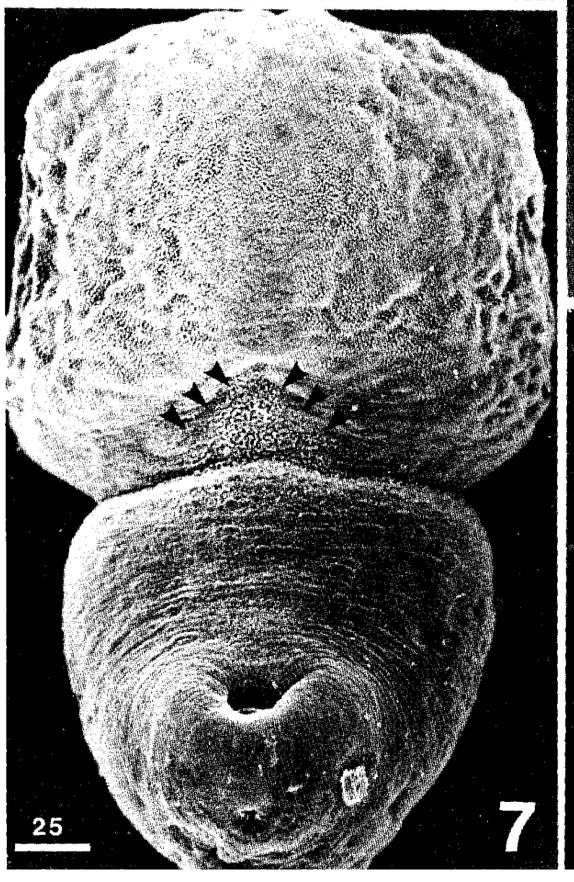
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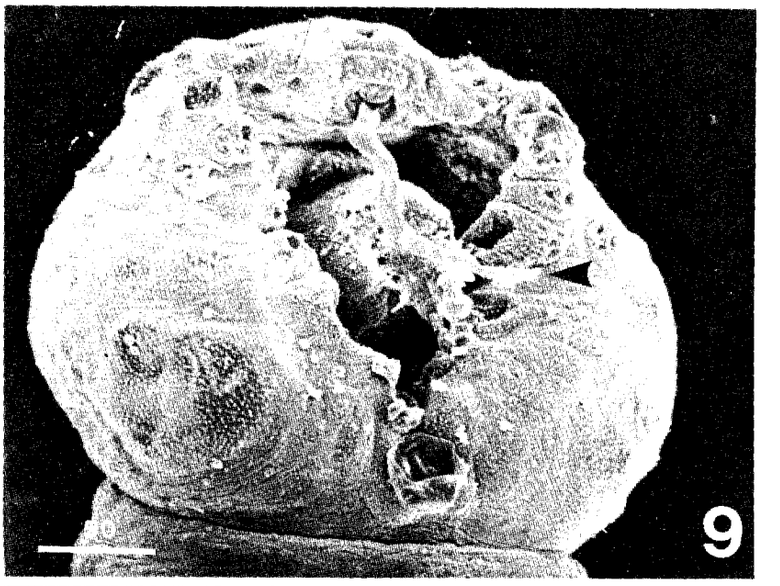
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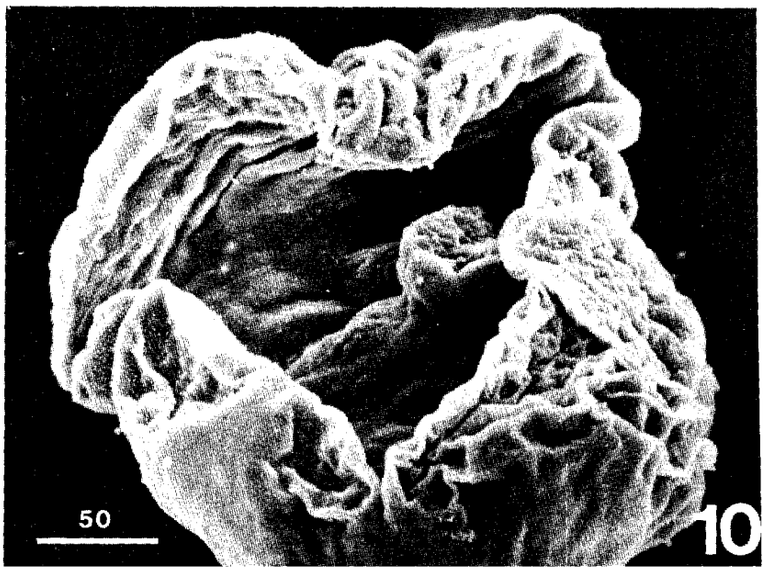
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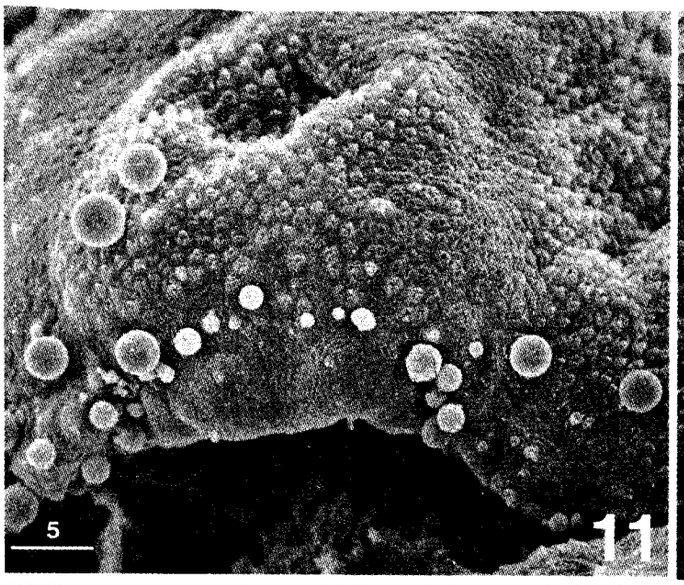
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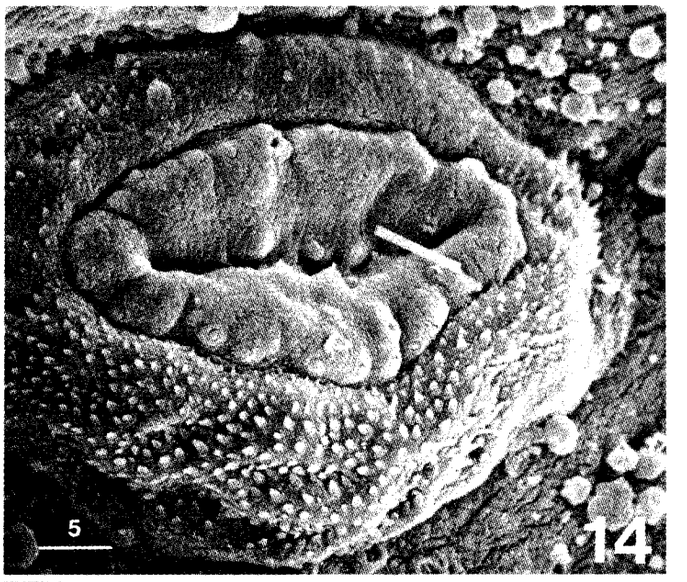
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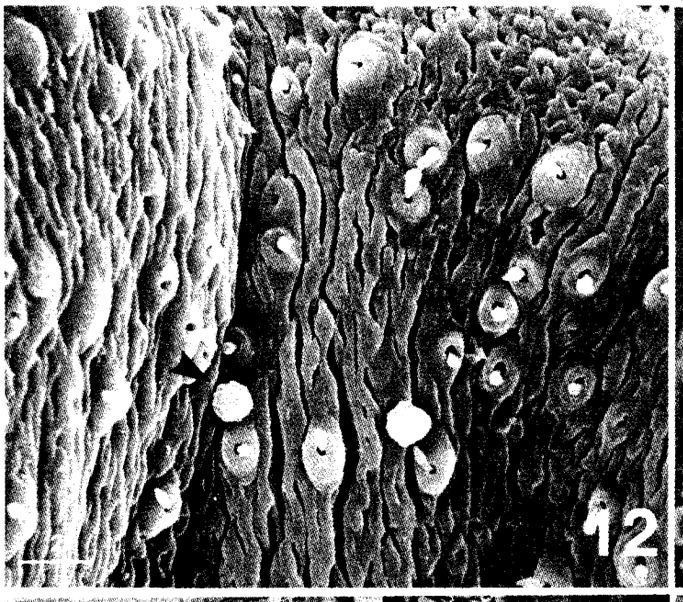
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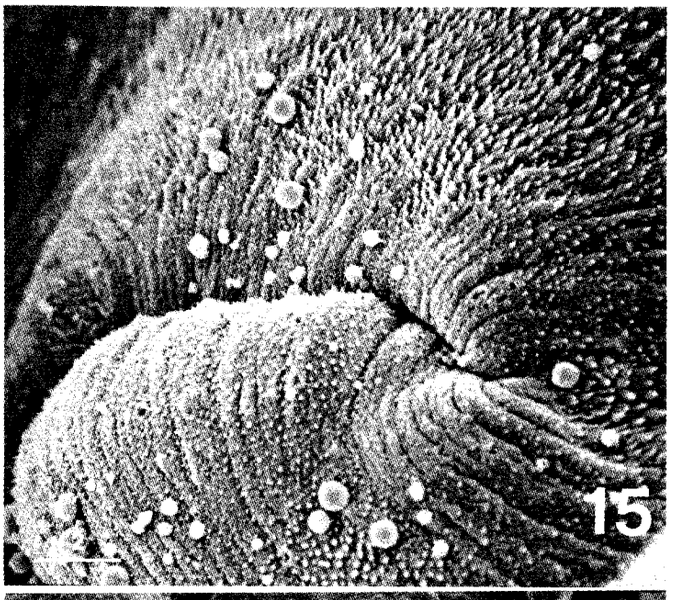
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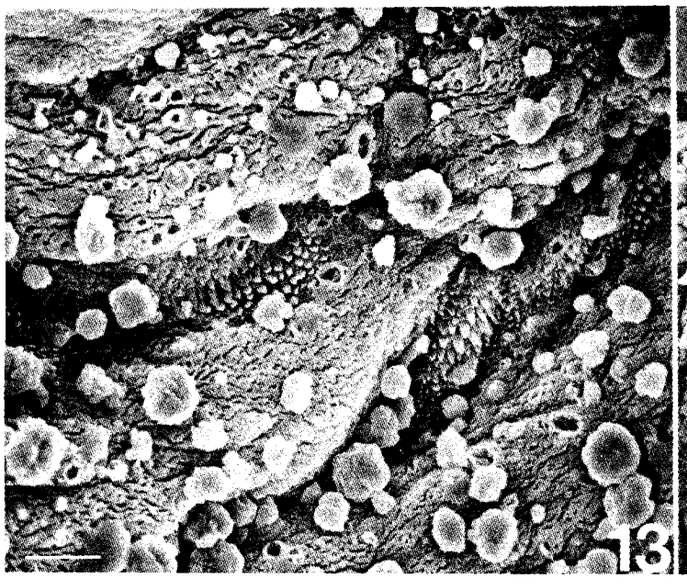
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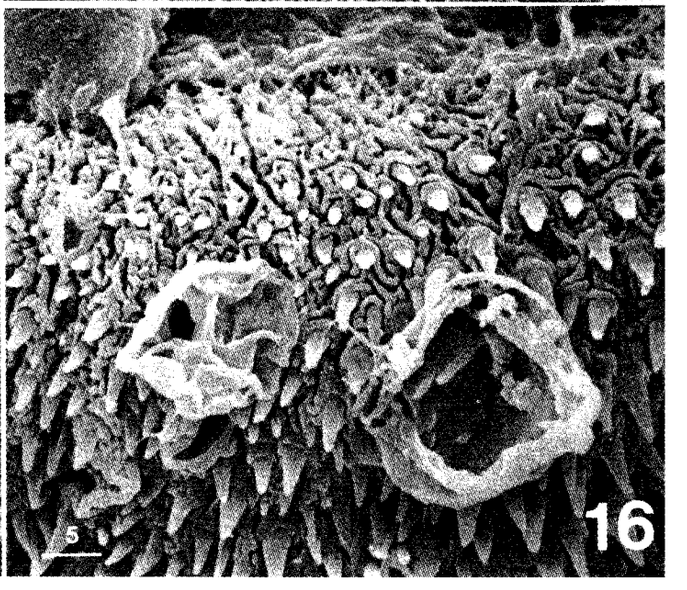
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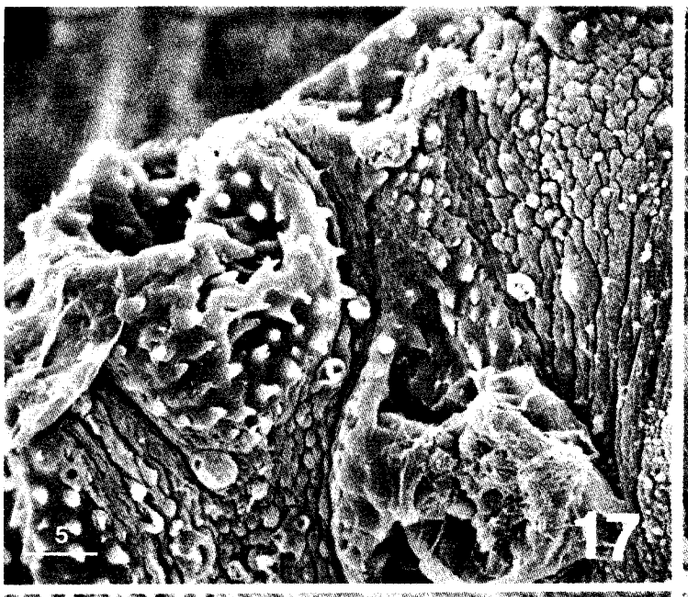
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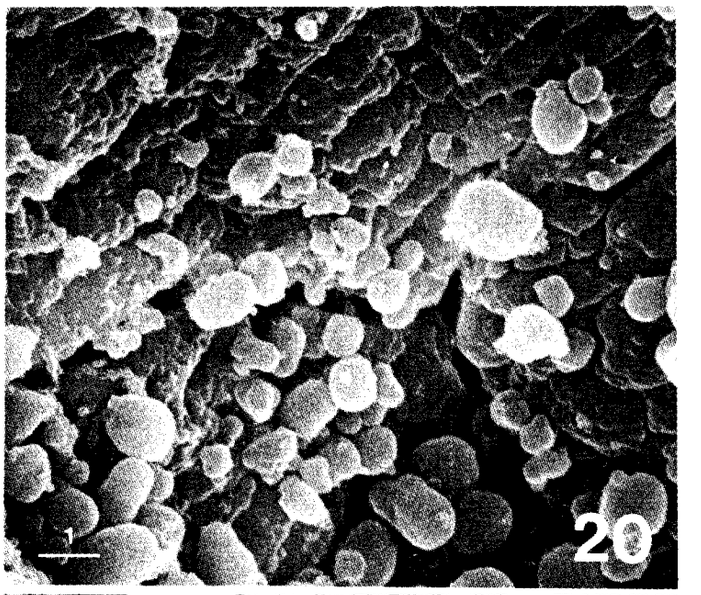
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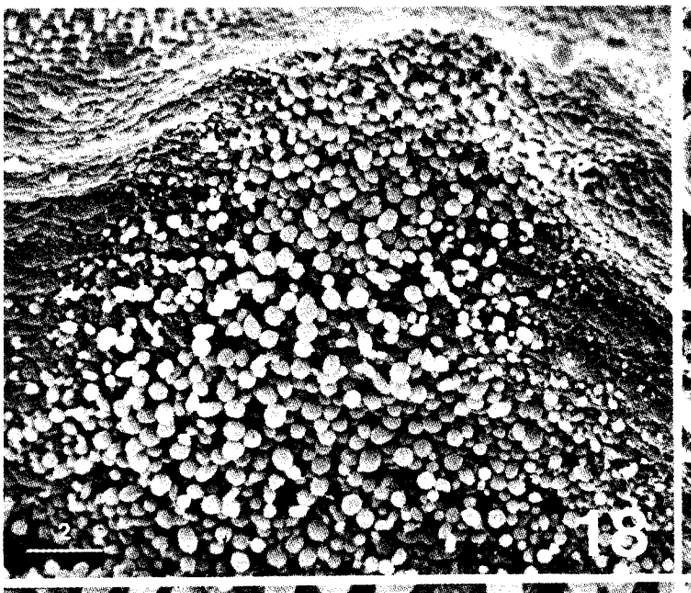
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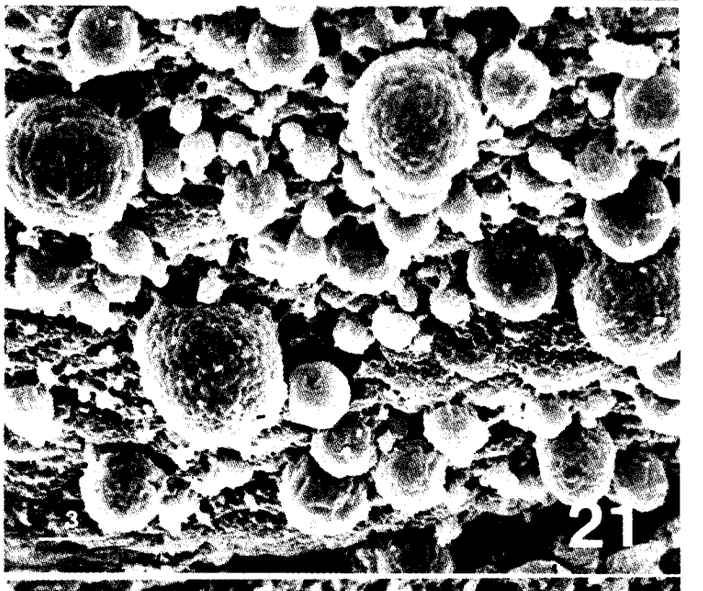
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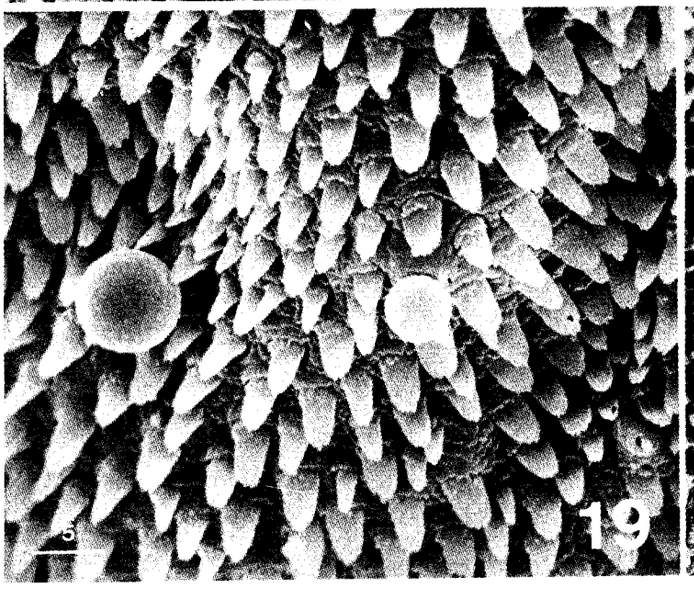
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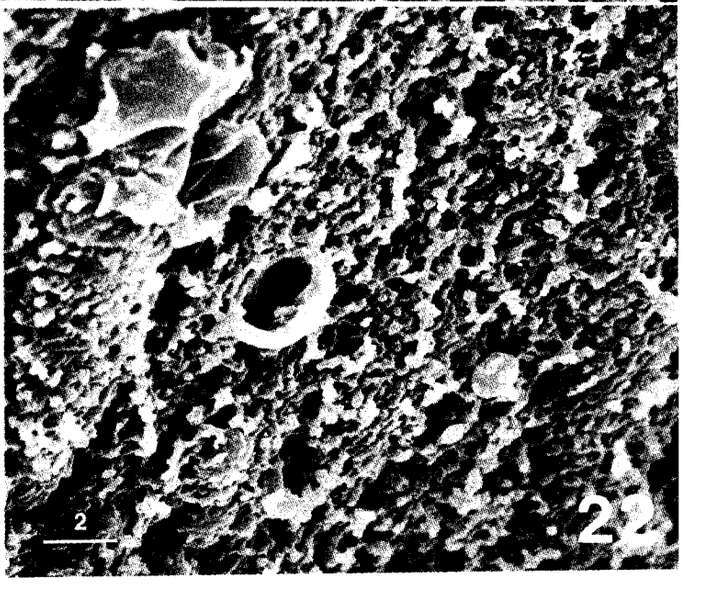
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＝ 국문초록 ＝

*Fibricola seoulensis*에 대한 Praziquantel의 試驗管內 作用

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李　純　炯

Praziquantel(디스토시드)이 시험관내에서 *Fibricola seoulensis*에 대하여 어떻게 作用하며 殺蟲效果는 어떤지를 알아보고자 이 硏究를 시행하였다. 蟲體는 뱀에서 분리한 被囊幼蟲을 感染시킨 흰쥐에서 7～9일 成長한 것을 回收하여 사용하였고 0.01－100 μg/ml 여러가지 농도의 praziquantel이 들어있는 배양액(Tyrode solution)에 넣고 24시간동안 경시적으로 蟲體의 死滅여부와 光學 및 電子顯微鏡的인 蟲體形態의 변화를 관찰하였다.

결과는 다음과 같다.

1. 殺蟲效果를 나타내는 praziquantel의 최저농도는 0.1 μg/ml이었고 접촉 30分後 모든 蟲體가 死滅하였다. 그 이상의 농도에서는 즉시 蟲體가 수축하고 死滅하였다.

2. 光學 및 電子顯微鏡으로 관찰한 蟲體形態의 변화로서는 表皮(tegument) 및 表皮下層(subtegumental layer)의 심한 水胞形成(bleb formation)과 水胞의 파열 등으로 인한 表皮 파괴, 변성 등 蟲體 外形의 변화가 가장 특징적이었다. 表皮의 파괴는 蟲體表面에 모두 일어났으나 특히 體前半部(forebody)의 外側(주로 腹面)과 前後半部 이행부위의 背面에서 가장 뚜렷하였다. 口吸盤과 腹吸盤의 거리가 이완되어 확장된 것도 특이한 現象의 하나이었다. 그러나 皮棘(tegumental spines)이나 感覺乳頭(sensory papillae)는 表皮構造物중 가장 적게 손상되는 것으로 나타났다.