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PREVENTIVE ACTION OF FUMAGILLIN ON THE DEGREE OF INFECTION WITH *Nosema apis* IN THE DIGESTIVE TRACT OF BEES

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The presence of Nosema apis in the digestive tract of bees and the preventive effect of fumagillin on their appearance were investigated pathohistologicaly. Three groups of bees were formed. Group A was artificially infected with N.apis spores in sugar syrup. In parallel with the infection the bees of Group B - were administered sugar syrup containing 0.015 g Fumagillin DCH. Group C was not artificially infected and served as a control. Organs such as the crop, midgut, small intestine, Malpighian tubes, rectum, and rectal papillae were taken from all three groups 4, 9, 12, and 16 days following artificial infection.

It was established that Nosema apis parasites were localized exclusively in the epithelial cells of the midgut and that fumagillin had a preventive effect by slowing down parasite development and stabilizing the system of peritrophic membranes.

Key words: nosema disease, prevention, pathohistology, fumagillin.

INTRODUCTION

All over the world, including our country, nosema disease is one of the very common diseases of bees. All adult members of the bee society are susceptible (workers, drones and queen bees).

Bees are infected per/os with *Nosema apis* spores, which sprout in the midgut. The spores release polar filaments, which penetrate into midgut epithelial cells, where they divide intensely and multiply. During histopatho-logical examinations of tissue of artificially infected bees, Bahrmann (1965) studied the midgut, peritrophic membranes, small intestine, Malpighian tubes, rectum and rectal papillae. He detected *N.apis* growth stages and spores in midgut epithelial cells and in orally placed cells of Malpighian tubes. Development stages and spores of *N.apis* in the small intestine were extremely rare, and none were found in epithelial cells of the rectum or rectal papillae. In addition to finding parasites in certain parts of the digestive tract, the author also observed changes in the epithelial cells. Bailey (1981) and Liu (1984), observed this parasite only in epithelial cells of the midgut.

The adequate application of medicines is important for apicultural science and practice because of the vast damage caused by nosema disease. Numerous investigations all over the world have shown that fumagillin was the only substance which met all the required parameters (Furgala and Boch, 1970; Van Steenkiste and Jacobs, 1980; Sugden and Furgala, 1979; Popa, 1962). Sometimes there is a dilemma whether to apply fumagillin preventively or therapeutically. Several authors consider that fumagillin has a preventive effect (Furgala and Gochnauer, 1969; Cmejla, 1955; Benecke, 1968; Gochnauer and Furgala, 1969; Furgala et al., 1973).

While all agree that parasites are found in the midgut, results presented so far show disagreement regarding the presence of N.apis in other parts of the digestive tract. Moreover, there is certain controversy about the preventive effect of fumagillin. Therefore, we decided to determine, the localization of *Nosema apis parasites* in certain tissues following artificial infection of bees with *N.apis* spores and to determine the effect of fumagillin treatment.

MATERIAL AND METHODS

Nosema apis spores

In order to obtain *N.apis* spores, we caught bees from hives of infected societies. They were sacrificed just before maceration and distilled water (1ml) was added to the mortar for each bee abdomen. The macerate with spores was then filtered through double gauze and *N.apis* spores were counted in the filtrate in Spencer's chamber using the method of Cantwell (1970). It was found that there were 4 million spores per bee, i.e. per 1ml of suspension. The spore suspension was stored in a refrigerator at 4^0C and used for artificial infection.

Bees and fumagillin

Healthy (non-infected) young bees *(Apis mellifica carnica)* from one society were taken from frames with an open brood which had larvae up to 4 days old. They were maintained in cages made according to Rothenbuhler and Kulicevic (1973) and there were 30 bees in each cage.

Fumagillin DCH, consistsed of the active substance fumagillin and a carrier (Chinoin-Budapest).

Experimental groups

Three groups of bees were formed marked as A, B and C. Each group had 5 cages of 30 bees each.

Group A. Bees of this group were infected artificially with *N.apis* spores added to sugar syrup. The stored suspension of *N.apis* spores was diluted in a plastic bottle (feeder) with sugar syrup in a ratio of 1:1, so that the spore concentration was 1 million per bee. Feeders were placed in the corresponding opening on the back wall of the cage. The syrup with *N.apis* spores was used up by the bees in 4 days and they were fed with pure sugar syrup from that time on.

Group B. This group of bees served for investigations of the preventive effect of fumagillin, i.e. Fumagillin DCH was applied at the same time as the infection. The sugar syrup was prepared in the same way as for the previous group and with the same concentration of *N.apis* spores (1 million/bee). Fumagillin DCH (0.015 g) was added to this syrup. Bees used up this syrup in 4 days and then they were fed with pure sugar syrup.

Group C. This group of healthy bees was not artificially infected and served as controlsThey were offered sufficient quantities of syrup throughout.

All three groups were kept in a thermostat at 31°C and bees had drinking water available during the experiment.

Pathohistological examinations

Digestive organs were taken from all three groups 4, 9, 12, and 16 days after artificial infection. Exenteration of organs was carried out under a biological magnifying glass (magnification 50x). Decapitated bees were placed on their back, fixed with pincers and exenteration was performed by pulling the back targit. The following organs were examined: crop, midgut, small intestine, Malpighian tubes, rectum and rectal papillae. Immediately following exenteration, the organs were placed in fixators in 10% neutral formalin, Bouin and Carnoy solutions. Following fixation and embedding in paraffin, series of tissue sections were cut on a microtome 2-6 μm thick, and stained according to Goldner or using the PAS (Periodic-Acid-Schiff) technique.

RESULTS

Honey crop

Examination of crop tissue sections of all three groups did not reveal the presence of spores or parasite growth stages in epithelial cells. Crop epithelial cells were clearly defined and no degenerative or lytic processes were observed in them. Normal secretory function was established in sections stained using the PAS method.

Midgut

Clear pathohistological differences were observed between groups A and B in comparison with group C in midgut sections. The results are presented according to groups and time of sacrifice of the bees.

Group C

Examinations of midgut sections of bees of the control group did not reveal the presence of *N.apis* growth stages or spores in any of the samples taken 4, 9, 12, and 16 days after the beginning of the experiment.

Group A

Significant changes were observed 4 days after artificial infection. In addition to the aboral segment, parasite growth stages and spores were also found in the oral segment of the midgut. Desquamation of epithelial cells was noted. Moreover a certain number of preserved desquamated cells filled with N.apis growth stages and spores were found in the gut content. In addition to these preserved desquamated cells, we also observed desquamated cells which were lysed, so that the released spores were in the gut content.

Moreover, homogenization of peritrophic membranes occured, primarily in the aboral segment of the midgut. The stratification of peritrophic membranes was no longer recognizable, and they were observed in the form of wider or narrow homogenous masses (Figure 1)

Examinations of sections obtained 9 days following infection showed that almost all epithelial cells were abundant with parasite growth stages and spores. The presence of spores in epithelial cells prevailed in most sections, so that growth stages were difficult to observe. Midgut epithelial cells were taken over by the

Figure 1. Midgut.Homogenization of peritrophic membranes

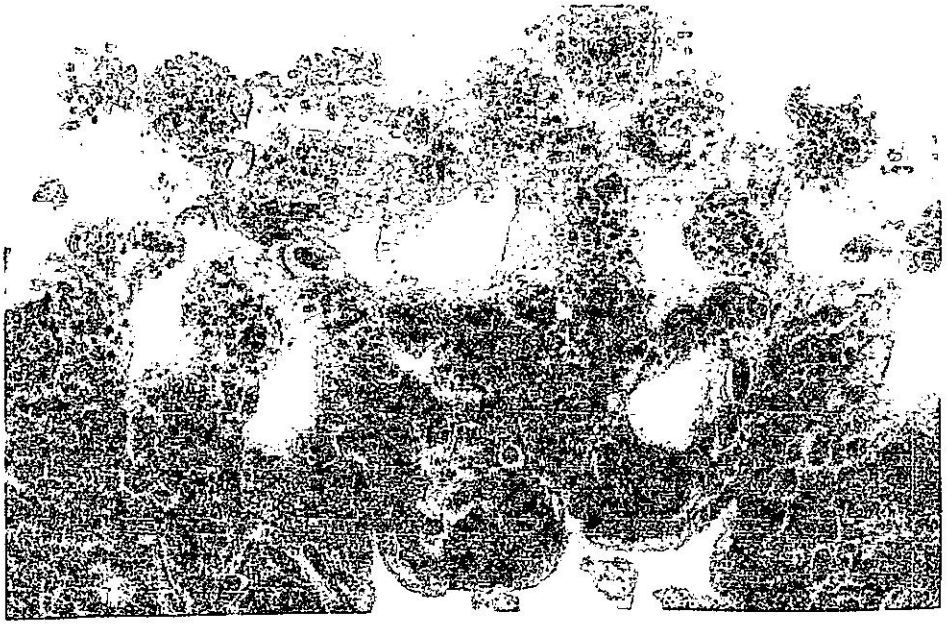

Figure 2. Midgut. Predominace of spores dominate in epithelial cells

parasite from the basal to the apical end (Figure 2). A considerable number of desquamated epithelial cells in the gut lumen were lysed. The lysed cells released a large number of spores and parasite growth stages which almost completely filled the gut lumen. The released spores were in the form of bundles but without any specific order. Peritrophic membranes were homogenized, and only their fragments were observed in the aboral area of the midgut.

Twelve days following infection, initial gaps were already seen in the epithelium due to the loss of epithelial cells (Figure 3). This epithelial cell loss was due in part to desquamation, and also in part to cell lysis. A large number of desquamated cells was observed in the gut lumen, as well as scattered spores which had reached the gut content from lysed epithelial cells. The basal membrane was well preserved. No activation of cells from regeneratory crypts was observed. The circular and longitudinal musculature of the midgut wall was clearly visible and without any changes.

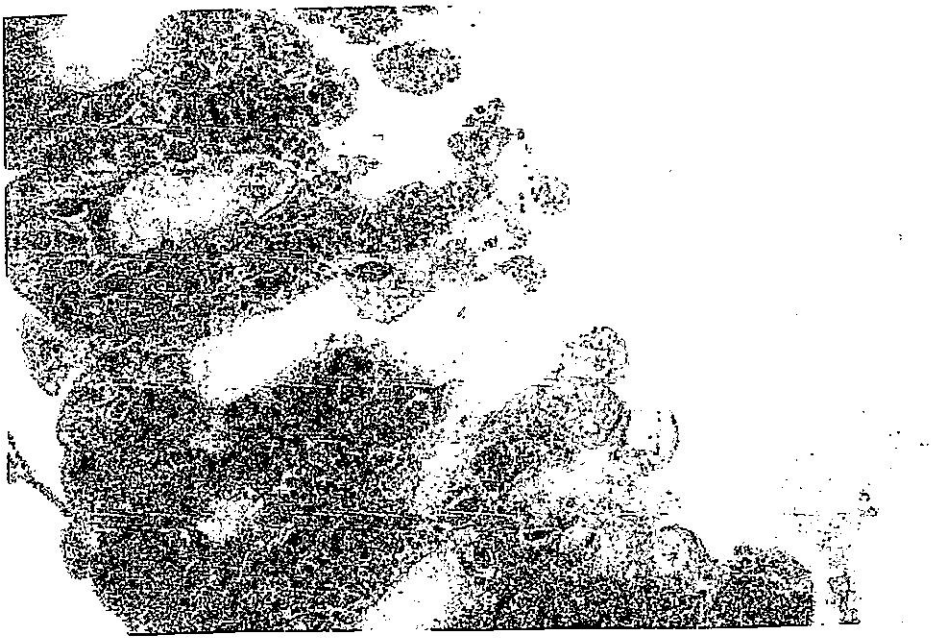

Figure 3. Midgut. Initial gaps in epithelium

Sixteen days after infection, the epithelium was reduced to only one row of cells in some places, which were filled with spores and growth stages of parasites (Figure 4). In places, epithelial loss was observed, so that the basal membrane was bare. Penetration of parasite growth stages or aggregated spores through the basal membrane or gut wall musculature was not observed in any preparation. N. Apis growth stages and spores were seen in certain epithelial cells of regeneratory crypts. The destruction of regeneratory crypts was also observed.

Figure 4. Midgut Scant epithelium

Peritrophic membranes were in the form of fragments, and were usually missing from places adjacent to the epithelium, so that wider gaps were visible between the gut content and the epithelium.

Group B

Up to 9 days after infection it was observed that a small number of cells was taken over by N.apis growth stages and spores, and in most sections they were present mostly in the aboral segment.

Examinations of midgut sectioons obtained 9 days after artificial infection and administration of fumagillin showed the presence of growth forms and spores in a somewhat greater number of epithelial cells. Growth stages and spores took over certain cells close to the gut lumen. Among these cells, we could clearly observe cells without parasite growth stages or spores. In certain segments, primarily in the aboral part, we established initial homogenization of peritrophic membranes (Figuru 5).

Twelve days after the beginning of the experiment, all midgut segments were affected, but the aboral part of the midgut contained a larger number of spores and parasite growth stages. Individual lysis of cells filled with parasite growth stages and spores was also observed. The gut content was found to contain a certain number of rejected and lysed cells, so that a small number of spores were in the gut content.

Sixteen days after the beginning of the experiment, almost all epithelial cells were filled with N.apis growth stages and spores. A large number of epithelial cells were lysed, so that bigger gaps were observed in certain gut segments. No

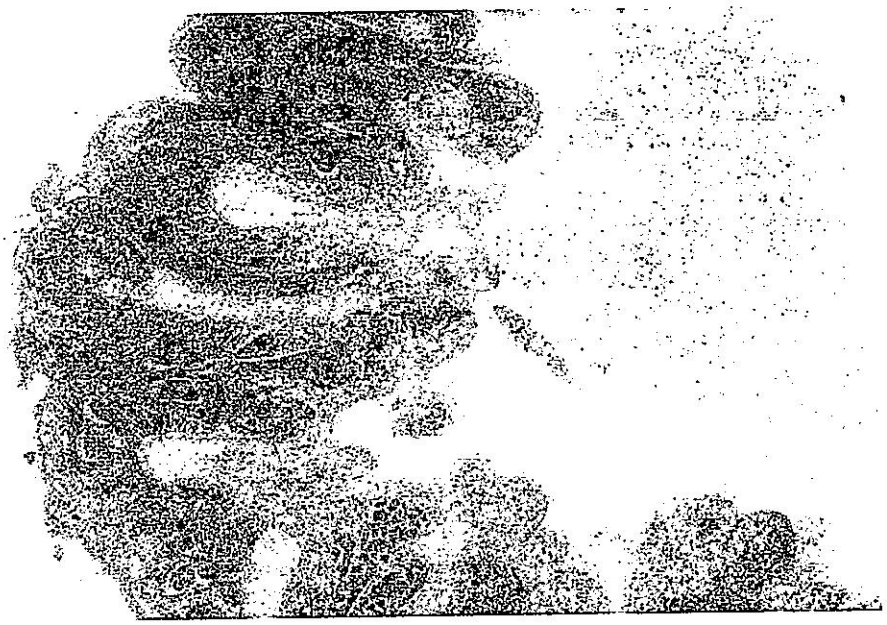

Figure 5. Midgut. Initial homogenization of peritrophic membranes

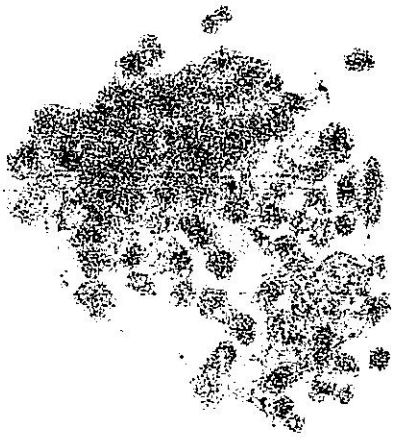

Figure 6.Midgut. Spores in midgut lumen

activation of regeneratory crypts was seen nor the taking over of regeneratory crypt cells by parasite growth stages or spores. Peritrophic membranes were homogenized, and fragmented in some places. As a result of the desquamation and lysis of cells filled with parasite growth stages and spores, a large number of spores was observed in the gut content (Fig. 6). The basal membrane was preserved, as well as the musculature.

Small intestine

Examinations of tissue sections of all three groups did not reveal the presence of *N.apis* growth stages or spores in small intestine epithelial cells. These cells were clearly defined, their nuclei were clearly distinguished, and they did not show any signs of karyopycnosis, karyorrhexis, or karyolysis.

Malpighian tubes

Examinations of tissue sections of Malpighian tubes of all three groups did not reveal any *N.apis* growth stages or spores in epithelial cells, or in the lumen of these vessels. No growth stages and spores were found in those parts of Malpighian tubes which enter the small intestine, either. Clearly defined epithelial cells were observed on Malpighian tubes, with large and sometimes eccentrically placed nuclei which did not show any changes. The vessel lumen was empty or sometimes filled with excreta.

Rectum

Examinations of rectum sections of all three groups did not show the presence of *N.apis* growth stages or spores in epithelial cells. The epithelial cells were elongated, with faintly visible nuclei and a narrow belt of protoplasm. The rectum wall did not exibit any pathological changes, and it was completely preserved.

Rectal papillae

These glandular structures on the rectum wall were also unchanged in all three groups. Glandular cells with fusiform and round nuclei, without any changes, were clearly observed. Sections stained using the PAS technique were PAS positive in all groups and all periods of sampling after the artificial infection.

DISCUSSION

Examination of organs of artificially infected healthy bees (group A) with N.apis spores were aimed at indicating which organs of the digestive tract undergo changes, since there have not been a sufficient number of such investigations so far.

It was found that infection progresses rapidly, since parasites were found in cells of the midgut oral segment 4 days after infection. Such results coincide with the findings of Bahrmann (1965)., We established maximum infection after 9 days when almost all epithelial cells from the basal to the apical part were filled with parasite growth stages and spores, wheras Bahrmann (1965) found maximum infection of epithelial cells with parasite growth forms and spores 10 days after artificial infection.

Our findings completely coincide with those of Bahrmann (1965) concerning the time of occurrence of initial gaps in the epithelium, changes in regeneratory crypts, and the preservation of the basal membrane. Moreover, we did not find a single case where the basal membrane, or the circular and longitudinal

musculature was penetrated, even at the time of the most intensive infection with the parasite. If parasite growth forms had in some case penetrated the basal membrane or musculature of the midgut wall, they would have been found in the hemolymph, and would have reached other bee organs through the hemolymph.

The results showed that regeneratory crypts remained preserved for a long time after infection, and that they were invaded by parasite growth stages and spores only 16 days after infection, which also corresponds to the findings of Bahrmann (1965).

Changes of peritrophic membranes occured very early, already 4 days after infection. The first changes were observed in the homogenization of peritrophic membranes, primarily in the aboral part, and later on their number was reduced and they became fragmented. In comparison with changes in the midgut of infected bees, samples obtained from control group C showed complete preservation of regeneratory crypts, as well as the formation of peritrophic membranes. Epithelial cells are thus constantly renewed from regeneratory crypts of non-infected bees, and peritrophic membranes are constantly formed as a protective belt outside the epithelial cells.

Thus the following takes place in group A as a result of infection and parasite action - epithelial cell lysis, degeneration of regeneratory crypts, scant formation and lack of peritrophic membranes. As a result of such changes in the midgut wall of bees with nosema disease, the passage of food through the midgut is quicker and it is utilized less, which inevitably leads to trophic changes in the organism, and thus also a shorter bee life (Rinderer and Elliot 1977, Cmejla 1954, Bailey 1976, Hassanein 1952)

Contrary to the midgut of bees of group A, which exibited intensive changes caused by the *N.apis* parasite, no alterations were observed in other organs of the digestive tract.

Thus, tissue sections of the crop did not show any pathohistological changes in the epithelial cells and their secretory function was preserved. In the small intestine, no parasite was observed in the epithelium and the structure of epithelial cells was clearly visible. The rectum wall was fully preserved, without any pathological alterations. *N.apis* growth stages and spores were not found in Malpighian tubes, or in rectal papillae. The lumen of Malpighian tubes was empty or sometimes filled with excreta, which indicates preserved vessel function. Glandular cells of rectal papillae completely preserved their appearance with fusiform and round nuclei, and PAS staining also showed the preservation of their function.

Bahrmann (1965) found growth stages and spores in Malpighian tubes in certain bees, and very rarely in very infected bees also in small intestine epithelial cells. He found that structural changes took place in rectal papillae, leading to an altered function, and he sometimes observed cell enlargement in the rectum epithelium.

Our results indicate that changes which occur in the digestive tract only cover midgut epithelial cells and that the bee midgut can in fact be considered as the exclusive place for parasite development, which was confirmed Bailey (1981) and Liu (1984).

In group B, the process also advanced, regardless of the presence of fumagillin. It can clearly be seen that our conclusion is correct from the examination of midgut sections of bees taken 9 days after the beginning of the

experiment. However, the changes which take place at that time (a greater number of cells with *N.apis* growth stage and spores, initial homogenization of peritrophic membranes, but no presence of released spores and growth stages in the gut content which means that lysis of infected cells has not occurred yet) still essentially differ from those in group A, since in that group at the same time interval, almost all midgut epithelial cells were filled with parasite growth stages and spores. A large number of epithelial cells were pushed out of the epithelium, a large number of spores and growth stages from lysed cells were observed in the midgut lumen, and peritrophic membranes were homogenized so that only their fragments were visible in the aboral part. Individual cell lysis did not take place in group 3 until 12 days after infection and the application of fumagillin and consequently, fewer spores were observed in midgut content, while at the same time in group A, initial gaps occurred in the epithelium due to loss of epithelial cells.

The weakening of the effect of fumagillin, or even an end of its effect, was observed in bees in group B in examinations of midgut wall sections taken 16 days after the beginning of the experiment. Due to epithelium cell lysis, we observed greater gaps in certain segments of the midgut, and peritrophic membranes were fragmented in some places. These changes, however, were still less intense than those visible at the same time interval in group A, where a bare basal membrane occurred in some places, as well as the penetration of parasites into certain epithelial cells of regeneratory crypts, which also disintegrated, while peritrophic membranes were observed only in the form of fragments.

Thus the results for group B showed that the simultaneous application of fumagillin with *N.apis* spores cannot prevent parasite growth, but it significantly slowed parasite growth. The infection of bees treated with fumagillin never reached the same level as in bees which were artificially infected but did not receive fumagillin. This confirms the results of Cmejla (1954). Thus our pathohistological examinations show that fumagillin acts by slowing down parasite growth in midgut epithelial cells and stabilizing the system of peritrophic membranes.

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PREVENTIVNO DELOVANJE FUMAGILINA NA STEPEN INFEKCIJE *Nosema apis* U DIGESTIVNOM TRAKTU PCELA

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SADRŽAJ

U ovom radu je patohistološkim ispitivanjem praćeno preventivno dejstvo fumagilina na pojavu *Nosema apis* u digestivnom traktu pčela. U tom cilju bile su formirane tri grupe pčela:

A grupa -pčele veštački inficirane sporama *N. apis* u šećernom sirupu;

B grupa -pčele veštački inficirane sporama *N. apis* u šećernom sirupu koji je sadr'avao 0,015 g Fumagillina DCH;

C grupa - grupa zdravih pčela koja nije bila veštački inficirana

Od sve tri grupe pčela uzimani su sledeći organi: medna voljka, srednje crevo, tanko crevo, Malpigijevi sudovi, rektum i rektalne papile i to: 4, 9, 12 i 16 dana posle veštačke infekcije. Ustanovljeno je da je lokalizacija parazita *Nosema apis* ograničena isključivo na ćelije epitela srednjeg creva pčela i da fumagilin ima preventivni efekat zbog toga što usporava razvoj parazita i stabilizuje sistem peritrofnih membrana.