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Endosymbiotic Alga from Green Hydra under the Influence of Cinoxacin

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ABSTRACT. Cinoxacin (Cxn) showed a strong effect on the endosymbiotic alga *Chlorella*; it was significantly damaged. Changes in algal color, position, structure and ultrastructure were found. In some algal cells ultrastructures were completely destroyed. The antichloroplastal and antimitochondrial effect was especially expressed. Damage to the thylakoid system of chloroplasts was more pronounced with increasing Cxn concentration. Some of the mitochondria were swollen and some of them were completely destroyed. From the evolutionary point of view, the correlation between antibacterial, and antichloroplastal and antimitochondrial effect of Cxn points to the evolutionary connection of chloroplasts and mitochondria with eubacteria.

Green hydra (*Hydra viridissima* PALLAS 1766) is a simple metazoan organism. It lives in the endosymbiosis with unicellular green algae of *Chlorella* genus that are placed inside its gastrodermal myoepithelial cells (Burnett 1973). Each alga is placed in a symbiosome (Douglas 1995). Hard cell wall and specific huge kidney-like chloroplast characterize the round-shaped algae.

Green hydra endosymbiosis is often used as a test organism for studying the influence of different agents like pesticides, toxic metals and mutagens on the organisms (Kalafatić *et al*. 1991; Kalafatić 1997). They induce different significant morphological, cytological and histological changes. The effect of antibiotics has been observed on bacteria, the flagellate *Euglena gracilis* and also on green hydra itself (Ebringer *et al*. 1993, 1996; Kalafatić *et al.* 2001; Kovačević *et al.* 2001).

Cinoxacin (Cxn) belongs to the quinolone group of chemotherapeutics; it acts as a bactericide by inhibiting the bacterial DNA gyrase (Wiedemann and Heisig 1994).

As it normally affects bacteria the deleterious effect of Cxn on chloroplasts and mitochondria could present a contribution to the origin of eukaryotic cell.

Presuming that chloroplasts and mitochondria are of bacterial origin (Krajčovič 2002), in this work we studied the toxic effect of Cxn upon the ultrastructure of an endosymbiotic green alga and tried to relate the results to the origin of chloroplasts and mitochondria in the eukaryotic cell.

MATERIALS AND METHODS

Individuals of green hydra were originally collected from Maksimir lakes in Zagreb (Croatia). They were mass-cultured and kept in glass dishes in clean aquarium water and maintained at 21 °C; the photoperiod regime light/dark was 14/10 h. Twice a week, they were fed with *Artemia salina* nauplia. After each feeding, hydras were transferred into clean aquarium water. Undamaged hydras of similar size and developmental stage without buds were chosen for the experiment. They and also endosymbiotic *Chlorella* were treated with 14.2, 62.5 and 117.6 mg/L aqueous solution of Cxn (*Lilly Deutschland*).

Thirty individuals were treated with each concentration of Cxn; one group served as control. They were exposed to the above concentrations for 1, 2 and 3 d and, afterwards, they were washed out and transferred into clean aquarium water. This procedure of transferring them into clean aquarium water was repeated every day during the 21-d experiment in which the organisms were not fed. The experiment was repeated three times.

For cytological analysis, two most damaged individuals from each concentration of antibiotic were chosen and fixed in Bouin's fixative immediately after 1, 2 and 3-d treatment; control organisms were fixed at the same time. Fixed material was dehydrated in an ethanol series and embedded in paraffin. Paraffin

blocks were cut on a microtome at 7 μm. After deparaffination, sections were stained with 0.1 % toluidine blue (pH 4.4). Then the preparations were embedded in Canada balsam and dried at room temperature for 14 d. Dried slides were examined with a binocular light microscope (*Reichert*). Micrographs were made with Pentax camera using *Kodak* Gold 200 film.

Standard preparation methods were used for conventional transmission electron microscopy (cTEM). Animals were treated for 3 d with 62.5 and 117.6 mg/L of water solution of Cxn. Immediately after the treatment they were fixed in 1 % glutaraldehyde (pH 6.9) buffered with 0.01 % sodium cacodylate buffer and postfixed in 1 % osmium tetroxide buffered with the same buffer. Preparations were dehydrated, immersed in acetone and araldite and cut with a glass knife on the ultramicrotome. Sections were stained with 4 % uranyl acetate and lead citrate (Reynolds 1963). Micrographs were made with electron microscope *Zeiss* EM10A.

RESULTS AND DISCUSSION

Morphological and cytological changes. There were no changes observed after a 1-d treatment; after the 2-d treatment with the lowest concentration initial changes in algal morphology were observed. Endosymbiotic algae were lighter than the control and did not have clearly contrasted cell walls. In 62.5 mg/L Cxn algae were spread all over the gastrodermal myoeptihelial cells. In 117.6 mg/L algae were pale, spread along the entire gastrodermal myoepithelial cells but closer to the mesoglea.

In organisms treated for 3 d with 14.2 and 62.5 mg/L Cxn algae could not be distinguished from cell organelles; in 117.6 mg/L Cxn algae were scarce in number and spread along the entire host cell. They were difficult to spot and were degenerating.

Cytological and ultrastructural changes. Endosymbiotic algae from green hydras treated with 62.5 mg/L Cxn kept their natural shape and size as the control. The algal cell wall was preserved. A perialgal space was seen and symbiosomes were well visible. Vacuoles were filled with different membrane and osmiophilic figures. Many ribosomes were visible in the cytoplasm. Nucleus and nucleolus were not affected and did not differ from those in the control group. The chloroplast structure was disturbed, showing irregularities in the thylakoid system. Plastoglobules were larger than in the control. Most of the mitochondria were swollen. The pyrenoid was expressed well. Damages to the membranes of gastrodermal myoepithelial cells were noticed (Figs 1A, 1B).

Algae treated with a higher concentration of Cxn were highly damaged, mixed with the organelles that fell apart, and could not be distinguished from them. They were located in severely damaged gastroderm of green hydra. The tough cell wall was wrinkled and thinned. Remains of perialgal space were noticeable. Cellular ultrastructures were not clearly discerned. The number of ribosomes in the cytoplasm was decreased compared to the control group, and to the group of organisms treated with 62.5 mg/L Cxn. Many granular osmiophilic structures were present in vacuoles. Chloroplasts were in the stages of degeneration, with extremely swollen thylakoids. Plastoglobules increased in number. They were huge and abundant (Fig. 2). All mitochondria were completely destroyed.

The toxic nature of Cxn on the structures of endosymbiotic algae of *Chlorella* genus in laboratory conditions was demonstrated. It was established that toxic effects of Cxn caused changes to morphological, cytological and ultrastructural features in endosymbiotic *Chlorella*. The damaging effect depended on the dose, period of exposure and the extent of damages to the membranes of gastrodermal myoepithelial cells of green hydra. As the concentration of Cxn increased, the effects on structures were more expressed. Also comparable was the damage to endosymbionts. Changes in algal morphology noted with light microscopy could be compared with cTEM level of structural analysis.

Toxicity of Cxn resulted in mortality and degradation of algae at two higher concentrations. The greatest morphological changes were observed in the change of color from green to pale. As the result of cytoskeletal damages of gastrodermal myoepithelial cells of hydra, algae spread all over the cells. It is known that pesticides also cause these damages. By regeneration and re-assembling of the endosymbiosis normal order inside gastrodermal myoepithelial cells of hydra was established (Kalafatić and Kopjar 1994).

The most expressed ultrastructural changes were observed after a 3-d treatment, especially in the highest used concentration considering all aspects of structural damages. Cxn caused significant ultrastructural changes on the unicellular endosymbiotic alga. The targeted organelles, chloroplasts and mitochondria were the most affected. Since it is presumed that these organelles originate from eubacteria, these antichloroplastal and antimitochondrial effects could be expected (Krajčovič 2002). The antichloroplastal effect was visible by the disturbed structure of the thylakoid system in the chloroplasts. Most mitochondria were also

swollen or even completely destroyed. Due to the degradation of chloroplasts plastoglobules increased in size and number; as more chloroplasts were damaged, the number and size of plastoglobules increased.

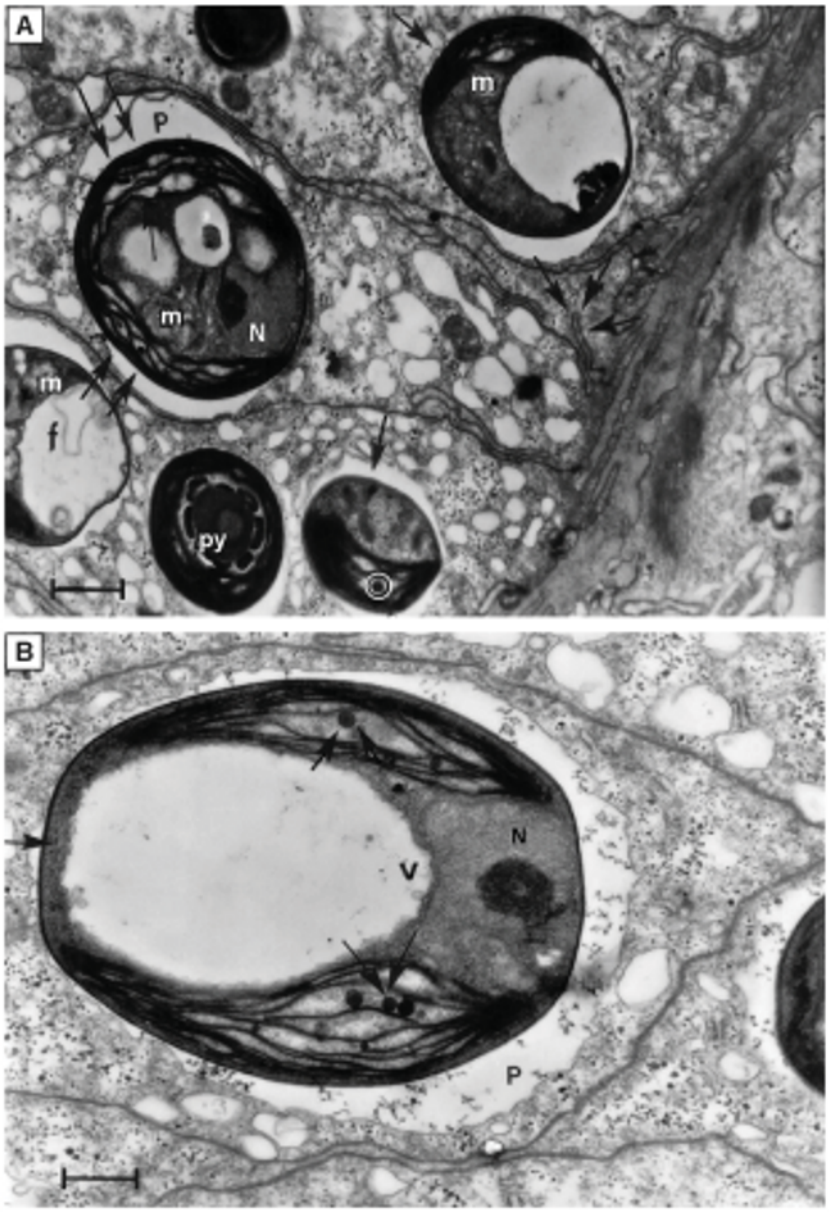

Fig. 1. Electron microscopy of gastrodermal layer of green hydra with endosymbiotic *Chlorella* (*arrows*), treated with 62.5 mg/L of aqueous solution of Cxn for 3 d and immediately fixed. **A**: irregularities of thylakoidal system of chloroplasts (*double arrows*), damages to membranes of gastrodermal myoepithelial cells (*three arrows*); f – osmiophilic figures in the vacuole; m – swollen mitochondria, N – nucleus with nucleolus, P – wide perialgal space, py – pyrenoid, *circle* – plastoglobules increased in size; *bar* = 1 μm. **B**: plastoglobules increased in size (*double arrows*) and wider perialgal space (P); V – vacuole, N – nucleus with nucleolus; *bar* = 0.5 μm.

With higher-concentration treatment also the algal cell wall was disturbed; it was thinned and wrinkled. This suggests that the toxic effect upon algal structures is extremely high. This could also be observed as the perialgal space in treated individuals was wider than in control. This can be considered to be one of many efficient protective mechanisms in the examined endosymbiosis. Ribosomes were also affected at the higher concentration. With higher intensity of described changes more membrane and osmiophilic structures were present in the vacuoles or granular osmiophilic structures appeared. These membrane structures occur by degradation of organelles (Kalafatić *et al*. 2001); they are also evident in *E. gracilis* after treatment with fluoroquinolone derivates (Polóny *et al*. 1998).

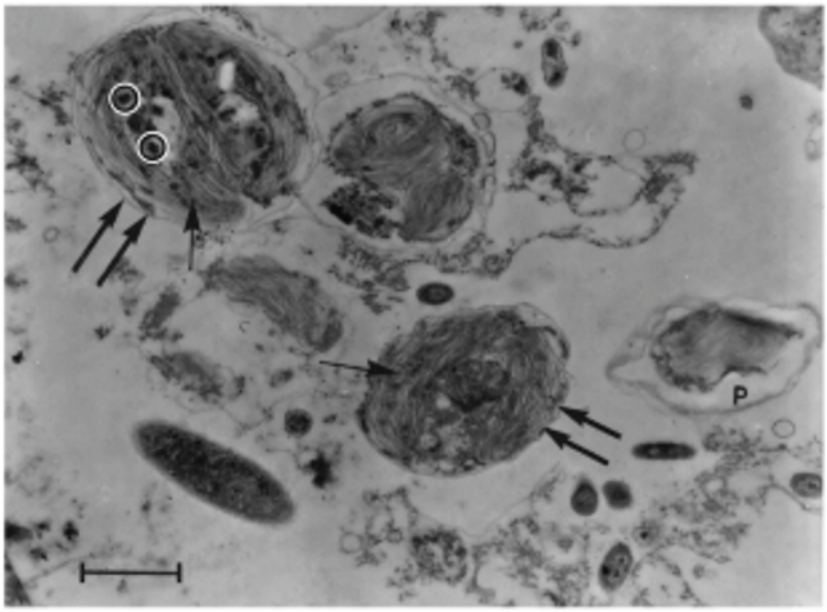

Fig. 2. The same treatment as in Fig. 1 but with 117.6 mg/L Cxn; wrinkled and thinned cell wall (*double arrows*) and chloroplasts in the stage of degeneration with extremely swollen thylakoids (*arrow*); *circles* – huge plastoglobules increased in number, P – retaining wide perialgal space; *bar* = 1 μm.

By analyzing the structures in endosymbiotic hydra–alga relationship under the influence of Cxn we established a better insight into the structural and microstructural changes that occurred. From the evolutionary point of view, this correlation between antibacterial, and antichloroplastal and antimitochondrial effects of Cxn points to an evolutionary connection of chloroplasts and mitochondria with eubacteria. Since the matter of endosymbiosis is one of the most important questions in biology, an application of microstructural analysis in this field is required.

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