Zoodiversity, **55**(5):431–438, 2021
DOI 10.15407/zoo2021.05.431

UDC 594:591.437.616.995.1

SUBMICROSCOPIC CHANGES IN THE HEPATOPANCREAS OF FRESHWATER MOLLUSKS INFECTED WITH PARTHENITES OF TREMATODES *ECHINOPARYPHIUM ACONIATUM* (ECHINOSTOMIDA) AND *PLAGIORCHIS ELEGANS* (PLAGIORCHIIDA)

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Submicroscopic Changes in the Hepatopancreas of Freshwater Mollusks Infected with Parthenites of Trematodes *Echinoparyphium aconiatum* (Echinostomida) and *Plagiorchis elegans* (Plagiorchiida). Zhytova, O., Kot, T., Huralska, S., Andreieva, O., Moroz V.— The study contains the results of the electron microscopic research of the hepatopancreas of *Lymnaea stagnalis* (Linné, 1758) molluscs infected with *Plagiorchis elegans* (Rudolfi, 1802) Braun, 1902 and *Echinoparyphium aconiatum* Dietz, 1909 trematodes. With a high degree of invasion, fibrous connective tissue growth between lime and liver cells was observed. The number of vacuoles in the cell cytoplasm increased, and the structural organization of the plasma membrane was disrupted. Heterochromatin content decreases in the nucleus, karyorrhexis could occur. The cytoplasm contained single organelles and a large number of electron-dense granules, some cells were destroyed. At a high degree of invasion of *L. stagnalis* by partenites and cercariae of *P. elegans*, the nature of the destructive changes in hepatic and lime cells of the hepatopancreas had same orientation as in mollusks with parasitic trematode *E. aconiatum*. However, the severity of the destructive changes in the hepatopancreas acini of mollusks infected with trematode *P. elegans* was much smaller, as evidenced by the absence of complete destruction of hepatic and lime cells.
Key words: freshwater mollusks, trematodes, liver cells, lime cells, acinus.

Introduction

In the life cycle of the vast majority of trematodes, freshwater gastropods are intermediate hosts, due to the formation of their initial life cycle, which coincides with the period of formation of modern freshwater mollusk fauna (Shakarbaev et al., 2013; Zhytova, 2015; Akimova, 2016). The links that exist between the components of the trematode-freshwater gastropods system are practically not disturbed; physiological balance is established in the system. However, no matter how smooth these relations are, they are still built on the principle of dynamic equilibrium (Kornyushin, 2011; Zhytova, 2015).

Histopathological studies have been used for investigation the parasite-host relationship in the trematode-mollusk system long enough. Sufficient data was collected on morphofunctional, histopathological changes in the hepatopancreas of freshwater and marine mollusks infected with trematodes (Stadnichenko, 2005; Usheva, Frolova, 2006; Stadnichenko, Leichenko, 2010; Zhytova, Khomych 2011; Choubisa et al., 2012; Nacheva, Sumbaev, 2013; Akyildiz et al., 2019). Parasite invasion is the cause of significant destructive changes in this mollusk organ. Various trematode species, due to the peculiarities of their reproduction and the degree of adaptation to the organism of a certain host species, cause different degrees of damage (Rizk et al., 2014). It was revealed (Zhytova, 2015) that the severity of microscopic changes in the hepatopancreas of freshwater gastropods infested with parthenites and cercariae of trematodes directly depends on the intensity of invasion and the type of the trematode life cycle, depending on the generation (redia or sporocyst) that follows the mather sporocyst.

A sufficient number of papers is devoted to the electron microscopic study of the hepatopancreas of mollusks infacted with various species of trematodes (Adam et al., 1995; Luchtel et al., 1997; Silva, 2003; Rizk et al., 2014; Paviotti-Fischer et al., 2019). However, information on the effect of these parasites on the host organism at the subcellular level is still insufficient; in particular, this concerns the comparative aspect of the influence of specific species of trematodes with different life cycles on the hepatopancreas of the host mollusk. This prompted us to study profound changes in the hepatopancreas of mollusk *Lymnaea stagnalis* (Linné, 1758), infested with one of the most widespread in the Ukrainian Polissia trematodes *Echinoparyphium aconiatum* Dietz, 1909 and *Plagiorchis elegans* (Rudolphi, 1802) with a high degree of invasion. These studies made it possible to analyze the degree of changes in the hepatopancreas of freshwater mollusks in the case of redioid or sporocystoid types in trematode life cycle.

Material and methods

For electron microscopic studies, the hepatopancreas from *L. stagnalis*, both free from parasites (control) and infested with parthenites and cercariae of redioid (*E. aconiatum*) and sporocystoid (*P. elegans*) trematodes was selected. The effect of parthenitis and cercariae of trematodes on *L. stagnalis* hepatopancreas was studied at a high degree of invasion.

The infection of mollusks with *L. stagnalis* was determined by the emission of cercariae (Zhytova, Khomych, 2011; Zhytova, 2015). After the end of the first stage of the experiment, the hepatopancreas was removed from the body of the mollusk and examined, starting from its color and consistency. Assessment of the hepatopancreas degree of lesion was carried out visually according to certain criteria: weak invasion — damage by parasites up to 1/10; moderate — from 1/10 to 1/2; high invasion — more than 1/2 of the organ volume (Kirichuk, Stadnichenko, 2010). Hepatopancreas from 15 specimens of *L. stagnalis* (shell height 40–56 mm), 5 specimens of each category (uninfected and highly infested) was selected for the research.

Electron microscopic studies were carried out on the basis of the laboratory of electron microscopy of the National Medical University named after A. A. Bogomolets (Kyiv). When making sections for electron microscopy, hepatopancreas was crushed in a drop of fixative, 2.5 % glutaraldehyde solution, additional fixation of the material was carried out using Caulfield's reagent (based on a 1 % Os3O4 solution) during 2 h at 40 °C; then it was thoroughly washed with distilled water and dehydrated in ethanol of increasing concentrations (700, 800, 900, 960, 1000), and later in acetone for 15 min. Next, the material was impregnated with acetone and epoxy resins (a mixture of eponym and araldite), gradually transferring it to glasses with different resin ratios (from 3 : 1 to 1 : 1 and 1 : 3, respectively), keeping for 1 h in each mixture. In the final stages, the material was embedded in resin (Epon-Araldite processing method). Polymerization took place at a temperature of 56 °C for 7–10 days (Karupu, 1984). After targeted orientation the tissue was sectioned with a Reichert Jung Ultracut E Ultramicrotome into semi-thin sections, which were then stained with toluidine blue and contrasted with a saturated solution of uranyl acetate in 70 °C ethyl alcohol and lead citrate for 15 min each. Sections were examined and photographed using a TEM-125K electron microscope.

Research results and discussion

The purpose of our electron microscopic studies was to identify pathological disorders in the hepatopancreas of *L. stagnalis* caused by a high degree of invasion by parthenitis and cercariae of the trematode *E. aconiatum*. For comparison, the structure

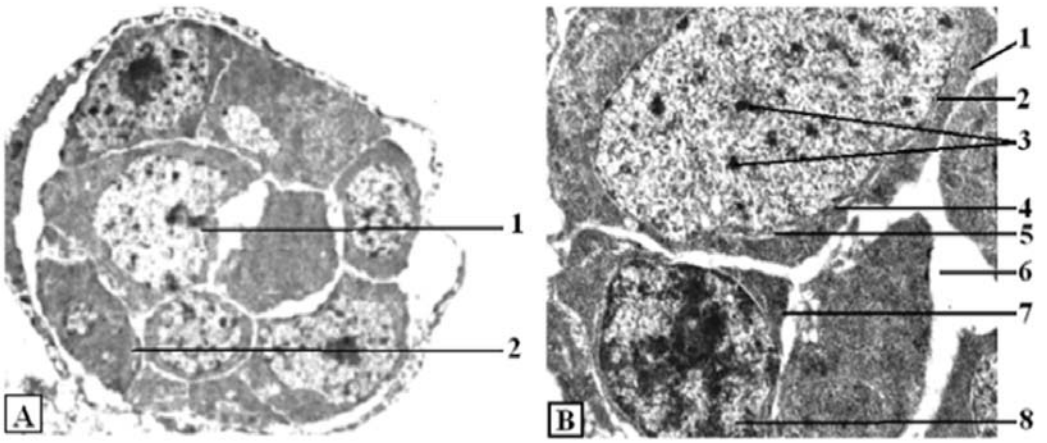

Fig. 1. Acinus of *L. stagnalis* hepatopancreas: A: Cells of *L. stagnalis* hepatopancreas acinus: 1 — hepatic cell; 2 — lime cell. (Electronogram ×4800); B: Hepatic and lime cells of a *L. stagnalis* hepatopancreas fragment undamaged by trematode parthenitis: 1 — hepatic cell; 2 — nucleus; 3 — heterochromatin; 4 — pore in the nuclear envelope; 5 — perinuclear space; 6 — the lumen of the acinus; 7 — lime cell; 8 — the nucleus of the lime cell. (Electronogram ×13000).

of the hepatopancreas of uninfected mollusks *L. stagnalis* was studied. The hepatopancreas of such mollusks had a brownish-brown color; its consistency was moderately dense. It consisted of the acini surrounded by loose fibrous connective tissue in which collagen fibers prevailed over reticular ones. The acini of the *L. stagnalis* hepatopancreas were predominantly oval in cross section, less often rounded. The wall of acini was formed by the basement membrane with two types of epithelial cells: hepatic and lime (fig. 1, A). Hepatic cells were elongated-oval or round, or irregularly polygonal. The contours of these cells were uneven; their membrane had a characteristic structure. The nuclei were rounded, elongated-oval, located in the basal part of the cell. The contours of the nucleus were equal. The nuclear envelope was formed by two elementary biological membranes, with perinuclear space of uneven thickness between them (fig. 1, B). Nucleoplasm was of low electron density. Heterochromatin in the form of lumps, grains of different electron density and size was determined in the nucleoplasm. A small portion of the heterochromatin was attached to the inner surface of the inner membrane of the nuclear envelope.

The nucleolus was predominantly one, but there could be two of them; they were located on the periphery of the nucleus, sometimes in its center. The electron density of the cytoplasm was average. It contained many mitochondria, mainly oval, ribosomes, vacuoles of low electron density, a cell center located near the nucleus, the Golgi complex, granular and agranular endoplasmic reticulum. Liver cells were larger than lime ones. The apical parts of the liver cells reached the lumen of the acini.

Unlike liver cells, lime cells were low, multifaceted, pyramidal, less often elongated-oval, they did not reach the lumen of the acinus. Structurally, lime cells were similar to liver cells. The nuclei of lime cells could be elongated-oval, round and pyramidal in shape, their contours were uneven, and the electron density was much higher than that of the hepatic cell nucleus. The perinuclear space was well expressed. The nucleoplasm contained significantly more heterochromatin than the liver cells. The nucleolus was predominantly one, located in the center of the nucleus. A significant amount of heterochromatin was fixed to the inner surface of the inner membrane of the nuclear envelope. In the cytoplasm, there were many oval and small mitochondria, the Golgi complex, ribosomes. We did not observe lime granules. The endoplasmic reticulum was well expressed, elements of its granular and agranular form were revealed.

Our electron microscopic studies of hepatopancreas tissues of *L. stagnalis* mollusks with a high degree of invasion by parthenites and larvae of *E. aconiatum* revealed the proliferation of fibrous connective tissue not only between the hepatopancreas acini, but also between lime and hepatic cells (fig. 2, A). Destructive changes in the hepatopancreas of *L. stagnalis* with a high degree of invasion by parthenitis and cercariae of *E. aconiatum* were enhanced due to long-term reproduction of parthenitis of trematodes. Fragments of destroyed liver cells and cellular debris were visible between the cells of the acini. Intact hepatic cells had an indistinct structure of the plasma membrane and a large number of vacuoles. The nucleus in some of these cells appeared to be spotty due to the formation of clumps of heterochromatin. There were also liver cells in which the nucleus was electronically transparent, its membrane was fragmented or absent (fig. 2, B).

In some cells, the nucleus broke down into fragments (karyorrhexis). The nucleolus was displaced to one of the poles of the nucleus or was not noticeable. The content of heterochromatin decreases; it was almost not found near the nuclear envelope. The cytoplasm contained solitary organelles and a large number of electron-dense granules of a round and elongated shape. Liver cells in the last stages of destruction were identified; the presence of destructed nuclei and vacuolization of the cytoplasm testified to this. In most lime cells, the amount of cytoplasm was significantly reduced; it surrounded the nucleus with a thin strip, which became irregular, elongated due to invaginations. In most lime cells,

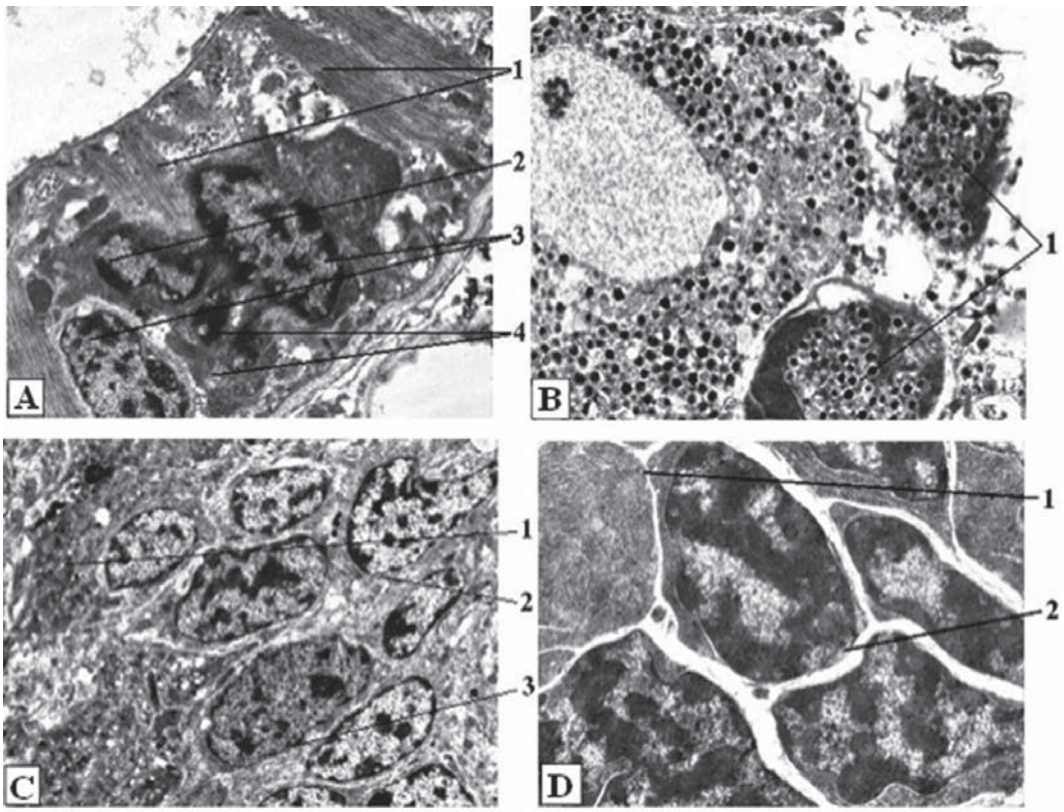

Fig. 2. Changes in the cells of the *L. stagnalis* hepatopancreas acinus with a high degree of invasion with parthenitis: A: Walls of a hepatopancreas acinus of a mollusk infected with *E. aconiatum*: 1 — collagen fibers; 2 — hepatic cell; 3 — lime cells; 4 — karyorrhexis. (Electronogram ×1000); B: hepatic cells of the hepatopancreas of a mollusk infected with *E. aconiatum*: 1 — fragments of a destroyed hepatic cell. (Electronogram ×10000); C: Lime cells of the hepatopancreas of the mollusk infected with *P. elegans*: 1 — interlobular fibrous connective tissue; 2 — hepatic cell; 3 — lime cell. (Electronogram × 6500); D: Cells of the hepatopancreas acinus of the mollusk infected with *P. elegans*: 1 — hepatic cell; 2 — lime cell. (Electronogram ×15000).

the amount of cytoplasm was significantly reduced; its thin strip surrounded the nucleus, which acquired an irregular, elongated shape due to invaginations. In separate lime cells, the nuclei were reduced in size, placed marginally (near plasmalemma).

As a rule, such nuclei form protrusions, which suggest the presence of karyorrhexis. The accumulation of heterochromatin was observed throughout the nucleoplasm. An extreme manifestation of destructive changes in lime cells was the processes of vacuolization and karyolysis of their nuclei. In the latter, the amount of heterochromatin decreased, the nucleolus disappeared, and the nucleoplasm became electronically transparent. Heterochromatin was found at the surface of the inner membrane of the nuclear envelope. The cytoplasm was electron-dense. Its microclasmatous outgrowths separating from the cells were noted, what led to a decrease in their size. At the same time, lime cells with an increased volume of cytoplasm were observed, what changed relation between the nucleus and the cytoplasm. The cytoplasm of such cells was of moderate electron density. Mitochondria had lysed matrix and cristae. The cytoplasm contained small vacuoles with and without granules. Lime cells differed in the number of granules, which could be a sign of their different functional state. At the same time, the presence of a significant number of lime cells with an insignificant volume of cytoplasm, and the absence of granules in it, suggested that with a high degree of invasion, the processes of synthesis and secretion in them were reduced in comparison with the norm (control).

So, our electron microscopic studies of *L. stagnalis* hepatopancreas, infested with parthenites and larvae of *E. aconiatum*, showed microscopic changes in most of the organ.

The detection of significant changes in the microstructure of *L. stagnalis* hepatopancreas recorded at a high degree of invasion (*E. aconiatum*) made it necessary to obtain comparative data on changes in the microstructure of *L. stagnalis* hepatopancreas infected with parthenites and larvae of the trematode *P. elegans* with the same degree of infection.

With a high level of *L. stagnalis* infection with parthenites and cercariae of *P. elegans*, the nature of destructive changes in the hepatic and lime cells of the hepatopancreas had the same direction as in the case of mollusks invasion with *E. aconiatum* trematode. However, the severity of destructive changes in the acini of the hepatopancreas of mollusks infected with *P. elegans* trematode was much less, as evidenced by the absence of complete destruction of hepatic and lime cells (fig. 2, C; fig. 2, D). Heterochromatin, which in the form of clusters was located both at the cell's periphery and throughout the nucleoplasm, predominated in the liver cells. The cytoplasm varied in volume and contained few organelles. The nuclei of lime cells had slightly higher electron density than the nuclei of hepatic cells. Lime cells differed somewhat in the size of the cytoplasm. The number of inclusions in these cells, even with a large volume of cytoplasm, was insignificant. Also, with a high level of infection with *L. stagnalis* partenits and *P. elegans* larvae, we did not find a continuous proliferation of collagen fibers between the cells of the acinus.

The manifestation of the pathogenic effect of parasites on the host organism depends on the relationships formed between them in the process of ontogeny and phylogenesis. The intensity of this effect depends on the direct effect of the trematodes on the intermediate host, the mollusk, and on the nature of its reaction to the presence of helminths.

The presence of parasites in the host body leads to changes in its organs and organ systems (Nevyadomska et al., 2007; Choubisa et al., 2012). Having settled mainly in the hepatopancreas of mollusks, trematodes use its tissues for nutrition and poison the host body with the products of their metabolism, what undoubtedly has a negative effect of the parasite on the intermediate host.

Redia cause mechanical damage to the tissues of the host mollusks hepatopancreas (Stadnichenko, 1977). In the intestines of redia, we observed in large numbers fragments

of hepatopancreas tissues, separate whole cells, and cellular detritus, which is consistent with the data of A. P. Stadnichenko (Stadnichenko, 1972) and S. L. Choubisa (Choubisa, 2008).

The degree of destruction of the hepatopancreas of mollusks largely depends on the species of Trematoda. According to T. A. Ginetsinskaya (Ginetsinskaya, 1968) sporocysts of *Cercaria roscovita* Stunk. caused the destruction of 80 % of hepatopancreas tissues in two months, while those of *C. lebouri* Stunk. only 15 % of the entire mass of this organ was destroyed in eight months. According to N. E. Serbina (Serbina, 2008), parasitizing redioid species of trematodes requires more energy from the host than sporocystoid.

According to our electron microscopic studies of *L. stagnalis* hepatopancreas, *E. aconiatum* trematode, in comparison with *P. elegans*, caused greater destruction of the hepatopancreas structure in mollusks. Our information on changes in tissues and cells of the hepatopancreas infected with *L. stagnalis* is confirmed by other studies (Stadnichenko, 1972; Souza et al., 1995). In mollusk *Viviparus viviparus* (Linné, 1758) infected with *Neoacanthoparyphium echinatoides* (de Filippi, 1854), under the mechanical impact of parasite larvae on the glandular epithelium, the liver cells were destroyed more than lime ones. In hepatic cells of the affected hepatopancreas, changes in the topography of their nuclei, which moved to the medial part, were noted. Not only cells adjacent to parasites, but those that were distant from them were subjected to destruction. The damage of liver cells was also noted in mollusks *Bithynia siamensis goniomphalus* Morelet, 1866, infected with trematode *Opisthorchis viverrini* (Adam et al., 1995) According to C. P. Souza (Souza et al., 1995), no proliferation of fibrous connective tissue was observed in the digestive gland of mollusks *Biomphalaria tenagophila* and *B. straminea* infected with the trematode *Schistosoma mansoni*. Some researchers (Silva, 2003) noted a significant destruction of *acini* and presence of cells with a large number of vacuoles in the hepatopancreas of *L. columella* (Say, 1817) infected with larvae of trematode *Echinostoma paraensi* Lie and Basch, 1967.

In mollusks infected with parthenites and larvae of redioid trematodes (*E. aconiatum*), we identified virus-like particles, which in shape and size (12.5 μm) were attributed to Baculoviruses group (baculovirus, or rod-like viruses) of the polyhedrosis subgroup, in particular, cytoplasmic polyhedrosis (Zhytova et al., 2013). The detection of virus-like particles in *L. stagnalis* infected with *E. aconiatum* is probably due to the fact that the parasitization of virus-like particles (cytoplasmic polyhedrosis) in mollusks infected with redioid trematodes is a manifestation of a significant weakening of the animal body defenses and the transition of the virus from a latent state to an active one. This case, in our opinion, is a manifestation of an opportunistic infection.

Conclusions

Thus, the results of our electron microscopic studies showed that infection with parthenitis of redioid trematode *E. aconiatum* leads to significant destructive changes in the hepatopancreas of the host *L. stagnalis*. At the same time, changes in the hepatopancreas acini of *L. stagnalis* infested with parthenites of sporocystoid *P. elegans*, are significantly less, as evidenced by the absence of complete destruction of hepatic and lime cells. In the future, studies of other species of trematodes should be expanded in order to study the pathogenicity of their parthenitis and larvae on various species of mollusks — intermediate hosts for biological monitoring of the state of populations of parasites and their hosts and preserving the biodiversity of aquatic organisms in water bodies of Ukrainian Polissia.

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Received 27 December 2020
Accepted 1 September 2021