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ФІЗІОЛОГІЯ ЛЮДИНИ І ТВАРИН

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VIABILITY OF LOWER CRUSTACEANS UNDER THE INFLUENCE OF BIOLOGICALLY ACTIVE SUBSTANCES OF BIOTECHNOLOGICAL WATER FROM THE MEDICINAL LEECH (*HIRUDO VERBANA*)

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The organism of the medicinal leech produces a complex of unique biologically active substances that are allocated into the aquatic environment with waste products and exhibit a wide number of therapeutic effects. The aim of the work was to study the influence of biologically active substances of biotechnological water from the leeches in artificial ecosystems on the survival of lower crustaceans (*Daphnia magna* Straus). We determined the acute lethal toxicity of biotechnological water from the medicinal leech on the test organism. Index of acute toxicity, compared with controls, in the first hours of the experiment (4–6 hours) is 3,3% and further increases, and starting from the third day (after 72 and 96 hours) acute toxicity was observed at the level 60,0 and 69,0%, respectively. Daphnids that were cultivated in biotechnological water from the medicinal leech differed morphologically from the control; in the initial stages of biotesting they had larger average body size and showed greater activity.

Keywords: biotesting, medicinal leech, biologically active substances, test-organism, acute toxicity.

The organism of the medicinal leech (*Hirudo medicinalis*, Linnaeus, 1758; *H. verbana*, Carena, 1820; *H. orientalis*, S. Utevsky et Trontelj, 2005) produces the complex of unique biologically active substances (BAS), that do not have analogues and are necessary in modern medicine and veterinary medicine and show the wide spectrum of therapeutic effects [4, 6, 9]. It is also recognized that an extract of the medicinal leech's saliva is very similar to antibiotics, but does not show side effects [4]. BAS of medicinal leech outflow into the water environment in which they are contained, with other waste products: guano, urine, mucus and desquamated cuticle remains. It is known that the biotechnological water from the medicinal leech includes: blood proteins of the host, hirudin, enzymes (hyaluronidase, triglyceridase, elastase, apyraze), amino acids (tryptophan, glutamic acid, alanine, lysine, leucine, etc.), biogenic elements (potassium, natrium, phosphorus) and microelements (iodine, bromine, sulfur, selenium) [6]. However, obviously, the spectrum of these substances is much wider and is not studied enough yet. The presence of these components shows a high biological value of biotechnological water from the medicinal leech. There are reports which suggest using externally biotechnological water from the medicinal leech for the purposes of prophylaxis and cure [6]. Besides, the medicinal leech is the important component of aquatic and terrestrial communities. Its effect on biotic relationships in these communities, except trophic relationships, has not been studied yet. So the research of the influence of BAS of the biotechnological water from the leeches in artificial ecosystems by the example of lower crustaceans (*Daphnia magna* Straus*, Cladocera, Crustacea*) is important.

Materials and methods

Daphnia, invertebrate animals, plants are often used as test-objects in bioindication of natural waters. The advantages of Daphnia over other types of animals include the fact that their species composition and abundance correspond to the level of environment saprobity, they have high sensitivity level to the changes in environmental quality, they are available and low-cost materials, experiments with Daphnia are of relatively short duration, test objects are easy to cultivate, organisms maintenance is carried out in a strictly defined environment, they reproduce themselves rapidly, capable of parthenogenesis reproduction.

Experimental studies were carried out on the basis of teaching and research laboratory of cellular and organism biotechnology of the faculty of Biology of Zaporizhzhya National University. The effects of exogenous BAS of medicinal leech on the performance of the viability of lower crustaceans – *Daphnia magna* Straus to test the acute lethal toxicity of cultural water were investigated. Daphnia was obtained through acyclic parthenogenesis at least in the third generation. The initial population of Daphnia was fed once a day – by the suspension of green algae (Chlorella – *Chlorella vulgaris* Beijer), grown on 10% Tamiya substratum, and once a week – by the suspension of baker's yeasts.

For the experiments the following water was used: water from centralized water supply, dechlorinated, by three-days sedimentation (control); biotechnological water after culturing *H. verbana* species of leeches aged 5 months in 3 L bottles in the number of 40 specimens in five days – without dilution (experiment) were used. Before using, the biotechnological water was filtered through paper filters to remove large particles of waste products of leeches (mucus, food molting etc.). The experiment was set in three repetitions, the temperature of the analyzed samples was 20±2ºC, the oxygen concentration at the beginning of testing was at least 6 mg/dm^3 [2].

The study was conducted with a view to acute toxicity of selected samples, biotesting procedure lasted 96 hours. The analysis of daphnids' viability was determined in the following intervals: after 2, 4, 6, 12, 24, 48, 72, 96 hours from the start of the experiment. The experiment was considered to be valid if the mortality of the test-objects in the control samples was less than 10%. During the experiment, daphnids were not fed, the environment was not aerated, and biological testing was carried out in diffused light. In each of the experimental and control samples with the volume 100 ml, 10 daphnids up to 24 hours (6–24 hours), which were fed for 2–3 hours prior to testing were placed. At various stages of biotesting the number of alive daphnids was visually counted. Daphnids were considered alive if it moves freely in the water or pop up from the bottom of the vessel for 15 seconds after a light shaking, and the rest daphnids were considered dead. On the basis of counting the number of alive daphnids in the control and experiment we determined the arithmetical means that were used to calculate the number of daphnids deaths in the experiment relative to the control (test-parameter, the index of acute toxicity, toxicity index) by the formula [5]:

$$A = (X_c - X_e) \times 100 / X_c \%$$

where: A – the number of dead daphnids in experiment relative to control (toxicity index), %; X_c – the arithmetic mean of the number of alive daphnids in control specimens; X_e – the arithmetic mean of the number of alive daphnids in experiment specimens. The accuracy of parallel measurements ranged from 5–10%. Samples of water were considered such that have acute lethal toxicity if A–index was 50% or more [2, 5].

The statistical processing of the experimental data was performed using the application package SPSS v. 20.0, using parametric and nonparametric statistical methods. Checking the normality of the distribution was carried out using one-sample Kolmogorov-Smirnov test, at the normal distribution using parametric statistical methods – statistical significance of differences between experiments and controls were evaluated by Student two-sample criterion (t-test for independent samples. The differences of the results were considered valid if P>95%, p<0.05.

Results and discussion

For evaluation of the quality of water physics-chemical, hydrobiological indicators, toxicity for living organisms were defined using techniques of biological indicators. However, physical and chemical methods for assessing water quality do not always provide adequate information about its suitability for practical use. Biological methods are less accurate than chemical, but more informative, by reference to determine the overall toxicity of water and its adverse effects on aquatic organisms. By the definition, after L.P. Braginskiy [1], the test-objects are «... replacements of complex chemical analysis, which allow you to establish quickly the toxicity (toxicity, hazards) of water pollution (“yes” or “no”) whether it is due to the presence of a substance or a complex of substances».

Daphnia is a bioindicator organism that belongs to the lower crustaceans, namely cladoceran crustaceans. By nature of nutrition they are filter-feeders organisms, they naturally feed with water bacteria, unicellular algae, detritus, dissolved organic matter [7].

At the beginning and after the experiment we determined a pH-value (pH) and dissolved oxygen in the studied samples [5], which were 7,4 and 8,5 mg/dm^3 respectively at the beginning of the experiment in control samples; 8,1, and 7,0 mg/dm^3 respectively in experiment. At the end of the experiment (after 96 hours) a pH was 7,6 and oxygen was 8,2 mg/dm^3 in control samples, when in experiment pH was 8,1 and oxygen was 6,6 mg/dm^3. These figures are subject to the requirements of methods, so testing is correct. The results of the determination of the acute toxicity of biotechnological water from the medicinal leech on the organism of the test-object (*D. magna* Straus) are presented in table 1. Indicator of acute toxicity of biotechnological water from the medicinal leech without dilution, compared with controls, in the early hours of the experiment (4, 6 hours) is 3,3% and further increases to 6,7% after 12 hours, to 33,3% after 24 hours, to 43,3% after 48 hours, and to 60,0 and 69,0% after the 3rd day of cultivation (at 72 and 96 hours respectively), which can be observed at acute toxicity.

Table 1

The number of alive specimens of *Daphnia magna* Straus *(Cladocera*, *Crustacea)* in the culture water from the biotechnology medicinal leech

Time of cultivation, hours	Control, 3 repetitions , M±m	Cultural water at the biotechnology of medicinal leech, 3 repetitions	
		M±m	A, %
2	10±0	10±0	0
4	10±0	9,67±0,333	3,3
6	10±0	9,67±0,333	3,3
12	10±0	9,33±0,333	6,7
24	10±0	6,67±0,333*	33,3
48	10±0	5,67±0,333*	43,3
72	10±0	4,0±0,577*	60,0^{AT}
96	9,67±0,333	3,0±0,577*	69,0^{AT}

Note: A – the number of dead in the daphnia experiment relative to control (a measure of acute toxicity), %; * – reliable differences compared to control at p≤0,001; AT – definition of outcome – acute toxicity, M – mean; m – standard error mean.

So as acute toxicity testing of biotechnological water from the medicinal leech without dilution was over 50%, according to the performed procedure a quantitative assessment of the toxicity of biotechnological water dilution of 1 : 2 and 1 : 3 (biotechnological water: defecated faucet water). In a dilution of 1 : 2 we found acute lethal toxicity, which was 50% in 96 hours and at a dilution of 1 : 3 – minimum lethal toxicity was found, which was 10%. When diluted 1 : 3, Daphnia looked lively and healthy, compared with controls, which may indicate a stimulating effect of toxicants at low concentrations [3].

Acute toxicity to daphnids shows a set of symptoms, which are observed visually, such as mortality rates (sinking to the bottom of bottle, convulsions, immobilization, death), reflex and behavioral responses (rotation around its axis, the disordered movements), reduced heart rate [3]. It is clear that any substance at sufficiently high concentrations and prolonged exposure can affect aquatic organisms, revealing damaging and depressing effect.

The morphological features of Daphnia after experiment were observed under the microscope, using slides with holes. The results are presented in table 2. At the end of the biotesting (after 96 hours) 29 out of 30 daphnids that were cultured in control samples were available for morphological studies, and 12 of 30 daphnids that were cultured in biotechnological water from the leeches, including 9 alive and 3 visually dead. Visually there were recorded the differences in sizes, active movements of the test organisms, that is why we analyzed not only indicator of acute toxicity, but also morphological characteristics of daphnids, such as body size (length, width, mm), motor activity, the presence of food in the digestive system. It should be noted that within two days of biotesting we noted an increase of the size of daphnids in the experimental samples of cultures.

Table 2

The morphometric parameters of Daphnia after biotesting (after 96 hours of cultivation) in experimental and control samples

Group	Statistical indicators	Length of the body, mm	Width of the body, mm
Control, n=29	M±m (SD)	1,58±0,034 (0,185)	0,85±0,026 (0,145)
	Variance (dispersion)	0,034	0,021
	CV, %	11,71	17,05
	CI (95%)	1,51–1,65	0,80–0,90
Experiments, n=9	M±m (CB)	2,18±0,028* (0,084)	1,55±0,038* (0,115)
	Variance (dispersion)	0,084	0,013
	CV, %	3,85	7,42
	CI (95%)	2,12–2,24	1,47–1,63

Note: * – reliable differences at p≤0,001; M – mean; m – standard error mean, SD – standard deviation, CV – coefficient of variation, CI – confidence interval of the difference. Index with the lowest CV has the least variability.

Daphnids (29 specimens) that were cultivated in defecated faucet water (control), after 96 hours from the start of the experiment had the following morphological characteristics: the study under the microscope showed active body movements, antennas, digestive system was filled with food, periodic discharge of feces was observed. Morphometric parameters: length of the body – mean ± standard error mean (standard deviation) – 1,58±0,034 (0,185) mm, with confidence interval (95%) – 1,51–1,65 mm, width of the body – 0,85±0,026 (0,145) mm, the confidence interval – 0,80–0,90 mm.

Daphnids (9 specimens) that were cultivated in the cultural water from the medicinal leech (experiment), after 96 hours from the beginning of the biotesting had the following morphological characteristics: unlike control, weak, gestures were noted, antennas mainly were folded, sometimes eye movements, small amount of food in the digestive system (more rarely absent) were observed; there was a discharge of feces, but less intense than in the control. Morphometric parameters were statistically significantly higher than control (p≤0,001): length of the body was – 2,18±0,028 (0,084) mm, with confidence interval (95%) – 2,12–2,24 mm, width of the body – 1,55±0,038 (0,115) mm, with confidence interval – 1,47–1,63 mm.

In addition, in experimental samples we analyzed morphological state of daphnids (3 specimens) which didn't show visually vital signs, according to the methodology such daphnids were considered to be dead. Their morphological features include: visual immobilization, under the microscope: isolated body movements were observed in one of them; there was a small amount of food in the digestive system. Two others were immobilized; the food in the digestive system was practically absent. Average size of their bodies was in the range: length – 2,04–2,28 mm, width – 1,32–1,74 mm.

Increase of average body size of studied daphnids, compared with control ones can be explained, firstly, by the fact that in the early stages of cultivating daphnids were fed with additional food (detritus, bacteria, dissolved organic matter) available in biotechnology water from the medicinal leech and secondly, by a direct stimulating effect of BAS of medicinal leech on the metabolism of Daphnia tissue cells, which coincides with the data of positive therapeutic effects of hirudotherapy in humans [4] and small cattle [8]. However, most of the BAS are dose-dependent, their effects depend on the dose and duration of action, that is why with increasing of dose and duration of action, stimulating effect was replaced by inhibitory one, and simultaneously with the stimulating effect daphnias' die-off was observed. This dose-dependent and prolonged action of BAS in subsequent cultivation (3–4 days) is replaced by acute toxicity and leads to the elimination of daphnids, starting with the most sensitive individuals. So, gradually substances depot that provided compensatory reaction in daphnids exhausted and accumulation of toxic intermediate and final by products and accumulation of toxins caused the transition of physiological changes into pathology and shellfish death. One of the reasons for the subsequent death of daphnids may be a violation of microbial symbiocenosis, including intestinal. It was found that among BAS of biotechnological water from the medicinal leech, there is a matter of antibiotic-like activity [4]. Representatives of pathogenic and saprophytic microflora are sensitive to it [10]. Taking into account, that daphnids were in the biotechnological water, the high concentration of BAS, which always influences on the metabolism of cells and intestinal microflora, thereby inhibits microbial symbionts. Toxic effects recorded by biotesting, provide an assessment of all the ingredients that affect the physiological, biochemical and genetic features of test organisms [3]. Taking into account the complexity of influence of various substances that are contained in biotechnological water from the leech, it is difficult to speculate on the nature of the size increase on the stages of Daphnia biotesting. However, further studies will allow setting amidst BAS of the biotechnological water from the medicinal leech, some stimulating and inhibiting components. It will be important for the determination of their effects on biotic relationships in biocenosis with subsequent application of cultural water and its components in preventive and curative medicine and veterinary medicine.

Conclusions

1. Acute toxic effects of biotechnological water from the medicinal leech to daphnia were revealed: after 72 hours toxicity index was 60,0%, after 96 hours – 69,0%. This suggests that exogenous BAS of medicinal leech contain toxic ingredients that affect directly – the metabolism of cells or indirectly – through bacteriostatic effect on symbionts, and thus affect the cladoceran crustaceans.

2. The increase of average body size of studied daphnids, compared with controls, indicates the presence of stimulating substances in biotechnological water from the medicinal leech, and substances that can serve as an additional source of food.

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ЖИТТЄЗДАТНІСТЬ НИЖЧИХ РАКОПОДІБНИХ ПІД ВПЛИВОМ БІОЛОГІЧНО АКТИВНИХ РЕЧОВИН БІОТЕХНОЛОГІЧНОЇ ВОДИ З-ПІД МЕДИЧНОЇ П'ЯВКИ (*HIRUDO VERBANA*)

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Організм медичної п'явки продукує комплекс унікальних біологічно активних речовин, які виділяються у водне середовище разом із продуктами життєдіяльності і проявляють широкий спектр терапевтичних ефектів. Метою роботи було дослідження впливу біологічно активних речовин біотехнологічної води з-під п'явок у штучних екосистемах на виживання нижчих ракоподібних (*Daphnia magna* Straus). Визначали гостру летальну токсичність біотехнологічної води з-під медичної п'явки щодо тест-об'єктів. Показник гострої токсичності, порівняно з контролем, вже в перші години досліду (4–6 годин) становить 3,3% і в подальшому зростає, а починаючи з третьої доби (через 72 та 96 годин) відзначається гостра токсичність на рівні 60,0 і 69,0%, відповідно. Дафнії, які культивувались у біотехнологічній воді з-під медичної п'явки, відрізнялися морфологічно від контрольних: на початкових етапах біотестування вони набували більших середніх розмірів тіла і проявляли більшу активність.

Ключові слова: біотестування, медична п'явка, біологічно активні речовини, тест-організм, гостра токсичність.

ЖИЗНЕСПОСОБНОСТЬ НИЗШИХ РАКООБРАЗНЫХ ПОД ВЛИЯНИЕМ БИОЛОГИЧЕСКИ АКТИВНЫХ ВЕЩЕСТВ БИОТЕХНОЛОГИЧЕСКОЙ ВОДЫ ИЗ-ПОД МЕДИЦИНСКОЙ ПИЯВКИ (*HIRUDO VERBANA*)

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Организм медицинской пиявки производит комплекс уникальных биологически активных веществ, которые выделяются в водную среду вместе с продуктами жизнедеятельности и проявляют широкий спектр терапевтических эффектов. Целью работы было исследование влияния биологически активных веществ биотехнологической воды из-под пиявок в искусственных экосистемах на выживание низших ракообразных (*Daphnia magna* Straus). Определяли острую летальную токсичность биотехнологической воды из-под медицинской пиявки в отношении тест-объектов. Показатель острой токсичности, по сравнению с контролем, уже в первые часы опыта (4–6 часов) составляет 3,3% и в дальнейшем возрастает, а начиная с третьих суток (через 72 и 96 часов), отмечается острая токсичность на уровне 60,0 и 69,0%, соответственно. Дафнии, которые культивировались в биотехнологической воде из-под медицинской пиявки, отличались морфологически от контрольных: на начальных этапах биотестирования они приобретали более крупные размеры тела и были более активными.

Ключевые слова: биотестирование, медицинская пиявка, биологически активные вещества, тест-организм, острая токсичность.