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Structural changes of loach embryos during embryogenesis under the influence of Flurenizyd

Zmiany strukturalne zarodków piskorza podczas embriogenezy
przy działaniu flurenizydy

Summary. With the aim of exposing the harmful influence of Flurenizyd, the studies investigated the ultrastructure and morphology of embryos of loach *Misgurnus fossilis* L. in early stages of development and larval development on the first days of incubation environment, which contained this antibiotic.. It was shown that the antibiotic at the concentrations of 1 mM, 5 mM, 15 mM caused a high death rate of embryos of loach already on the first day of its application. It was established that Flurenizyd caused structural disturbances of the mitochondria and the cytoplasmic net, in addition to causing an increase of the number of lysosomes in the embryos of loach at the stage of development of 2 blastomeres. The degree of these changes is dose dependent. Flurenizyd at the concentrations of 0.05 mM, 0.15 mM and 5 mM at the stage of development of 64 blastomeres leads to dystrophic changes. It was shown that the antibiotic at the concentration of 0.05 mM and 0.15 mM causes necrotic changes, while at the maximal investigated concentration the influence of Flurenizyd on the structural changes of blastomeres is less visible.

Key words: loach embryos, embryogenesis, Flurenizyd, ultrastructure

INTRODUCTION

Research of influence of various compounds on living organisms, in particular on development of embryos, is important, as it is known that ultrastructural changes, during early embryogenesis and morphofunctional rejection in development of larvae, result in violation of functioning of adult organism, and it gives an opportunity to reduce the degree of harmfulness of this substance.

From 2000 in medical practice use the new class of medications – derivatives of fluorene (tricyclic aromatic kernel). To them belong the known antiviral preparations

Florenalum and Amiksyn [Petrukh 1997, 2008, 2011, Mihalik 2013]. Florenalum is bisulfite connection of 2-fluorenonilglioksal, that neutralizes the action of herpes simplex, virus herpes zoster and is used in ophthalmology for the treatment of viral diseases of eyes. Amiksyn (Tylorone dihydrochloride, 2,7-bis-[2-(diethylamine)-ethoxy]-fluorene-9-on) is a low-molecular inductor of endogenous interferon, antiviral means and immunomodulator effective against all causative agents of sharp respiratory viral infections. A search among fluorenes, high-efficiency substances of the wide spectrum of action, resulted in creation of Flurenizyd (N-9-fluoreniliden-N'-izonycotynohydrazide) – preparation of antimicrobial, antiphthisic, antichlamydial, immunomodulated, antioxidant, hepatoprotective, anti-inflammatory, antiviral action [Petrukh 2003a, 2008]. The antiviral action of Flurenizyd is studied *in vitro* and *in ovo* in relation to the virus of flu of bird (VFB) as Sprout/34 (H7N1) and to the virus of illness of Njukasla. The nearest analog of Flurenizyd, after an action and structure, Amiksyn is, that differs in pharmacological properties. Indexes of antiviral action of Flurenizyd on the reproduction of virus of flu of birds in the systems of *in vitro* and *in ovo* exceed such for Amiksyn.

Flurenizyd is Ukrainian preparation (registration certification № of P.10.00/02305 from 12.10.2000) – produced as powder, pills and suppository vaginal [Petrukh 2003b, 2008].

Without regard to the attained successes in relation to the application of Flurenizyd, with a therapeutic aim, his operating remains unknown on healthy cells. Thus, research on the influence of Flurenizyd on the morphofunctional state of bioblasts of loach is an important question.

MATERIALS AND METHODS

The object of our researches were embryos of freshwater fish of loach *Misgurnus fossilis* L. A loach is widely used for research of row of problems of the modern biology of development, including in embryology, biochemical, biophysical, cytological and other researches [Tupper *et al*. 1970, De Laat *et al*.1974, Kafiani and Malenkov 1976, Kostyuk 1978, Belousow *et al.* 1990]. He belongs to the family of loach of *Cobitidae*, row of carps *Cypriniformes*, the superorder of bonefishes of *Teleostei* [De Laat *et al*. 1974, Tupper *et al*. 1970].

For an experiment used the ovules of loach *Misgurnus fossilis* L., that got and impregnated after the method by Neyfah and Timofeeva [1978]. In the laboratory terms of fishes retained in a refrigerator at 4–5°C. For the receipt of caviar, a gonadotropin was intramuscular entered females after 24–48 hours to the realization of the experiment. Depending on a season and sizes of female, the dose of hormone presented from 250 international units (February–June) to 500 (beginning from October) [Kostomarova 1975, Goida 1996, Yaremkevych *et al.* 2009]. Ovulation came through 36–40 hours in the temperature range from 19 to 20°C. Male decapitated, testis had ground down and inundated by the defended tapwater. The impregnations of caviar conducted in Petri dish *in vitro*, adding the suspension of sperm. For the satisfactory impregnation of caviar a contact with sperm presented from 5 to 10 min [Bozkova and Chaylahyan 1977, Sanagurskiy 2008]. The then impregnated caviar was washed from sperm and incubation

at 21–22°C in the solution of Holtfreter [Goida 1993]. The stages of development controlled by sight under the binocular microscope of MBS-9.

Researchers conducted on the embryos of loach, that answered: to the first division of zygote (2 blastomeres); fourth (16 blastomeres); sixth (64 blastomeres); eighth (256 blastomeres); tenth (1024 blastomeres). Through 5–10 min after an impregnation zygote is washed incubation in solution of Holtfreter (from 20 to 22°C), that contained solution of Flurenizyd (used new synthesis professor Petrukh in the Lviv National Medical University of the name of Danylo Galychina substance) in concentrations 0.01; 0.05; 0.15; 1; 5; 15 mM. In obedience to the State Pharmacopeia of Ukraine Flurenizyd at first dissolved dimethylsulfoxide, (as he in this substance easily soluble) in correlation 1:2 (the eventual concentration of dimethylsulfoxide in solution did not exceed 0.9%), whereupon led to the solution of Holtfreter to the corresponding concentrations [Petrukh 2008]. Morphological and ultrastructural researchers of experimental and control objects conducted after the generally accepted methods [Kostomarova 1975, Goida 1996, Yaremkevych 2009]. Watching embryos and larvae carried out by means of binocular microscope of MBS-9 with the photographic camera.

Experiments on a survival conducted with embryos, that incubation in the control solution (solution of Holtfreter) and in the solution, that contained Flurenizyd, during five twenty-four hours from the day of impregnation to ten twenty-four hours after hatching. The environment of incubation was changed each fifteen minutes.

Ultrastructural researchers conducted on the stages of development of embryos of 2 blastomeres, 64 blastomeres and to a 10 division (1024 blastomeres), for the actions of Flurenizyd in concentrations 0.05; 0.15 and 5 mM. The investigated embryos of loach were fixed by a 1.5% solution of glutar aldehyde in a 0.2 M cacodylat buffer (pH = 7.2) at 4°C, during 1 hour. Standards were washed by a cacodylat buffer and additionally fixed 2% solutions of oxide osmium in the same buffer during 1 hour (4°C). Preparations washed from fixing and dehydrated in the growing concentrations of ethyl spirit (50%, 70%, 90% and 100%). Additionally dehydrated in the two changes of the oxide propylene and placed in the epoxid resin of Epon 812 [Hinsburg 1968].

For getting cuts, we used ultramicrotome of UMTP-6, with a diamond knife, contrasted 2% solutions of uranyl acetate, during 15 min and additionally the lead citrate by Reynolds [Golichenkov 1996].

Cuts were examined and took pictures by means of electronic transmission microscope of PEM-100.

RESULTS AND DISCUSION

Conducting a test of the survival of embryos of the loach for incubations in an environment from Flurenizyd, we are set the dose-dependent decreasing of their viability, comparatively with control (Table 1). Was shown that on the first stage of development the percent of survival of embryos of the loach decreased approximately in two times at the action of Flurenizyd in the concentration of 0.01 mM, 0.05 mM, 0.15 mM (Table 1). Insignificant decreasing of survival of embryonic objects of the loach, at the influence of the investigated factor, it is reduced to the second time of experience (0.01 mM and 0.05 mM – in 3 times, 0.15 mM – in 5 times, comparatively with control). At influence

of Flurenizyd of low concentrations percent of surviving pre-larvae decreased on the third day of his action in 6 times (in the concentration of 0.01 mM and 0.15 mM) and in 12 times – under the action of antibiotic in a concentration 0.05 mM. Percent of survival of pre-larvae falls in 16 times under the actions of antibiotic in the most lowest investigated concentration (0.01 mM) on a 4th day, and already the pre-larvae of loaches died on the 5th day. The investigated embryos died in an environment with Flurenizyd in the concentration of 0.05 mM and 0.15 mM already after the third day of development. It was noticed, that an antibiotic in the concentration of 1 mM, on the first day of development, led to lowering of the percent of living individuals in 13 times, while on a 2nd day – in 62 times. After the second day the alive embryos not found at the influence of Flurenizyd in the concentration of 1 mM. It followed to mark that the embryos of loach did not live out to the second day of development for the actions of antibiotic in the concentrations of 5 mM and 15 mM. Test on a survival in the control group of embryos comports with data of literature [Zyn *et al*. 2014]. It is needed to mark that in control larvae lived so long to the 10th day of development, after that, without corresponding additional forage, they perished.

Thus, we concluded that Flurenizyd in the concentration of 1 mM, 5 mM, 15 mM predetermines the high death rate of embryos of loach already on the first day of its action that testifies to considerable harmful influence on germinal cells. It is known that Flurenizyd is able to penetrate through cellular membranes, pass to the nucleus and act on the processes of vital functions through influence on DNA [Bodnarchuk *et al*. 2016], that explains the negative dose-dependent act of this antibiotic on the viability of embryonic objects.

Table 1. Influence of solution of Flurenizyd is on the survival of embryos of loach
Tabela 1. Działanie roztworu flurenizydy na przeżywalność zarodków piskorza

Days/ Dni	Living individuals/ Przeżywalność (%)						
	control kontrola	0.01 mM	0.05 mM	0.15 mM	1 mM	5 mM	15 mM
1	75	38	46	35	6	0	0
2	62	21	20	12	1	0	0
3	62	10	5	10	0	0	0
4	48	3	0	0	0	0	0

A morphological analysis showed that Flurenizyd in the concentration of 0.01 mM, 0.05 mM and 0.15 mM conduces to deceleration of development of embryos. The unclear contours of blastomeres were found on a 10th division stage of embryos development, that testified to a violation of structure of cellular membranes. Flurenizyd in the concentration of 0.15 mM in rare cases determined the excessive division of cells, revealed on the tenth division stage. It confirms the ability of the investigated antibiotic to influence on DNA in embryos. It is known that on the stage of a 10 division of blastomeres a mitotic index falls and grows morphogenic activity of nucleus. There is a desynchronization of the division of blastomers [Zyn 2014]. Such unlimited increasing the number of cells for the actions of Flurenizyd in the concentration of 5 mM and 15 mM,

already the stage of eighth division (Fig. 1) was set. It is needed to mark that Flurenizyd in the concentration of 1 mM, 5 mM, 15 mM leads to more expressed deceleration of processes of development of embryos, comparatively with an antibiotic in lower investigated concentrations.

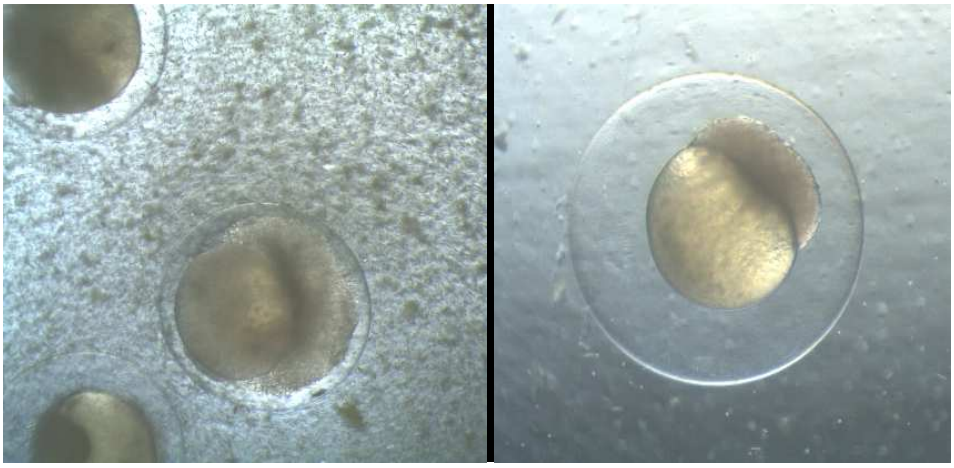

Fig. 1. The embryos of loach on the stage of the eighth division at the influence of Flurenizyd in the concentration of 5 mM (a) and in control (b)
Rys. 1. Zarodki piskorza w etapie ósmego podziału przy stężeniu flurenizydy 5 mM (a) i kontroli (b)

The analysis of control electrogram showed that on the stage of two blastomeres in the embryos of loach prevail the wrong form of different size of mitochondria, with well noticeable cristae located in parallel, that comports with data of literature [Zyn 2014]. A matrix is electronic dense. It was found the smooth cytoplasmic net (sCPN) that testifies to the low level of biosynthetic processes prevails in the hyaloplasm of the cell. It was found lysosomes, vitelline granules, peroxisomes with sharp-edged membranes. The matrix of peroxisomes had dense content electronic. It is needed to mark that on the stage of 2 blastomeres we found single peroxisomes in the cell. In the hyaloplasm of the germinal cells, in a greater degree, there are primary lysosomes (shallow membrane blisters are filled by an anhistous substance that contains active sour phosphatase) and less – secondary (Fig. 2a).

It was set, that the Flurenizyd in the concentration of 0.05 mM on the stage of 2 blastomeres predetermines the swelled mitochondria, destruction of their cristae (Fig. 2b). The matrix of mitochondria became light optically, that testified to the change of content of his components, and also about violation of power processes in a cell. As well as in a norm, prevailed sCPN, and content of free polyribosomes increases in a hyaloplasm. Already on this stage of development the Flurenizyd, in the concentration of 0.05 mM, here and there led to violation the structures of membrane round vitelline granules. The incubation of embryos in the conditions of presence of the Flurenizyd, in the concentration of 0.15 mM, led to more expressed violations of the structure of mitochondria, on the stage of development of 2 blastomeres (Fig. 2c).

The cristae were destroyed in the considerable part of mitochondria's. Their matrix characterized lowering of electronic density and as a result appeared transparent zones.

The loosening of external membranes was found in separate areas of mitochondria. Such changes led to the destruction of mitochondria. Vitelline granules lost the clearness of membranes that testifies to a violation of structure of lipid layer. The cytoplasmic net of blastomeres was not visible as result destruction. The number of lysosomes increased in a hyaloplasm, that testifies to an increase proteolytic activity of cells.

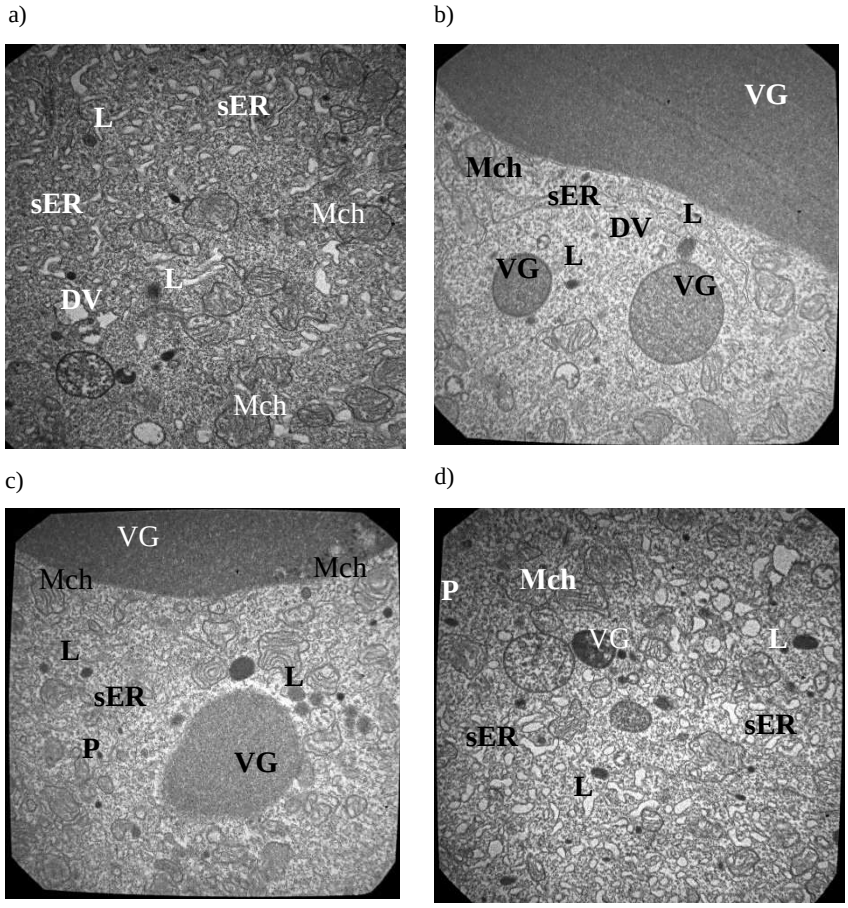

Fig. 2. An ultrastructure of the loach embryo on the stage of development of 2 blastomeres in control (a), at the influence of Flurenizyd in the concentration of 0.05 mM (b), 0.15 mM (c), 5 mM (d). Magnification 1 : 10 000. Mch – mitochondria; sER – smooth endoplasmatic reticulum, L – lysosome, P – peroxisome, DV – digestive vacuole, VG – vitelline granule

Rys. 2. Ultrastruktura zarodka piskorza w stadium 2 blastomerów w kontroli (a) przy stężeniu flurenizydy 0,05 mM (b), 0,15 mM (c), 5 mM (d). Powiększenie 1 : 10000. Mch – mitochondria; sER – gładkie retikulum endoplazmatyczne, L – lizosoma, P – peroksysoma, DV – wakuola pokarmowa, VG – granulka żółtkowa

Flurenizyd in the concentration of 5 mM caused considerable vacuolization, the degeneration of cisterns of the endoplasmic reticulum (Fig. 2d). In a hyaloplasm, thus, prevails smooth endoplasmatic reticulum (sER), that testifies to the weak biosynthetic processes of cells. The electronic density of mitochondria's matrix was decreased, however internal (cristae) and external membrane of mitochondria looked well. There was also many of mitochondria with an increased electronic densities and lost cristae. On electrograms was found digestive vacuoles and increased number of lysosomes. We also found areas with the irreversible degenerative changes of hyaloplasm. There were present peroxisomes. It is known from the sources of scientific literature the vacuolization of germinal cells shows the late result of necrosis of cell. It is the consequence of the violation of stream of Na^+, K^+ and molecules of water inside/outside of the cell, the exit of ions of Ca^{2+} and blocking of its transport in the cellular depots of blastomeres [Mandzynets *et al*. 2011].

Thus, Flurenizyd predetermines structural violations of mitochondria and endoplasmic reticulum, causes the increasing of a number of lysosomes at the loach embryos on the stage of development 2 blastomeres, and a degree of expressed of these changes is dose dependent. The amount of violations of the structure of organoids correlates with a test on a survival, where was set that Flurenizyd in the concentration of 5 mM causes 100% death of embryos of loach on the first day of development.

In control blastomeres on the 10th division of mitochondria acquired an oval form with clearly expressed cristae located in parallel, that correspond with data of literature [Zyn *et al.* 2014]. The membrane surrounds vitelline granules had correct outlines. It was found digestive vacuoles, peroxisomes, prevailed smooth endoplasmatic reticulum (Fig. 3).

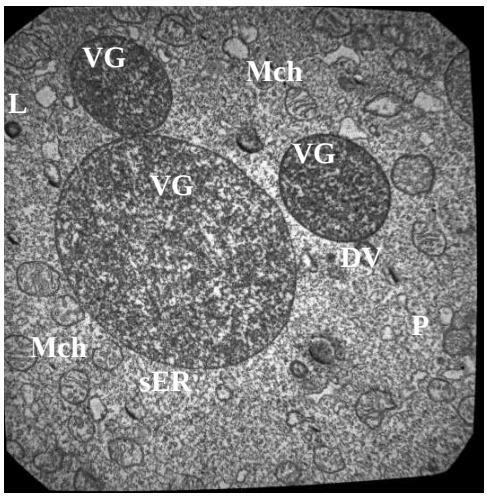

Fig. 3. An ultrastructure of the embryo of loach on the stage of a 10 division. Control. Magnification 1 : 10 000. Mch – mitochondria; sER – smooth endoplasmatic reticulum, L – lysosome, P – peroxisome, DV – digestive vacuole, VG – vitelline granule

Rys. 3. Ultrastruktura zarodka piskorza w stadium 10 podzialu. Kontrola. Powiększenie 1 : 10 000. Mch – mitochondria; sER – gładkie retikulum endoplazmatyczne, L – lizosoma, P – peroksysoma, DV – wakuola pokarmowa, VG – granulka żółtkowa

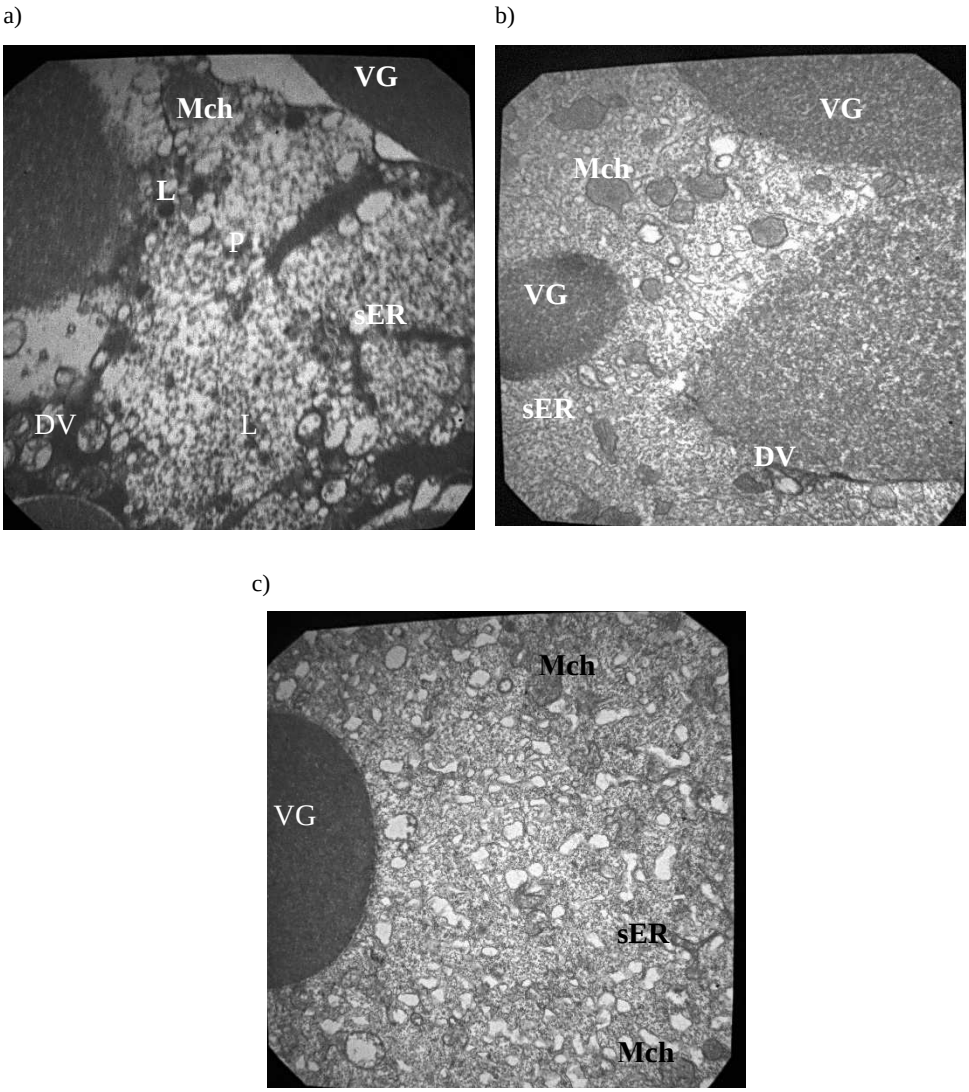

Fig. 4. Ultrastructure of embryo of loach on the stage of 64 blastomeres at influence of Flurenizyd in concentration 0.05 mM (a), 0.15 mM (b), 5 mM (c). Magnification 1 : 10 000.
Mch – mitochondria; sER – smooth endoplasmatic reticulum, L – lysosome, P – peroxisome, DV – digestive vacuole, VG – vitelline granule
Rys. 4. Ultrastruktura zarodka piskorza w stadium wpływu 64 blastomerów przy stężeniu flurenizydy 0,05 mM (a), 0,15 mM (b), 5 mM (c). Powiększenie 1 : 10 000. Mch – mitochondria; sER – gładkie retikulum endoplazmatyczne, L – lizosoma, P – peroksysoma, DV – wakuola pokarmowa, VG – granulka żółtkowa

On the stage of development of 64 blastomeres, Flurenizyd in the concentration of 0.05 mM caused the violation of structure of membranes of vitelline granules, that are the source of feed for an embryo (Fig. 4a). A number of digestive vacuoles, lysosomes and

peroxisomes grew in a hyaloplasm. The content of vacuoles had a low electronic density that testified to the active process of lysis [Mandzynets *et al.*2011]. The structure of ER was violated, that was expressed through the diffuse type of hyaloplasm. Under actions of such low concentration of antibiotic, mitochondria acquired a spherical form that testifies to their considerable swelling and lost the parallel location of cristae, and sometimes even and their disappearance. The swelling of mitochondria testifies about violation of water-salt exchange, power processes in cells. Next to such cells there were blastomeres with the external membrane of mitochondria that forms invaginations, and had unclear outlines, their matrix was optically transparent, that specifies on structural-functional changes. The cisterns of sER broaden. In the sight prevailed sER and free polyribosomes.

On the stage of 64 blastomeres in an environment with Flurenizyd of the higher investigated concentration (0.15 mM) the membrane around the vitelline granules loosened, mitochondria acquired an angular form cristae (Fig. 4b) was badly looked. A matrix was optically dense. We met mitochondria with a light matrix. The structure of CPN remained destroyed.

Flurenizyd in the concentration of 5 mM led to the analogical structural changes as the action of Flurenizyd in the concentration of 0.15 mM. However, in this case, it was found considerable vacuolization of cytoplasm and destruction of membranes of cellular organoids appeared on the stage of development of 64 blastomeres that testified to the great degenerative changes (Fig. 4c).

a) b)

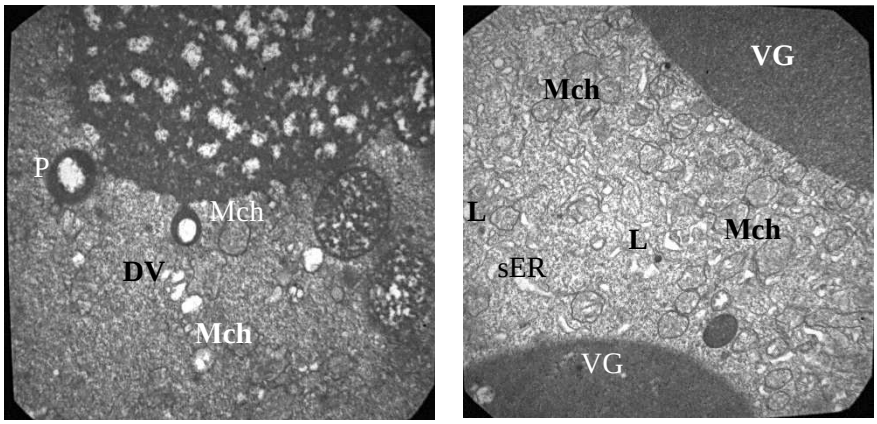

Fig. 5. Ultrastructure of embryo of loach on the stage of a 10 division at influence of Flurenizyd in concentration 0.15 mM (a), 5 mM (b). Magnification 1 : 10000. Mch – mitochondria, sER – smooth endoplasmatic reticulum, L – lysosome, P – peroxisome, DV – digestive vacuole, VG – vitelline granule

Rys. 5. Ultrastruktura zarodka piskorza w stadium 10 podziału przy stężeniu flurenizydy 0,15 mM (A), 5 mM (b). Powiększenie 1 : 10 000. Mch – mitochondria; sER – gładkie retikulum endoplazmatyczne, L – lizosoma, P – peroksysoma, DV – wakuola pokarmowa, VG – granulka żółtkowa

To the 10th division, Flurenizyd in the concentration of 0.05 mM predetermined the damage of mitochondria, the matrix of that was optically opaque (turbid), cristae disorganized. It was found the mitochondrias with the signs of swelling. The membranes of

organoids were loosened, that testified to the violation of processes of lipoperoxidation in cells. On this stage of development, the antibiotic in the higher investigated concentration (0.15 mM) led to the considerable damages of all organelles of the cell (Fig. 5a). The large digestive vacuoles appeared vitelline granules collapsed, mitochondria difficult found, ER and vehicle of Golgi apparatus were not structured. It is known that the changes of intracellular organoids with a very high electronic density can testify to the processes of denaturation of intracellular proteins and contraction of colloids, that go out outside a physiology norm (that and is characteristic for necrosis) [Kovalyschyn *et al.* 2007].

It is needed to mark, that ultrastructural changes of blastomeres were less expressed on the stage of 10 division (Fig. 5b) under action Flurenizyd in the concentration of 5 mM. The hyaloplasm of the cell contained sER with light extended cisterns. Mitochondria's were oval form, but their an external membrane was deformed, although had well-expressed contours. The mitochondria were with the transparent content. There were single lysosomes and digestive vacuoles. It is known that beginning from a ninth division there is a desynchronization process in the nucleus and mitotic index falls, RNK is intensively synthesized and activated morphogenic function of the nucleus. Probably, on this stage of development of embryos of loach, Flurenizyd in a high concentration activates the protective processes in cells, while the low concentrations lead to their oppression. These results correlate with intensity of processes of lipoperoxydation, where it was shown by us, that at influence of Flurenizyd in a concentration 5 mM content of TBK-active products decrease in relation to control, comparatively with the action of Flurenizyd in the concentration of 0.05 mM and 0.15 mM where content of secondary products of lipoperoxydation increasecomparatively with control [Bodnarchuk 2016].

Thus, Flurenizyd of the investigated concentrations, on the stage of development of 64 blastomeres, lead to the dystrophic changes. Antibiotic in the concentration of 0.05 mM and 0.15 mM causes necrotic changes, comparatively with Flurenizyd of the maximal investigated concentrations, under its influence of that structural changes of blastomeres are less expressed.

CONCLUSIONS

1. Flurenizyd in the concentration 1 mM and higher predetermines high death rate of embryos of loach already on the first day of his influence, comparatively with the action of antibiotic in a concentrations 0.01; 0.05; 0.15 mM.

2. Antibiotic causes structural violations of mitochondria and endoplasmatic reticulum, the increase of an amount of lysosomes at the embryos of loach, on the stage of development of 2 blastomeres, and a degree of expressed of these changes is dose-dependent.

3. Development of embryos, at the presence in the environment of Flurenizyd, on the stage of 64 blastomeres, characterized by dystrophic pathology, the degree of expression increases with the concentration from 0.05 mM to 5 mM.

4. Flurenizyd in low concentrations predetermines necrotic changes in the cells of loach embryos on the stage of 10th division.

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Streszczenie. W celu udowodnienia szkodliwego wpływu flurenizydy zbadano ultrastruktury i morfologię zarodków piskorza *Misgurnus fossilis* L. w początkowych stadiach embriogenezy i larw w pierwszych dniach inkubacji w pożywce, która zawierała roztwór tego antybiotyku. Wykazano, że flurenizyda w stężeniu 1 mM, 5 mM, 15 mM powoduje wzrost śmiertelności zarodków piskorza w pierwszym dniu jej stosowania. Stwierdzono, że flurenizyda prowadzi do zaburzeń strukturalnych w mitochondriach i gładkiej siateczce śródplazmatycznej, powoduje wzrost liczby lizosomów w zarodkach piskorza w stadium 2 blastomerów. Stopień tych zmian zależy od dawki preparatu. Flurenizyda w stężeniach 0,05 mM, 0,15 mM i 5 mM na etapie 64 blastomerów prowadzi do zmian degeneracyjnych. Wykazano, że w stężeniu 0,05 mM i 0,15 mM powoduje zmiany martwicze, natomiast w maksymalnym stężeniu powoduje mniej wyraźne zmiany strukturalne blastomerów.

Słowa kluczowe: zarodki piskorza, embriogeneza, flurenizyda, ultrastruktura