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HEAT SHOCK PROTEINS AS BIOMARKERS OF FISH POLLUTION

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Heat Shock Proteins (HSP) belong to the natural biomarkers, which are important indicators for animal diseases diagnostics and / or instrument of analyzing the effects on organism of the habitat deteriorating factors. The contamination of water by heavy metals has adverse effect on fish organism. Even in a small quantity, such heavy metal as lead is very dangerous. The analysis of toxic effects of the lead ions on the level of expression of such heat shock proteins as HSP60, HSP70 and HSC70 family in leukocytes, liver, brain and gills of the scaly carp was the main goal of our investigation.

During 96 hours the fish were kept in the aqueous environment of a tank which additionally was supplemented with Pb $(CH_3COO)_2$. The control group of fish was maintained for the similar period of time under the same conditions, without lead acetate supplementation.

Concentration of HSP60, HSP70 was determinated by the dot-blot-analysis due to application of monoclonal antibodies against heat shock proteins SAB4501464 (Sigma, USA), [5A5] (ab2787) (Abcam, USA) and [1B5] (ab19136) (Abcam, USA). Detection of immune complexes was performed by using the commercial substrate solution for alkaline phosphatase - CDP-Star (Tropix, UK). Visualization was done by using X-ray film ECL HyperFilm (Amersham, USA) and a kit for films developing (Kodak). The images were processed using the software package GelPro (Version 3.1, USA).

The significant dose-depended increase ($p<0,001$) in all experimental groups of HSP60 and HSP70 concentrations in leukocytes, liver, gill and brain has been detected, applying dot-blot analysis. At the same time, significant changes in expression of HSC70 protein have not been established. It is the evidence that stress-proteins are the sensitive markers of toxic effects of excessive concentration of lead.

Key words: heat shock proteins, HSP60, HSP70, HSC70, dot-blot analysis, scaly carp, biomarkers, heavy metal, lead.

Онисковець М., Панас Н., Лопотич Н., Іванків М., Саламаха І. Білки теплового шоку як біомаркери забруднення риб

Білки теплового шоку (Heat Shock Proteins – HSP) належать до природних біомаркерів, і визначення їх кількості у тканинах або клітинах стає однією з цілей діагностики поширених захворювань тварин та/або аналізу впливу чинників, що порушують природне середовище існування. Для досліджень обрано найпоширеніші протеїни теплового шоку, які відносять до родини білків з молекулярною масою 60 та 70 кДа (HSP60 і HSP70). Актуальність таких досліджень визначається значною мірою зростанням антропогенного впливу на природні водойми, де для риб, як кінцевої ланки трофічного ланцюга, існує значна токсикологічна загроза. Проаналізовано токсичний вплив іонів Плюмбуму на рівень експресії HSP60, HSP70 та HSC70 у лейкоцитах, печінці, мозку та зябрах коропа лускатого.

За допомогою дот-блот-аналізу виявлено концентраційно залежне зростання ($p<0,001$) вмісту HSP60 і HSP70 за дії всіх досліджуваних концентрацій важкого металу. У лейкоцитах коропа вміст HSP60 і HSP70 максимально зріс у 15 і 98 разів відповідно порівняно з контролем. Найвищий рівень експресії цих білків зафіксовано у печінці риб. Слідові кількості HSP70 виявлено у зябрах риб контрольної групи, 2 ГДК Плюмбуму не зумовлювали істотних змін у вмісті досліджуваних білків, тоді як 5 і 50 ГДК спричиняли зростання досліджуваних показників майже у 5 і

15 разів для HSP60 та у 107 і 144 рази для HSP70. Водночас у мозку ефект при дії Плюмбуму не був таким вираженим порівняно з наведеними вище органами.

На підставі отриманих даних висунуто припущення про наявність кореляції між станом цілого організму і вмістом білків теплового шоку.

Отже, отримані дані свідчать про можливість використання стрес-білків як чутливих маркерів у реакції на токсичний вплив важких металів.

Ключові слова: білки теплового шоку, HSP60, HSP70, HSC70, дот-блот аналіз, короп лускатий, біомаркер, важкі метали, Плюмбум.

Problem setting. Nowadays, most aquatic biota is exposed to moderate and chronic multifactor pollution and adverse effects on these ecosystems are difficult to assess. Pollution caused by heavy metals represents one of the major factors of environmental stress in aquatic environments [2; 5].

That is why, during the last years, substantial efforts have focus on development of sensitive biomarkers for ecological risk assessment. Development of modern methods of aquatic biological resources quality estimation contributes to sorting out the problems of conservation and restoration of industrial fish populations in the natural environment [1].

By now, it is clearly established that biomarkers are biological parameters measuring alterations in behaviors, physiology, biochemistry, cell integrity, genomic structure and expression. Many researchers have focused their efforts to develop and apply biomarkers in ecotoxicology and, now, there is a consensus to consider that they provide useful informations in the ecological risk assessment [8; 15].

Heat shock proteins have been evidenced by Tissières et al. (1974) in salivary glands of the Diptera Drosophila melanogaster submitted to heat shock in laboratory conditions. The name of heat shock proteins proposed at that time has been conserved even if it has been further demonstrated that these proteins are drastically induced after different physical and chemical treatments, especially those that denature proteins [5; 6; 10].

The induction of HSP in cells of organisms exposed to stress represents a rapid and highly conserved response to proteotoxic insult. As this ubiquitous response, observed in all organisms studied from bacteria to human, can be induced by pathophysiological and environmental stresses [11; 13], it has been proposed to consider HSP induction as a biomarker tool for the early detection of environmental changes and ecological risks in aquatic biota [7; 8]

The impact of lead on concentration of HSP in fish tissues has not been studied enough. Such studies are extremely relevant and necessary for assessing the health status of fish during monitoring of aquatic biocenoses [16].

The analysis of toxic effects of lead on the level of expression of heat shock proteins with molecular weight 60 and 70 kDa was the main goal of our investigation. The last group of proteins includes HSP70, induced by stress factors, as well as HSC70, constitutively expressed by cells.

Presenting main material. During 96 hours the fish were kept in the aqueous environment of a tank which additionally was supplemented with Pb $(CH_3COO)_2$. The control group of fish was maintained for the similar period of time under the same conditions, without lead acetate supplementation. The tanks with oxygenated, running water were maintained at temperature of 18–20 C.

The blood was sampled by Pasteur pipette from the heart of fish. The tissues of liver, brain and gills were removed from the fish and washed by physiological saline solution. The samples were frozen in liquid nitrogen and stored until the laboratory treatment. All experimental procedures with animals were conducted in accordance with the European Convention for Animal Care.

Concentration of HSP60, HSP70 was determinated by the dot-blot-analysis due to application of monoclonal antibodies against heat shock proteins SAB4501464 (Sigma, USA), [5A5] (ab2787) (Abcam, USA) and [1B5] (ab19136) (Abcam, USA) and polyclonal goat antimice antibodies, conjugated with alkaline phosphatase («Tropix», USA).

According to the experimental procedure, samples of the tissues were defrostrated and used in a mix with physiological solution while preparing of homogenates has taken place. Simultaneously the protein concentrations were measured.

Namely, after defrosting, the tissue was lysed in the ten- fold volume of the lysis buffer, pH 7.4 (10 % N-laurylsarkosine, 10 μM phenylmethylsulfonyl fluoride, 10 μM N-ethylmaleimide in 0.01 M N-phosphate buffer, 0.001 % proteinase inhibitor cocktail – Sigma, Germany).

The samples were further centrifuged at 5200 g for 5 min. at 4 °C. The concentration of protein in the lysates was measured by Lowry's method. To make the volumes and concentrations of the total protein identical, the samples were diluted with the buffer, pH 7.4 (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM KCl).

Lysates were put on nitrocellulose membrane (Millipore) in a volume of 3 µl with concentration of total protein approximately 1–5 µg. To detect background indices, the lysis and delution buffers were applied on the membrane. The membrane was blocked for 1 hour by 5 % solution of casein.

After application of control and experimental samples, the membrane was incubated with antibodies against heat shock proteins SAB4501464 (Sigma, USA), [5A5] (ab2787) (Abcam, USA), and [1B5] (ab19136) (Abcam, USA) at PBS 90 min., as well as with polyclonal goat anti-mouse antibodies conjugated with alkaline phosphatase (Tropix, USA) – 1:5000 in PBS for 30 min. (*Fig. 1)*.

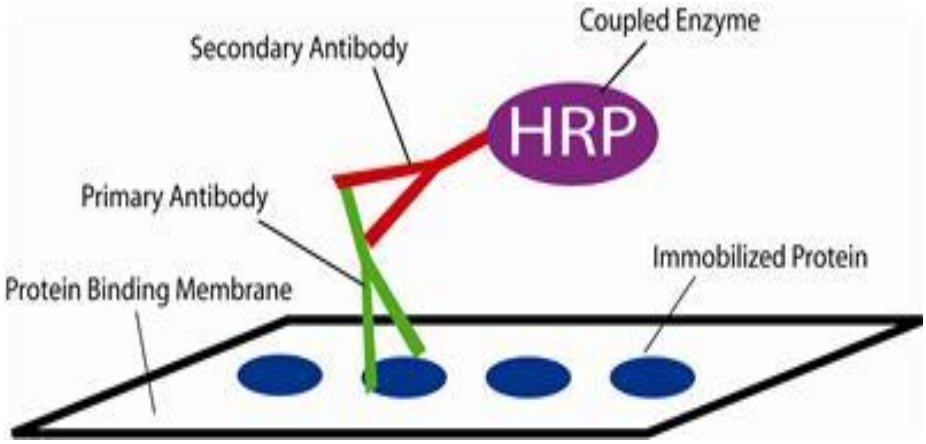

Fig. 1. Dot blot technique

Detection of immune complexes was performed by using a commercial substrate solution for alkaline phosphatase – CDP-Star (Tropix, UK). Visualization was done by using X-ray film ECL HyperFilm (Amersham, USA) and a kit for films developing (Kodak). Images were processed using the software package GelPro (Version 3.1, USA).

Heat Shock Proteins (HSP) belong to the natural biomarkers, which are important indicators for animal diseases diagnostics and / or instrument of analyzing the effects on organism of the habitat deteriorating factors. The contamination of water by heavy metals has adverse effect on fish organism.

HSP is a family of highly-conserved proteins that are required by the cell in all its processes of life, including adaptation to a huge number of cytotoxic factors, both xenobiotic as well as natural origine [6; 10; 12].

The obtained data evidences a significant role of these proteins in cell responses to the stress factors and infectious pathogens, abiotic stressors, high temperature and cold shock, such environmental pollutants as heavy metals [5].

It is known that pollutants interfere with organism integrity at the biochemical level with consequent adverse effects at the individual level such as growth, reproduction and survival. However, these biochemical parameters have a reduced long-term ecological relevance at the population and community levels. Indeed, such early warning biomarkers would be useful for detecting sublethal pollution before changes in community structure or species composition occur [1; 8; 16].

The contamination of reservoirs by such heavy metal as lead, which, even in a small quantity, provokes stress and morphofunctional changes in the organism of fish is the most dangerous [3; 5].

The analysis of toxic effects of the lead ions on the level of expression of such heat shock proteins as HSP60, HSP70 and HSC70 family in leukocytes, liver, brain and gills of the scaly carp was the main goal of our investigation.

The concentration-dependent growth ($p < 0.001$) of HSP60 and HSP70 in all experimental groups with applicable lead concentrations was detected applying dot-blot analysis. Comparing with the control group, the concentration of HSP60 and HSP70 in the white blood cells increased as much as 15 and 98 times, respectively. The highest level of the proteins expression (185.2±12.39 U.S. for HSP60 and 252.3±18.64 U.sup.v. for HSP70) was recorded in the liver of fish (Fig. 2, Fig. 3).

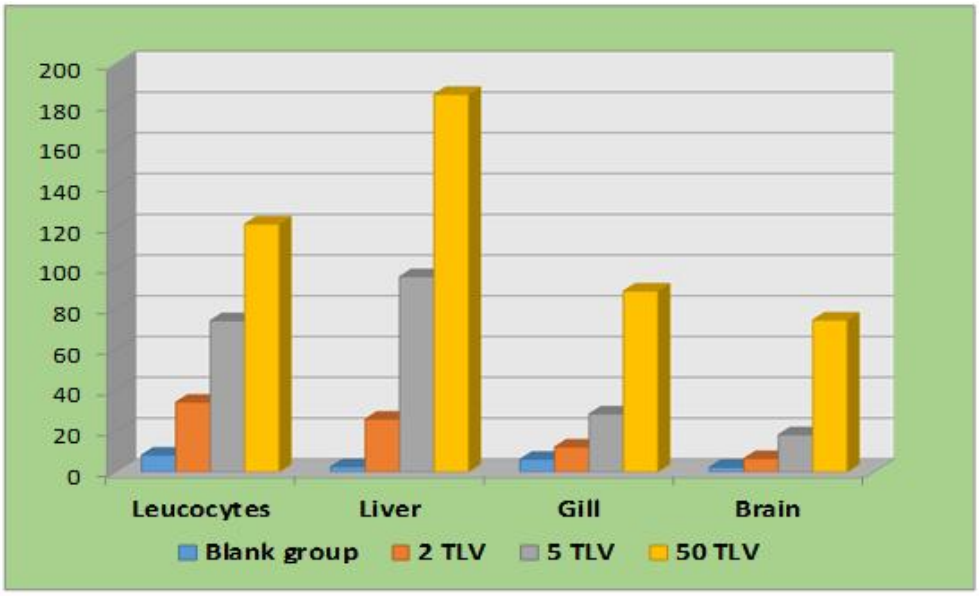

Fig. 2. The effects of the lead on concentration of HSP60 in blood leucocytes and tissues of scaly carps, c.units

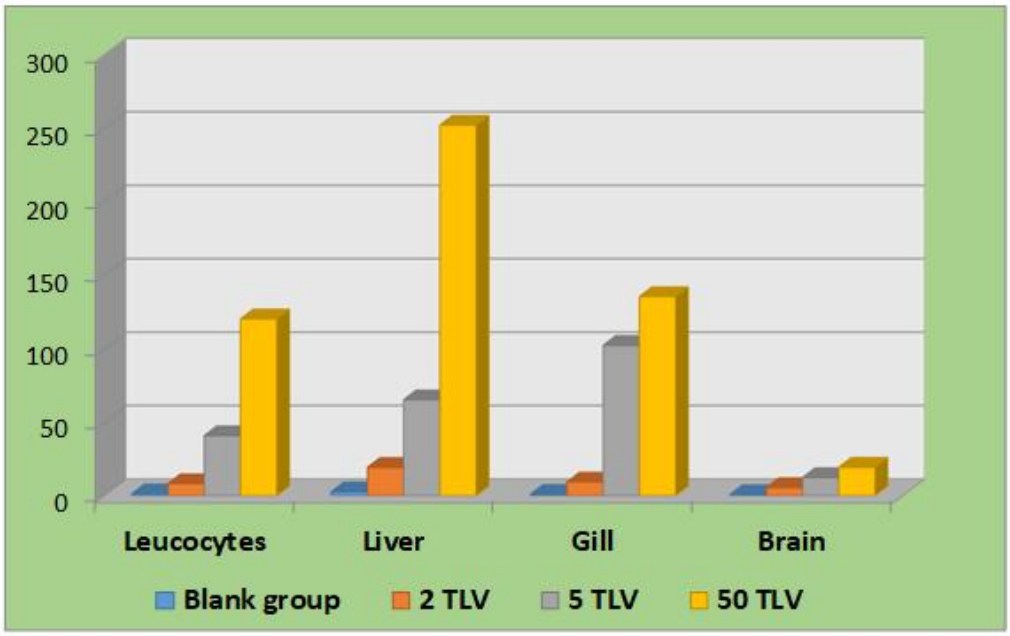

Fig. 3. The effects of the lead on concentration of HSP70 in blood leucocytes and tissues of scaly carps, c.units

Trace amounts of HSP70 were detected in the gills of the fish of the control group. The fish exposed to the lead effects in concentration of 0.2 mg/l was characterized by no significant changes in the content of the investigated proteins, while concentration of lead in 0.5 mg/l and 5 mg/l in water resulted in increase of the studied parameters to almost 5 and 15 times for HSP60 and 107 and 144 times for HSP70 (Fig. 3).

It was established that in comparison with the control group, none of the applied concentrations of lead brought on significant changes in the expression of HSC70 protein in the investigated organs of *Cyprinus carpio L.* (Fig. 4).

It could be explained by the fact that HSP60 and HSP70 belong to the group of stress-proteins affected by a wide range of stressors, in particular, the heavy metals. Moreover, HSC70 is involved in more

specific mechanisms of response on the deterministic stress-induced factors [3; 4]. Thus, the obtained data indicates that stress-proteins are possibly applicable as the sensitive markers of toxic effects of the lead.

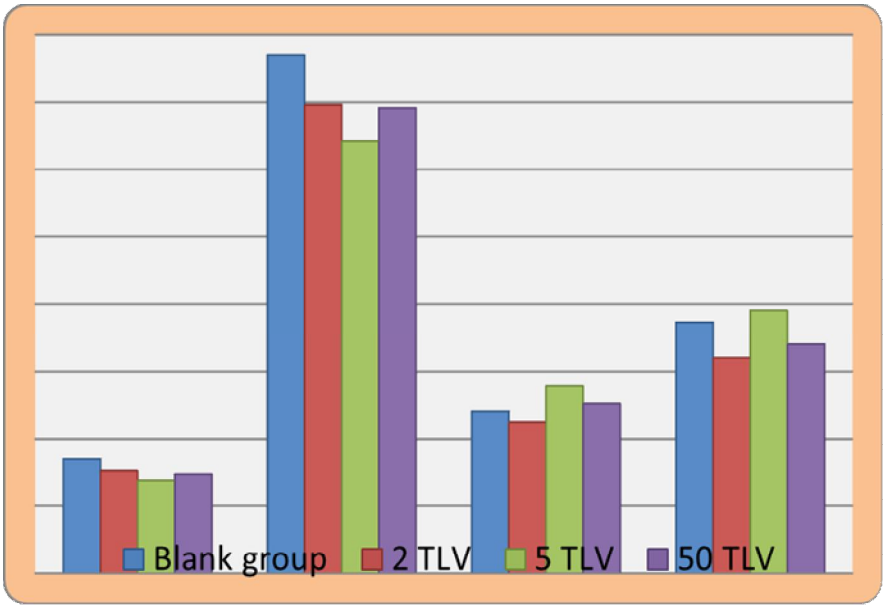

Fig. 4. The effects of the lead on concentration of HSC70 in blood leucocytes and tissues of scaly carps, c.units

Conclusions. The obtained data evidences a significant role of these heat shock proteins in cell responses to the stress factors and environmental pollutants (heavy metals). Concentration of HSP was determinated by the dot-blot-analysis due to application of monoclonal antibodies against heat shock proteins. The significant dose-depended increase ($p<0,001$) in all experimental groups of HSP60 and HSP70 concentrations in leukocytes, liver, gills and brain has been detected, applying dot-blot analysis. It could be explained by the fact that HSP60 and HSP70 belong to the group of stress-proteins affected by a wide range of stressors.

At the same time, significant changes in expression of HSC70 protein have not been established. HSC70 is involved in more specific mechanisms of response on the deterministic factors. Thus, it is the evidence that stress-proteins are the sensitive markers of toxic effects of excessive concentration of lead.

References

1. An L. H., Lei K., Zheng B. H. Use of heat shock protein mRNA expressions as biomarkers in wild crucian carp for monitoring water quality. *Environmental Toxicology and Pharmacology*. 2014. No 37 (1). P. 248–255.

2. Barata C. et al. The relative importance of water and food as cadmium source to Daphnia magna Straus. *Aquatic Toxicology*. 2002. No 61. P. 143–154.

3. Boone A. N., Vijayan M. M. Constitutive heat shock protein 70 (HSC70) expression in rainbow trout hepatocytes: effect of heat shock and heavy metal exposure. *Comparative Biochemistry and Physiology C: Toxicology and Pharmacology*. 2002. No 132 (2). P. 223–233.

4. Chen S., Brown I. R. Neuronal expression of constitutive heat shock proteins: implications for neurodegenerative diseases. *Cell Stress Chaperones*. 2007. No 12. P. 51–58.

5. Deane E. E., Woo N. Y. Impact of heavy metals and organochlorines on hsp70 and hsc70 gene expression in black sea bream fibroblasts. *Aquatic Toxicology*. 2006. No 79. P. 9–15.

6. Dzaman-Serafin S. Telatyńska-Mieszek B., Ciechanowski K. Heat shock proteins and their characteristics. *Pol Merkur Lekarski*. 2005. No 19 (110). P. 215–219.

7. De Jong L., Moreau X., Jean S., Scher O. & Thiéry A. Expression of the heat shock protein Hsp70 in chloride target cells of mayfly larvae from motorway retention pond: A biomarker of osmotic shock. *Science of the Total Environment*. 2006. No 366. P. 164–173.

8. De Jong L., Moreau X., Thiéry A. Expression of heat shock proteins as biomarker tool in aquatic invertebrates: Actual knowledge and ongoing developments for the early detection of environmental changes and ecological risks. Emma Morel and Camille Vincent. *Heat-Shock Proteins: New Research*. 2008. No 20. P. 375–392.

9. Efremova S. M. et al. Heat shock protein HSP70 expression and DNA damage in Baikalian sponges exposed to model pollutants and wastewater from Baikalsk Pulp and Paper Plant. *Aquat. Toxicol.* 2002. No 57. P. 267–280.

10. Evdonin A., Medvedeva N. The extracellular heat shock protein 70 and its functions. *Cytology*. 2009. No 51 (2). P. 130–137.

11. Feder M. E. & Hofmann G. E. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*. 1999. No 61. P. 243–282.

12. Hartl F. U. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*. 2002. No 295 (5561). P. 1852–1858.

13. Ito H., Inaguma Y., Kato K. Small heat shock proteins participate in the regulation of cellular aggregates of misfolded protein. *Nippon Yakurigaku Zasshi*. 2003. No 121. P. 27–32.

14. Mayer A. B., Hsp70 chaperone: cellular function and molecular mechanism. *Cell. Mol. Life Sci.* 2005. No 62. P. 670–684.

15. Oliva-Teles A. Nutrition and health of aquaculture fish. *Journal of Fish Diseases*. 2012. No 35 (2). P. 83–108.

16. Sharaf-Eldeen K. Accumulation of a 70 kDa stress protein in the Nile Tilapia, *Oreochromis niloticus*, and its use as a biomarker of Cu exposure. *Egypt. J. Auqat. Biol. & Fish*. 2006. No 10 (2). P. 19–31.

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