THE EFFECTS OF PURE MICROCYSTIN LR AND BIOMASS OF BLUE-GREEN ALGAE ON SELECTED IMMUNOLOGICAL INDICES OF CARP (*Cyprinus carpio* L.) AND SILVER CARP (*Hypophthalmichthys molitrix* Val.)

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Abstract

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The aim of this work was to study the effects of Microcystin LR and biomass of blue-green algae with known amount of Microcystin LR on immunological indices in juvenile carp and silver carp.

The pure Microcystin LR or biomass of blue-green algae with a known amount of Microcystin LR was applied to experimental fish. Pure Microcystin LR in the dose of 400 μg per kg of body mass was administered intraperitoneally, the biomass *per os* to carp and *per anus* to silver carp. The volume of Microcystin LR in single doses was 3, 300, 600 and 1 200 μg per kg of body mass. After 24 and 48 h blood was collected from fish by cardiac punction. The following immunological indices were determined: total leukocytes, leukocrit, differential leukocyte count, numbers of sIg+ and T cytotoxic lymphocytes and occasionally numbers of NK cells by means FITC protein A, HP lectin and monoclonal antibody anti CD 11b and phagocyte activity by luminol enhanced chemiluminiscence.

Decrease of total leukocyte count and leukocrit were characteristic changes due to the treatment. With carp this decrease occurred mainly in lymphocytes (Tcytotoxic and sIg+ cells), with silver carp mainly in myelocytes (neutrophilic myelocytes and metamyelocytes). With carp the most remarkable changes were observed after oral administration of biomass of blue-green algae containing Microcystin LR in the dose of 1 200 μg per kg of body mass. On the other hand, minor changes were observed after i.p. administration of pure toxin. These changes correspond with changes in phagocytic activity of cells: minor changes were detected in carp, while in silver carp the decrease of myelocyte cells brought a major decrease of phagocytic activity.

Cyanotoxins, leukocytes, lymphocytes, phagocyte activity

Toxins of blue-green algae (cyanotoxins) are substances of secondary metabolism. They are released into the water during the disintegration of blue-green algae. They are more toxic than toxins of higher plants and fungi and less toxic than bacterial toxins (Maršálek and Turánek 1996).

In human and veterinary medicine cases of health damage or poisoning have been known (Falconer 1989; Carmichael 1992). Cyanotoxins cause an impairment of the immune system, torpidity and complete weakness, vomiting and digestion problems, respiratory and allergic diseases, damage of the liver and other health problems. They also play an important role in process of cancerogenesis (Bell and Codd 1994).

The influence of cyanotoxins on fish following experimental intoxications or the impact of the environment containing cyanotoxins on fish have been studied by a number of authors

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using clinical, pathomorphological, histological, ultrastructural, haematological and biochemical methods (Bruno et al. 1989; Garcia 1989; Råbergh et al. 1991; Johnston et al. 1994; Rodger et al. 1994; Tencalla et al. 1994; Carbis et al. 1996ab; Bury et al. 1997; Navrátil et al. 1997ab).

Immunosuppressive effects of cyanotoxins have been explored and proven e. g. in mice and humans. Mundt et al. (1991) compared the influence of extracts from four various kinds of cyanobacteria and they describe the inhibition of the number of plaque-forming cells in mice after i.p. administration of higher concentrations of toxins. On the other hand, lower concentrations of toxins stimulated the immune system. To our knowledge, there is no data available on the impact of cyanotoxins on the immune system of fish.

The aim of this work was therefore to study the effects of Microcystin LR and biomass of blue-green algae with known amount of Microcystin LR on juvenile carp (*Cyprinus carpio* L.) and silver carp (*Hypophthalmichthys molitrix* Val.) immunological indices.

Materials and Methods

A total of 83 juvenile carp with average mass of 168g (110-380) and 44 silver carp with average mass of 1 077 g (304-1 660 g) were used in the experiment. Before the experiment fish had been kept at least for five days in laminated 1 m^3 tanks in the water at a temperature of 18 °C equipped with aeration. Fish were fed a commercial pelleted food for carp. The feeding had been always finished two days before the experiment. The experimental and control groups consisted of 6-8 individuals each. The experiments were finished in 24 and 48 h. For i.p. administration pure Microcystin LR (Veterinary Research Institute, Brno, Czech Republic) in the solution of HEPES in the dose 400μg per kg of body mass was used (1 ml containing 100 μg of Microcystin LR). For oral, and *per anus* administration (in silver carp) three kinds of biomass with known amount of Microcystin LR were used. The analysis of biomass was conducted by means of HPLC. Individual doses of biomass were administrated according to the content of Microcystin LR per os to carp in doses 3, 300, 600 and 1 200 μg per kg of body mass and per anus to silver carp in doses 3 and 300μg per kg of body mass. The volume of one-dose biomass was 3.0-3.6 ml. For the required volume the biomass was diluted with distilled water. To obtain the total dose of 1 200 μg of Microcystin LR per kg of body mass, the biomass was divided into two doses administered in 24 h intervals. For the experiments with doses 400, 600 and 1 200 μg of Microcystin LR per kg of body mass the fish were obtained in July (water temperature about 20 °C), and for the experiments with doses 3 and 300 μg of Microcystin LR per kg of body mass the fish were obtained in December (water temperature about 8 °C). The biomasses dosed in 3 and 300 μg of Microcystin LR per kg of body mass contained besides Microcystin LR also other unidentified substances. Leukocrit, total leukocyte count and differential leukocyte count were evaluated according to Svobodová et al. (1986). Furthermore, the lymphocytes were separated from the blood by discontinual gradient centrifugation and incubated with FITC protein A for the evaluation of numbers of sIg+ lymphocytes (Surolia et al. 1981) and with HP lectin for the evaluation of numbers of cytotoxic lymphocytes (Mattes and Holden 1981; Poros et al. 1983; Kovářů 1987). Further the monoclonal antibody anti CD 11b was used. In addition to monocytes and granulocytes, this antibody links to the external antigen of NK cells. It is a rat monoclonal antibody against mouse antigen CD 11b, which reacts in a specific cross way with fish (Palíková et al. 1998). The numbers of cells, HP lectin and protein A were selected on the basis of preceding studies (Palíková et al. 1995). The evaluation was conducted by fluorescence cell sorter - FACS. The evaluation of phagocyte activity by luminol enhanced chemiluminiscence were conducted by the modified method according to Kubala et al. (1996). Spontaneous and activated chemiluminiscence (CL) by opsonized zymozan were evaluated. Zymozan was opsonized by method according to Leino and Lillius (1992). The kinetics of chemiluminiscence was measured for 77 min at the temperature of 27 °C by means of Luminometr 1251. The measured data included peak of CL (mV), the time of peak (s) and the integral of CL (mV/s). Peak and integral of CL were transferred to 1000 of phagocytes. The statistic evaluation of the data was carried out by help of the hypothesis test of two independent groups (T - test). The statistic program STAT PLUS 1.01 was used for the evaluation.

Results

The results of our study are presented in Tables 1 through 4.

Minor or major decrease of total leukocyte counts (Table 1) and leukocrit were characteristic for changes in white blood cells (leukocrit corresponded with total

Table 1
Total leukocytes and absolute numbers of lymphocytes in 1 μl.10^3

			Total leukocytes					Numbers of lymphocytes				
			Microcystin LR μg·kg^{-1} of body mass									
			3	300	400ip	600	1200	3	300	400ip	600	1200
Carp												
24h	exp.	mean	39.0	45.1		36.1	48.6	35.3	39.6		22.8	29.1
		±SD	*19.5*	*19.6*		*13.5*	*15.2*	*19.4*	*19.7*		*10.9*	*8.1*
	cont.	mean	64.5	64.5		58.3	58.3	58.5	58.5		46.6	46.6
		±SD	*13.1*	*13.1*		*13.0*	*13.0*	*11.5*	*11.5*		*13.4*	*13.4*
48 h	exp.	mean	35.6	37.5	46.8	55.5	41.5	32.1	32.6	30.2	41.7	31.5
		±SD	*16.3*	*12.5*	*21.7*	*29.6*	*21.7*	*14.2*	*11.4*	*22.8*	*28.0*	*24.2*
	cont.	mean	70.1	70.1	74.3	28.3	58.3	65.7	65.7	63.0	46.6	46.6
		±SD	*29.9*	*29.9*	*31.3*	*13.0*	*13.0*	*27.7*	*27.7*	*30.4*	*13.4*	*13.4*
Silver carp												
24 h	exp.	mean	9.6	11.0				3.9	5.3			
		±SD	*4.2*	*3.1*				*2.4*	*2.0*			
	cont.	mean	17.6	17.6				2.9	2.9			
		±SD	*5.1*	*5.1*				*1.2*	*1.2*			
48 h	exp.	mean	9.4	8.0	19.8			1.7	1.4	3.1		
		±SD	*3.9*	*2.8*	*8.0*			*0.7*	*0.5*	*1.2*		
	cont.	mean	13.4	13.4	14.7			2.2	2.2	4.5		
		±SD	*3.1*	*3.1*	*4.6*			*0.5*	*0.5*	*1.5*		

$p \leq 0.01$

$p \leq 0.05$

leukocytes). With carp this decrease was affected mainly in lymphocytes (T cytotoxic and sIg+ lymphocytes –Tables 1 and 2), with silver carp mainly in myelocytes (neutrophilic myelocytes and metamyelocytes – Table 4). With carp major changes have been observed following per os administration of biomass of blue-green algae containing Microcystin LR in the dose of 1 200 μg per kg of body mass. On the other hand, minor changes have been observed following i.p. administration of pure toxin. These changes correspond with changes in phagocyte activity of cells: minor changes in carp, while in silver carp the decrease of myelocyte cells brought a major decrease of phagocyte activity (Table 3).

Discussion

From the results stated above it is obvious that the influence of water bloom of blue-green algae on fish is dependent not only on the amount of Microcystin LR in biomass but also on other unidentified substances present in biomass of blue-green algae (this is why biomass containing only 3 μg of Microcystin LR per kg of body mass had a similar effect as biomass containing higher amount of this toxin). Further it may be concluded that Microcystin LR alone damages mainly hepatopancreas and affects red blood cells, while the immune system is being influenced by other substances of biomass whose effect can be multiplied by their interaction. Certain differences are in the influence of biomass of blue-green algae to

Table 2
Absolute numbers of sIg+ and T cytotoxic lymphocytes in 1 μl.10³

			Numbers of sIg+ lymphocytes					Numbers of T cytotoxic lymphocytes				
			Microcystin LR μg·kg⁻¹ of body mass									
			3	300	400ip	600	1200	3	300	400ip	600	1200
Carp												
24h	exp.	mean	3.6	3.5		3.6	4.0	4.0	2.6		2.0	2.7
		±SD	*1.8*	*1.5*		*2.4*	*1.3*	*3.0*	*1.4*		*1.9*	*1.4*
	cont.	mean	6.0	6.0		9.1	9.1	5.3	5.3		20.4	20.4
		±SD	*2.3*	*2.3*		*5.1*	*5.1*	*2.4*	*2.4*		*16.9*	*16.9*
48 h	exp.	mean		4.4	3.9	2.4	3.8		2.8		2.0	3.8
		±SD		*2.5*	*4.1*	*1.6*	*3.3*		*2.0*		*2.3*	*5.7*
	cont.	mean		6.0	3.8	9.1	9.1		5.3		20.4	20.4
		±SD		*2.3*	*3.1*	*5.1*	*5.1*		*2.4*		*16.9*	*16.9*
Silver carp												
24 h	exp.	mean	0.4	0.3				1.1	1.2			
		±SD	*0.3*	*0.2*				*0.9*	*0.4*			
	cont.	mean	0.1	0.1				1.0	1.0			
		±SD	*0.1*	*0.1*				*0.9*	*0.9*			
48 h	exp.	mean	0.2	0.3	1.4			0.9	0.8			
		±SD	*0.2*	*0.3*	*0.3*			*0.4*	*0.4*			
	cont.	mean	0.1	0.1	1.1			0.7	0.7			
		±SD	*0.1*	*0.1*	*0.5*			*0.8*	*0.8*			

$p \leq 0.01$

$p \leq 0.05$

immune parameters in carp and in silver carp. The decrease of lymphocytes affected both species. These data correspond with data quoted in studies by Navrátil et al. (1997ab), Vajcová et al. (1997). While in carp leukocytes are decreased in lymphocytes, in silver carp the decrease affects especially myelocytes, while on the other hand lymphocytes seem to be stimulated. In lymphocytes of carp was the effect concentrated to specific immune response of cellular and humoral type. The greatest differences have been found in T cytotoxic lymphocytes which represent the most significant group of cytotoxic cells, which are part of specific cellular immunity (Šterzl 1993). Also specific humoral response has been affected which was reflected in the decrease of sIg+ lymphocytes. These results correspond to a certain extend with the results obtained in mammals (Mundt et al. 1991). This study has shown an immunosuppressive effect in specific immune response. Most likely in the immune area the effect of toxin in fish will be similar to that in mammals. However, this is just a rough comparison, because these authors used different methods for the finding out of immunosuppressive effects of cyanotoxins. Nevertheles in silver carp it has to be said that leukocrit and total leukocyte count were 3-5 times lower and i.e. in control groups. This fact might have been caused by a later catch of silver carp (water temperature about 8 °C) and seasonal dynamics in the amount of leukocytes connected with it. Also differential leukocyte counts of silver carp are of different character. After transferring differentional leukocyte counts into absolute numbers and comparing them with numbers of cells in carp it is shown that myelocyte cells are in a certain surplus while on the other hand the number of lymphocytes is drastically reduced. However, the question still remains whether this nonphysiological relationship is caused by stress or whether the relationship in

Table 3
The peak of chemiluminiscence of transferred phagocyte activity in m.V

			Spontaneous phagocyte activity					Activated phagocyte activity				
			Microcystin LR μg·kg^{-1} of body mass									
			3	300	400ip	600	1200	3	300	400ip	600	1200
Carp												
24h	exp.	mean	0.73	0.36				6.98	1.01		0.09	0.09
		±SD	*0.58*	*0.24*				*9.11*	*0.75*		*0.06*	*0.05*
	cont.	mean	0.48	0.48				3.26	3.26		0.07	0.07
		±SD	*0.39*	*0.39*				*5.19*	*5.19*		*0.06*	*0.06*
48 h	exp.	mean	0.25	0.26	0.10			1.58	1.35	4.14	0.11	0.07
		±SD	*0.16*	*0.13*	*0.07*			*1.16*	*0.59*	*0.90*	*0.06*	*0.05*
	cont.	mean	0.50	0.50	0.13			2.65	2.65	2.81	0.07	0.07
		±SD	*0.22*	*0.22*	*0.07*			*1.07*	*1.07*	*0.95*	*0.06*	*0.06*
Silver carp												
24 h	exp.	mean	0.66	0.32				44.65	26.78			
		±SD	*0.64*	*0.16*				*14.59*	*38.64*			
	cont.	mean	1.01	1.01				43.84	43.84			
		±SD	*0.68*	*0.68*				*35.08*	*35.08*			
48 h	exp.	mean	0.47	0.88	0.13			41.72	78.38	12.01		
		±SD	*0.31*	*0.36*	*0.04*			*32.48*	*14.86*	*5.76*		
	cont.	mean	1.41	1.41	0.24			43.64	43.64	7.48		
		±SD	*0.66*	*0.66*	*0.14*			*15.29*	*15.29*	*2.67*		

$p \leq 0.01$

$p \leq 0.05$

the winter season life in cold water in these fish brings about a decrease in the number of lymphocytes. The third variant could be a combination of both proceding variants. A certain difference can be noted in phagocyte activity of leukocytes. While in carp minor changes occurred more in the sense of decreasing of chemiluminiscence after 48 h, in silver carp major changes occurred already after 24 h, and after 48 h a certain increase related only to application of dose 300 μg of Microcystin LR per kg of body mass in biomass. These results in silver carp are most likely caused by a higher representation of more mature forms of neutrophilic granulocytes i.e. by a higher potential for releasing loose radicals after stimulation.

Vliv čistého microcystinu LR a biomasy sinic na vybrané imunologické ukazatele kapra (*Cyprinus carpio* L.) a tolstolobika (*Hypophthalmichthys molitrix* Val.)

Cílem práce bylo zjistit vliv microcystinu LR a biomasy sinic se známým obsahem microcystinu LR na imunologické ukazatele násady kapra obecného a tolstolobika bílého.

Pokusným rybám byl aplikován čistý microcystin LR nebo biomasa sinic se známým obsahem microcystinu LR. Aplikace čistého microcystinu LR v dávce 400 μg · kg^{-1}ž. hm. byla prováděna intraperitoneálně, biomasa byla aplikována kaprům perorálně a tolstolobikům peranálně. Obsah microcystinu LR v jednotlivých dávkách byl 3, 300, 600 a 1 200 μg · kg^{-1} ž. hm. Po 24 a 48 hodinách po aplikaci byla rybám odebrána krev. Byl stanovován počet leukocytů, leukokrit, diferenciální rozpočet, počet sIg+ lymfocytů

Table 4
Differential leukocyte counts of fish after application of 3 and 300 μg of Microcystin LR in biomass per 1 kg of body mass

Carp	3 μg	300 μg	Control
After 24 h	Mean ± SD (%)		
Blasts (mono-, myelo-, lympho-)	1.19 ± 0.86	0.94 ± 0.98	0.69 ± 0.83
Monocytes	0.63 ± 0.65	1.63 ± 0.86	1.13 ± 0.54
Neutrophilic myelocytes	2.06 ± 1.63	1.88 ± 0.96	1.56 ± 1.38
Neutrophilic metamyelocytes	4.50 ± 5.45	5.44 ± 3.46	3.31 ± 1.60
Band neutrophil	2.88 ± 2.12	2.38 ± 1.45	1.38 ± 1.17
Segmenter neutrophil	0.94 ± 0.13	1.50 ± 1.54	0.94 ± 0.95
Lymphocytes	87.75 ± 9.96	86.85 ± 4.92	91.00 ± 2.37
After 48 h			
Blasts (mono-, myelo-, lympho-)	0.81 ± 0.83	1.63 ± 1.71	0.44 ± 0.46
Monocytes	2.63 ± 1.76	1.38 ± 0.65	1.38 ± 0.84
Neutrophilic myelocytes	3.63 ± 1.90	3.50 ± 2.65	1.69 ± 1.90
Neutrophilic metamyelocytes	11.88 ± 1.54	4.44 ± 3.37	1.81 ± 1.39
Band neutrophil	0.88 ± 0.33	0.88 ± 0.93	0.81 ± 0.86
Segmenter neutrophil	0.19 ± 0.24	0.88 ± 0.82	0.63 ± 0.41
Lymphocytes	90.00 ± 3.94	87.13 ± 6.75	93.25 ± 3.92
Silver carp	3 μg	300 μg	Control
After 24 h	Mean ± SD (%)		
Blasts (mono-, myelo-, lympho-)	0.50 ± 0.71	0.75 ± 0.83	0.88 ± 0.60
Monocytes	1.13 ± 0.93	1.56 ± 1.26	3.75 ± 1.85
Neutrophilic myelocytes	23.13 ± 10.82	17.19 ± 7.86	53.75 ± 8.69
Neutrophilic metamyelocytes	21.50 ± 4.77	22.50 ± 11.34	21.13 ± 6.45
Band neutrophil	13.75 ± 4.35	6.44 ± 3.42	3.50 ± 1.87
Segmenter neutrophil	0.63 ± 0.86	0.31 ± 0.66	0.25 ± 0.66
Lymphocytes	39.38 ± 12.97	51.25 ± 18.26	16.75 ± 6.70
After 48 h			
Blasts (mono-, myelo-, lympho-)	2.00 ± 2.35	4.80 ± 4.58	2.00 ± 1.66
Monocytes	3.75 ± 2.22	2.00 ± 1.10	3.38 ± 2.29
Neutrophilic myelocytes	51.50 ± 10.23	34.00 ± 13.61	60.25 ± 5.47
Neutrophilic metamyelocytes	14.25 ± 5.45	28.20 ± 6.40	11.63 ± 4.55
Band neutrophil	8.25 ± 4.89	12.80 ± 3.66	5.38 ± 3.94
Segmenter neutrophil	0.13 ± 0.33	0.20 ± 0.40	0.50 ± 1.32
Lymphocytes	18.88 ± 9.01	18.00 ± 4.60	16.88 ± 3.55

$p \leq 0.01$

$p \leq 0.05$

a cytotoxických lymfocytů, případně NK buněk pomocí HP lektinu, FITC proteinu A a monoklonální protilátky anti CD 11b a fagocytární aktivita luminolem zesílenou chemiluminiscencí.

Změny v bílém krevním obraze byly charakterizovány menším či větším snížením celkového počtu leukocytů a leukokritových hodnot. U kaprů toto snížení postihovalo

zejména oblast lymfocytární (Tcytotoxické a sIg+ lymfocyty), u tolstolobiků zejména oblast myelocytární (zejména neutrofilní myelocyty a metamyelocyty). K nejvýraznějším změnám došlo u kaprů po perorální aplikaci biomasy sinic obsahující mikrocystin LR a to v dávce 1 200 $\mu g \cdot kg^{-1}$ ž. hm. Naopak k méně výrazným změnám došlo po intraperitoneální aplikaci čistého toxinu. S těmito změnami korelují i změny ve fagocytární aktivitě buněk. Zatímco u kaprů došlo k méně výrazným změnám, u tolstolobiků došlo v souvislosti se snížením myelocytárních buněk k výraznějšímu snížení fagocytární aktivity.

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