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In vivo effects of microcystins and complex cyanobacterial biomass on rats (*Rattus norvegicus* var. *alba*): Changes in immunological and haematological parameters

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ABSTRACT

Toxic cyanobacteria represent a serious health and ecological problem in drinking and recreational waters worldwide. Some previous toxicological studies investigated effects of isolated microcystins on laboratory rodents including mice and rats. However, much less attention has been paid to more realistic exposure situations such as the effects of MCs accumulated in food. The objectives of the present study were to provide a simple model simulation of the food chain in order to evaluate impacts of microcystins (MCs) on rat immune and haematologicalparameters. Impacts of feeding experimental rats with a diet containing fish meat with and without microcystins and complex toxic biomass have been studied during a 28 day exposure. Red blood cell parameters (RBC counts, haematocrit values, MCH, MCV and MCHC) showed significant differences in experimental groups ($p \leq 0.05$, $p \leq 0.01$) in comparison with the control group. We also detected an immunomodulatory effect in the experimental groups. NK cells and γδ+ T lymphocytes were significantly increased in peripheral blood in the group exposed to isolated microcystin in the food. Significant change in the ratio of CD4+ and CD8+ cells (increase of CD4+ and a drop in CD8+) was found in the group with added cyanobacterial biomass with low concentration of MCs. The greatest changes in lymphoid organs were observed in the same groups. There was an increase of spleen subpopulations of γδ+ T lymphocytes as well as of IgM+ lymphocytes (B lymphocytes) and CD8+ T lymphocytes. Indeed, the modulation of CD4+ and CD8+ of peripheral lymphocytes was associated with similar changes in thymic lymphocytic subpopulations. In summary, food containing fish meat with considerable doses of microcystins (or toxic cyanobacterial biomass) induces significant changes in RBC parameters and influence preferably innate part of the immune system represented by NK cells and by gamma-delta T cells, which are known to play role as a bridge between adaptive and innate immune response.

1. Introduction

Toxic cyanobacteria represent a serious health and ecological problem in drinking and recreational waters

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worldwide (Kuiper-Goodman et al., 1999). Besides their impact on overall water quality, cyanobacteria produce a range of bioactive and toxic metabolites with microcystins being the most widely studied (Welker and von Dohren, 2006). Bioaccumulation of microcystins in aquatic food chains has been discussed as well as possible human health impacts related to the presence of toxins in edible fish (Adamovsky et al., 2007). Various documented toxic effects of microcystins include chronic hepatocarcinogenicity (Eriksson et al., 1990), oxidative stress (Paskova et al., 2011) as well as modulations of hepatological parameters (Zikova and Kopp, 2008).

The immune system plays a major role in the overall health of both higher and lower vertebrates. Lankoff et al. (2004) using human and chicken peripheral blood lymphocytes, showed that microcystin-LR influences the production of IL-2 and IL-6 and decreases proliferation of T as well as B lymphocytes. Likewise, Hernandez et al. (2000) showed that small concentrations of cyanobacterial hepatotoxins, i.e. microcystin and nodularin, are able to significantly enhance the early adherence of resting human peripheral polymorphonuclears. Yea et al. (2001) showed that MC-LR, MC-YR and nodularin inhibit immune functions as *in vitro* polyclonal antibody forming cell (AFC) response and mitogen-induced lymphoproliferation in mice lymphocytes. In their study, the immunosuppressive effect was also demonstrated, and nodularin caused greater cellular effects on T lymphocytes. The immunosuppression was mediated, at least partly, through decreased IL-2 mRNA stability. Immunosuppression in mice was demonstrated also by Shen et al. (2003). These authors recorded the reduction of phagocytosis evaluated using the phagocytic index of peripheral phagocytes, the inhibition of LPS-induced lymphoproliferation and the dose-dependent decrease in the counts of antibody-forming cells after intraperitoneal injection of the cyanobacterial bloom extract containing MCs. In the later study, Chen et al. (2004) focused on *in vitro* determination of the effects of MC-LR on nitric oxide (NO) generation and some mice macrophage-related cytokines. The results demonstrated that NO production, mRNA levels of induced nitric oxide synthase, IL-1β, TNF-α were down-regulated by MC-LR in a dose-dependent manner and mRNA levels of GM-CSF and IFN-γ were also decreased. Shi et al. (2004) reported data on modulations of multiple cytokines following *in vivo* exposure of mice to the crude cyanobacterial extract. The results showed significantly decreased mRNA levels of TNF-α, IL-1β (pro-inflammatory cytokines) and IL-2, IL-4 and IL-10 (Th1/Th2-related cytokines), while the IL-6 level was unaffected. Teneva et al. (2005) investigated the influence of microcystin-LR on mouse B and T-lymphocyte subpopulations *in vitro*. These authors demonstrated clearly that MC-LR specifically induces apoptosis in mouse B cells, while the T cells were not affected. Kujbida et al. (2006) observed affection of human polymorphonuclear leukocytes after *in vitro* exposure of MC-LR even at concentrations lower than those recommended by the WHO as safety levels for chronic exposure. In their study, MCs influenced the direct chemotactic activity of polymorphonuclear cells, induced the oxidative burst in these cells and increased phagocytosis and killing of *Candida albicans*. Microcystins increased interleukin-8, cytokine-induced neutrophil chemoattractant-2αß in human and rat neutrophils, as well as extracellular reactive oxygen species (ROS) levels (Kujbida et al., 2008). Reactive oxygen species play a central role in the host defence and removal of cell debris. However, they can also cause tissue damage and injury (Jaeschke et al., 2002), and accumulation of activated neutrophils in the liver can greatly contribute to the pathogenesis of hepatic damage (Jaeschke and Hasegawa, 2006).

Some previous toxicological studies investigated effects of isolated microcystins on laboratory rodents including mice and rats (Zikova and Kopp, 2008). However, much less attention has been paid to more realistic exposure situations such as the effects of MCs accumulated in food such as fish meat. The objectives of the present study were to provide a simple model simulation of the food chain in order to evaluate impacts on Wistar laboratory rat physiology under different exposure scenarios. Impacts of feeding experimental rats with a diet containing fish meat with and without microcystins and complex toxic biomass have been studied during a 28 day exposure. The present study is focused on outcomes of haematological and immunological parameters.

2. Material and methods

2.1. Experimental animals and design

Experimental Wistar albino rats (males, 30 days old) were purchased from the commercial breeding company Anlab s.r.o. (Prague, Czech Republic), and acclimated for one week prior to the study under laboratory conditions. Animals kept in the experimental facility (23 °C, 12 h light/12 h dark, 60% humidity) were supplied *ad libitum* with an optimal diet for rats (i.e. mixture of wheat and starch, vitamins and minerals, lysine and soya oil meat).

As the study aimed at investigation of impacts of microcystins by simulating a simple food chain model, 25% (based on wet weight) of fish meat (carp muscle) was added to the feed. Preliminary experiments showed that the 25% content of fish meat did not affect food consumption by experimental animals. The complete feeding ration was formed on the basis of data on optimal nutrition of laboratory rats. The high content of nitrogen compounds in fish meat allowed for a maximum supplement of 25% of fish meat. The standard feeding ration was composed of wheat flour 33%, fish muscle 25%, starch 37.8%, mono potassium phosphate (MPK) 3%, mixture of vitamins 0.2%, and lysine 1%. Control feeding rations without fish meat contained a higher proportion of wheat, decrease in starch but the diet was supplemented with soya in order to maintain the nutritional value.

Following, different exposure variants were investigated according to feed, i.e.

A) Blank control (rats fed with optimal commercial diet without fish meat);
B) Control (rats fed with commercial diet with 25% of fish from the locality with no occurrence of cyanobacteria and microcystins);

C) Rats fed with commercial diet with 25% of fish from the locality with no occurrence of cyanobacteria and microcystins enriched with cyanobacterial bloom biomass (1% based on dry weight), which contained the final total nominal MCs concentration of 25,000 μg/kg of food (analysed total concentration was 26,572 μg/kg containing MC-LR 8829 μg/kg; MC-RR 15,425 μg/kg, MC-YR 872 μg/kg, MC-LF 671 μg/kg and MC-LW 775 μg/kg);

D, E) Rats fed with commercial diet with 25% of fish from the locality with no occurrence of cyanobacteria and microcystins enriched with MCs – externally added microcystins in two nominal doses corresponding to 700 and 5000 μg total MCs per kg feed wet weight (analysed MC content was 698 and 4902 μg/kg, respectively);

F) Rats fed with commercial diet with 25% of fish (common carp muscle) from the locality with heavy cyanobacterial bloom.

A total of 10 rats per exposure were kept in cages each containing 5 animals. On a random basis of tossing cage numbers, rats were allocated to exposure variants (two cages per treatment, randomly placed to avoid positional effects). Diet and drinking water were provided *ad libitum*. Food consumption was recorded on a daily basis. The study lasted 28 days. At the end, animals were weighed, anaesthetized for blood collection by cardiopuncture using heparinised syringes at the same time period (7.00 a.m.) to avoid pre-analytical variations due to circadian rhythm. After that, animals were euthanised and tissues collected for further analyses.

Weights of the major organs (liver, kidney, brain, thymus, spleen and testes) were measured. Experimental conditions were the same as described above for the period of acclimation.

Experiment was performed in compliance with the laws for the protection of animals against cruelty as approved by the Ethical Committee of the Mendel University Brno, Czech Republic.

2.2. Microcystin mixture preparation

The microcystin mixture for dosing of feed was prepared from a natural cyanobacterial bloom (dominated by *Microcystis aeruginosa*, collected during summer 2003 at the Nové Mlýny reservoir, Czech Republic). The freeze-dried material was repeatedly extracted with 50% methanol using sonication followed by solid-phase extraction on ODS cartridge (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, MA, USA). The final solution contained 139.6 μg total MCs/mL with three dominant MC variants (MC-LR 47.5 μg/L (34%), MC-RR 86.6 μg/L (62%) and MC-YR 5.6 μg/L (4%)), and it was stored at −18 °C until use.

2.3. Analyses of microcystins

Concentrations of microcystins in cyanobacterial samples used for the preparation of feed rations were analysed as described previously using high performance liquid chromatography HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) with Supelcosil ABZ Plus column (150 × 4.6 mm, 5 μm; Supelco) at 30 °C with UV detection (Babica et al., 2006). The binary gradient of the mobile phase consisted of (A) water and (B) acetonitrile. Both A and B contained 0.1% TFA. We used a linear gradient increase with 20% B at 0 min to 59% B at 30 min using the flow rate of 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector and MCs were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations.

The prepared rat tissues were analysed for MCs by the Agilent LC–MS/MS system with Agilent 6410 Triple Quad mass spectrometer (Kohoutek et al., 2010). Tissue (frozen sample; 0.5 g fresh weight) was homogenised three times with methanol (3 mL), sonicated in an ultrasonic bath for 30 min, and centrifuged at 2900 *g* for 10 min. Supernatants were pooled and extracted repeatedly (3 times) with hexane (1 mL) to remove lipids. Extract was evaporated at 50 °C, and the residue was dissolved in 300 μL of 50% aqueous methanol (v/v) and used for LC–MS/MS analyses.

Analyses were based on liquid chromatography mass spectrometry (MS/MS) with MRM (multiple reaction monitoring mode) using the HPLC apparatus Agilent 1200 series (Agilent Technologies, Waldbronn, Germany), which consisted of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 30 °C. The column was a Supelcosil ABZ + Plus RP-18 endcapped (5 μm) 150 × 4.6 mm i.d. (Supelco). A SecureGuard C18 (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM ammonium acetate in water, pH 4 (A) and acetonitrile (B). The binary pump gradient was linear (increase from 20% B at 0 min to 59% B at 30 min, then 90% B for 15 min); the flow rate was 0.4 mL/min 20 μL of individual sample was injected for the analyses. The mass spectrometer was an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, Waldbronn, Germany) with electrospray ionisation (ESI). Ions were detected in the positive mode. The ionisation parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350 °C; desolvation gas flow, 11 L/min. The transitions from the protonated molecular ion to a fragment of aminoacid Adda (unusual aminoacid present only in microcystins and related nodularins – (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) at *m/z* 135.2 and fragment at *m/z* 127.1 were monitored in multiple reaction monitoring (MRM) mode. Collision energies (CE, V) used for fragmentation were 50 V for MC-RR and respective conjugates, and 40 V for MC-YR and -LR and respective conjugates. Quantification of analyses was based on external standards of MC-RR, MC-YR, MC-LR in matrix (final extract of microcystin-free fish tissue). Method detection limit (MDL; per gram of tissue, fresh weight) was 3 ng/g in MRM mode.

2.4. Haematological parameters

Red blood cell counts (RBC count), haematocrit value and haemoglobin concentration and platelet counts were measured and following indexes were calculated: mean corpuscular volume (MCV), mean corpuscular haemoglobin

(MCH), and mean corpuscular haemoglobin concentration (MCHC). Haematological examinations were performed using a semiautomated electronic impedance analyser (MEK-5208K, Nihon Kohden, Japan).

2.5. Immunological evaluation

The rat liver and thymus were weighed and disintegrated using a fine nylon mesh. The contaminating red blood cells were lysed with ammonium chloride solution (154.4 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, all from Sigma–Aldrich, St. Louis, USA), leukocyte suspension was then washed with cell washing solution (CWS, phosphate buffered saline containing 1.84 g/L EDTA, 1 g/L sodium azide and 4 mL/L gelatin, all from Sigma–Aldrich) and the final leukocyte count was ascertained.

The peripheral blood leukocytes were prepared in the following way: Fifty microlitres of rat peripheral blood were collected and erythrocytes lysed with an ammonium chloride solution. Blood leukocytes were washed with cell washing solution and the final leukocyte count was ascertained. The total white blood cell count was ascertained using an autohaematology analyser (BC-2800Vet, Shenzhen Mindray Bio-Medical Electronics, Shenzhen, People's Republic of China). The 1×10^6 leukocytes were stained with two different mixtures of primary antibodies for 15 min at 4 °C. T-lymphocytes, B-lymphocytes and NK cells were distinguished by R-PE-conjugated anti-CD3 (clone eBioG4.18, eBioscience, USA), FITC-conjugated anti-IgM (clone HIS40, eBioscience, USA), AlexaFluor647-conjugated anti-CD161 (clone 10/78, Biolegend, USA) and unconjugated anti-CD8α (clone G28, Biolegend, USA) antibodies. Various T-lymphocyte subpopulations were distinguished by R-PE-conjugated anti-CD3, FITC-conjugated anti-CD4 (clone W3/25, Biolegend, USA), AlexaFluor647-conjugated anti-γδTCR (clone V65, Biolegend, USA) and unconjugated anti-CD8α antibodies.

Staining of anti-CD8α antibody with secondary PE-Cy5.5-conjugated goat anti-mouse IgG2a antibody (Invitrogen, USA) was performed for 20 min at 4 °C after washing with CWS. The cells were finally washed with CWS and measured immediately by FACS Calibur flow cytometer (Becton Dickinson, USA). At least 40,000 events were acquired per sample. Postacquisition analysis of data was performed using Summit software (DAKO, Denmark).

2.6. Histopathology examination

Samples of thymus and spleen were collected and placed in 10% buffered formalin during necropsy. Samples were treated using a routine histological technique and embedded in paraffin. Sections of 5 μm thick were made from the paraffin blocks, and these were stained with haematoxylin and eosin.

2.7. Statistics

Statistica for Windows® 7.0 (StatSoft, Tulsa, OK, USA) was used to compare differences among treatment groups using one-way analysis of variance (ANOVA) and post-hoc analysis of means by the LSD test. The homogeneity of variances was tested by Levene's test. In these cases, the non-parametric Kruskall–Wallis and Mann–Whitney tests were used for the comparison of treatment groups. Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively, for all tests.

3. Results

3.1. Daily intake of microcystins

Based on the food consumption, average doses of microcystins administered orally in food could be calculated for individual groups, and they are presented in Table 1. Although considerable doses of microcystin have been consumed by the animals, only minor MCs concentrations around limit of detection (3–5 ng/g f.w) have been detected in liver of six rats in group fed with biomass sample (group C) and in liver of 3 rats in group exposed to higher dose of isolated microcystin mixture (group E).

3.2. Haematological parameters

Because significant differences were found between groups A (conventional feed) and B (group fed with 25% of fish meat from the environment without cyanobacteria), the group B was taken as the control group for other investigations. The composition of diet in other groups (C–F) differed from the group B in other added components only (i.e. MCs, biomass or fish meat from the environment with cyanobacterial bloom).

Platelet counts ranged from 644.1 to 732.8 g/l and no differences between groups were found. Haemoglobin values ranged from 110.8 to 114.7 g/l without significant differences among groups. Interestingly, significant differences ($p < 0.01$) between the control group B and groups C and F, respectively, were found in RBC counts (average counts: B 5.82 T/l; C 5.22 T/l; F 5.39 T/l), haematocrit value (average values: B 0.39 l/l; C 0.33 l/l; F 0.35 l/l), MCH (average values: B 19.73 pg; C 21.27 pg; F 21.01 pg), MCV (average volumes: B 66.8 fl; C 63.1 fl) and MCHC (average concentrations: B 295.9 g/l; C 336.9 g/l; F 321.2 g/l), (Fig. 1 A–D).

3.3. Immunological parameters

With regard to immune system, histopathology of spleen and thymus as well as lymphocyte populations (in

Table 1
Average doses of microcystins administered orally in food (recalculated according to food consumption and rat weight). Exposures: A – control (without fish meat), B – control (with fish meat), C – fish + cyanobacterial biomass (MCs 25 mg/kg of feed), D, E – fish + pure microcystin (700 and 5000 μg/kg of feed), F – fish from locality with heavy cyanobacterial bloom.

Group	∑Received MCs (μg/kg b.w./day)	∑Consumed MCs (mg/rat/28 days)	∑Consumed MCs (mg/kg b.w./28 days)
A	0	0	0
B	0	0	0
C	3000	15.3	83.5
D	136	0.7	3.8
E	928	5.4	26.0
F	≤1	≤0.006	≤0.03

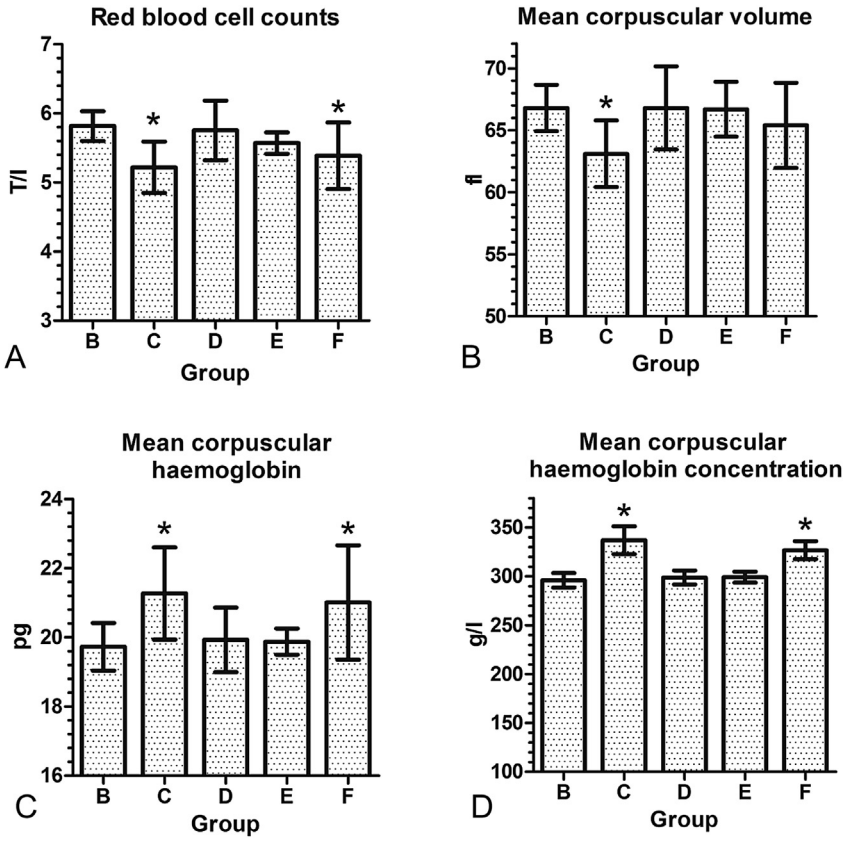

Fig. 1. Effects of feeding regimes with toxic cyanobacteria (28 day exposure) on selected hematological parameters in rats. A – red blood cells count (RBC count), B – mean corpuscular volume (MCV), C – mean corpuscular haemoglobin (MCH), D – mean corpuscular haemoglobin concentrations (MCHC). Asterisks indicate statistical significance from the control group B ($p < 0.01$), $N = 10$, graphs represent average with standard deviations. Exposures: B – control, C – fish + cyanobacterial biomass (MCs 25 mg/kg of feed), D,E – fish + pure microcystin (700 and 5000 μg/kg of feed), F – fish from locality with heavy cyanobacterial bloom.

peripheral blood, spleen and thymus separately) have been investigated. No significant histological changes have been observed in spleen or thymus.

3.3.1. Peripheral blood

The total leukocyte count was the lowest in group E (3.6 ± 2.3 G/l), in other groups the values were in the range of 5.0–7.8 G/l, but none of the differences was statistically significant. Table 2 also presents the values of lymphocyte subpopulations in peripheral blood. As it is apparent, the main differences from the control were observed in groups E and F. Significantly elevated % of NK cells (CD161+) was observed in group E (high dose of microcystin in the feed), and the same group has elevated also % of γδ+ cells. Increase in γδ+ cells was also apparent in group F but it was not significant with respect to high variability. Group F fed with feed containing fish meat from the environment with cyanobacterial bloom had decreased CD4+/CD8+ ratio (both decrease in CD4+ and increase in CD8+ were significant; Table 2).

3.3.2. Lymphoid organs

The values of lymphocyte subpopulations in spleen are presented in Table 3. The major differences in spleen lymphocyte subpopulations were observed in the group E (high dose of microcystin) with elevated % of IgM+, CD8+ and γδ+ lymphocytes. Less pronounced increase was found in γδ+ lymphocytes of group C (cyanobacterial biomass in the feed). Decrease in NK C161+ cells was recorded in group D. Lymphocyte subpopulations in thymus are shown in Table 4. Significant differences were found in groups E and F.

4. Discussion

Most published experiments on the influence of cyanotoxins in laboratory mice or rats deal with effects of isolated microcystins after intraperitoneal administration, i.e. fairly unnatural mode of exposure, and report adverse reactions that are approximately 10 times stronger than following oral administration. A single *per os* dose of microcystin-LR resulted in the LD_{50} of 5 and 10.9 mg/kg of body weight for rats and mice, respectively (Fawell et al., 1999; Yoshida et al., 1997). While the maximum dose of MCs used in the present experiment amounted from 3 to 5 mg/kg body weight per day, there were no cases of mortality or behavioural changes in experimental rats. These doses were near to those reported by Fawell et al.

Table 2
Lymphocyte subpopulations (%) in peripheral blood of rats exposed for 28 days to feed containing fish meat and toxic cyanobacterial samples. Asterisks (*, **) indicate statistical significance ($p < 0.05$ and $p < 0.01$, respectively) in comparison with the control group B. Exposures: B – control, C – fish + cyanobacterial biomass (MCs 25 mg/kg of feed), D, E – fish + pure microcystin (700 and 5000 μg/kg of feed), F – fish from locality with heavy cyanobacterial bloom.

	CD3+	IgM+	CD161+	CD4+	CD8+	γδ+	CD4+/CD8+
B	49.2 ± 10.7	46.4 ± 9.2	4.4 ± 2.0	62.5 ± 2.6	34.1 ± 2.7	6.6 ± 1.7	1.9 ± 0.2
C	49.1 ± 2.6	46.8 ± 2.3	4.1 ± 1.5	64.8 ± 3.1	31.9 ± 2.4	6.5 ± 2.5	2.1 ± 0.3
D	50.0 ± 7.2	45.6 ± 7.4	4.4 ± 1.6	64.2 ± 2.9	32.6 ± 3.5	5.5 ± 1.3	2.0 ± 0.3
E	42.3 ± 10.3	48.8 ± 10.6	8.9 ± 4.1**	59.4 ± 7.7	36.1 ± 7.2	11.7 ± 4.1**	1.8 ± 0.7
F	47.3 ± 14.9	47.9 ± 13.4	4.8 ± 3.6	56.0 ± 5.6**	39.9 ± 4.7**	10.1 ± 4.6	1.4 ± 0.3**

(1999). In the present study MCs were, however, administered in the form of complex feed that may contain compounds influencing MCs availability in gut or have positive effects on faster elimination of MCs. We assume that various blood cells (erythrocytes as well as lymphocytes) can be target for MCs, because MCs can easily cross the intestine-blood barrier and can enter various cells through OATP transporters (Zeller et al., 2012). Microcystin in human serum was first identified in a chronically exposed human population in China (Chen et al., 2007), but only hepatocellular damages were investigated. There is generally scarce information about how pure microcystins and complex cyanobacterial biomass effects blood cells and/or modulate immune system.

There were no significant differences in the platelet counts and haemoglobin values among experimental groups. On the other hand, RBC count, haematocrit value, MCH, MCV and MCHC showed significant differences in groups C and F in comparison with the control group. The most profound changes were observed in the group C that showed the highest drop in RBC count and subsequent alterations in all indexes dependent on the count of erythrocytes such as MCV, MCH and MCHC. The life cycle of erythrocytes differs from 80 to 120 days. Our experiment lasted 28 days. Therefore, we assume that the new erythrocytes (about 1/3–1/4) account for higher amounts of haemoglobin due to an adaptation mechanism. To our knowledge, only a limited number of studies reported impacts of cyanotoxins on haematological and biochemical parameters in rats or mice. There are mainly reports concerning the increase of liver enzymes (Gupta et al., 2003; Hermansky et al., 1990; Hooser et al., 1989; Rao et al., 2005). Clark et al. (2007) described an enzyme increase and the decrease of total proteins, albumin and glucose. Interestingly, similar changes in the red blood cells were found in fish after exposure to cyanotoxins (Navratil et al., 1998; Vajcova et al., 1998).

Considering the immunological parameters, there were changes in lymphocyte subpopulations of groups E (high dose of microcystin) and F (feed with meat from carps caught in cyanobacterial bloom-polluted pond), in particular. NK cells and γδ+ T lymphocytes were significantly increased in peripheral blood of group E. Undoubtedly, immunomodulatory effects were associated with the pure microcystin in the feeding ration. There was a significant change in the ratio of CD4+ and CD8+ in group F because of an increase of CD4+ and a drop in CD8+. However, some other nutritional components were probably responsible for the immunomodulatory effects because there was only minimal content of MCs in the feeding ration of this group of experimental rats. Likewise, the greatest changes in lymphoid organs were observed in groups E and F. There was an increase of spleen subpopulations of γδ+ T lymphocytes in group E as well as of IgM+ lymphocytes (B lymphocytes) and CD8+ T lymphocytes. The increase of γδ+ T lymphocytes can be associated with their release from the thymus where, apart from other changes, there was a statistically significant drop of CD4–8-lymphocytes. Indeed, the modulation of CD4+ and CD8+ of peripheral lymphocytes in group F was associated with similar changes in thymic lymphocytic subpopulations. It seems that the addition of MCs stimulated the immune system in group E rats.

Previous studies investigating the effect of cyanotoxins on T and B lymphocytes mostly report inhibition of lymphoproliferation, i.e. immunosuppressive action of cyanobacterial toxins (Lankoff et al., 2004; Shen et al., 2003; Yea et al., 2001). However, their experimental design was quite different. Lankoff et al. (2004) and Yea et al. (2001) studied mammalian lymphocytes and splenocytes, respectively, *in vitro*, while Shen et al. (2003) injected mice with the cyanobacterial extract containing MCs intraperitoneally. Apart from the different exposure route, it is not known which components of the extract were responsible for the observed immunosuppressive effect. Considering other organisms, we have described immunosuppressive effects on T cytotoxic and Ig+ lymphocytes in carp after peroral exposure (Palikova et al., 1998). The greatest effect was

Table 3
Lymphocyte subpopulations (%) in the spleen of rats exposed for 28 days to feed containing fish meat and toxic cyanobacterial samples. Asterisks (*,**) indicate statistical significance ($p < 0.05$ and $p < 0.01$, respectively) in comparison with the control group B. Exposures: B – control, C – fish + cyanobacterial biomass (MCs 25 mg/kg of feed), D,E – fish + pure microcystin (700 and 5000 μg/kg of feed), F – fish from locality with heavy cyanobacterial bloom.

	CD3+	IgM+	CD161+	CD4+	CD8+	γδ+
B	50.5 ± 11.3	49.6 ± 10.6	4.8 ± 1.5	54.6 ± 9.7	50.3 ± 10.7	17.8 ± 4.5
C	56.4 ± 19.2	59.1 ± 15.8	5.3 ± 1.2	63.2 ± 15.5	57.5 ± 19.3	22.3 ± 6.6*
D	45.6 ± 13.4	50.9 ± 12.2	3.2 ± 1.2*	56.3 ± 12.0	43.3 ± 13.9	18.4 ± 5.2
E	48.8 ± 10.5	82.3 ± 26.5**	5.2 ± 1.6	71.3 ± 23.9	65.1 ± 13.6**	27.8 ± 9.8**
F	43.5 ± 10.3	58.7 ± 11.2	4.9 ± 1.6	54.5 ± 10.8	52.6 ± 13.0	21.1 ± 8.1

Table 4
Lymphocyte subpopulations (10^6/organ) in the thymus of rats exposed for 28 days to feed containing fish meat and toxic cyanobacterial samples. Distribution of lymphocyte subpopulations is presented separately for "small" and "large" lymphocytes as discriminated by flow cytometric analysis. Asterisks (*, **) indicate statistical significance ($p < 0.05$ and $p < 0.01$, respectively) in comparison with the control group B. Exposures: B – control, C – fish + cyanobacterial biomass (MCs 25 mg/kg of feed), D, E – fish + pure microcystin (700 and 5000 μg/kg of feed), F – fish from locality with heavy cyanobacterial bloom.

	Of all lymphocytes	CD3+	CD4+ CD8+	CD4+ CD8−	CD4− CD8+	CD4− CD8−
Small lymphocytes						
B	128.9 ± 27.6	25.8 ± 6.0	223.8 ± 50.4	14.2 ± 3.0	2.9 ± 0.7	1.8 ± 0.5
C	148.0 ± 51.0	24.1 ± 8.1	240.6 ± 78.4	10.6 ± 4.9	2.7 ± 1.3	2.1 ± 1.5
D	134.0 ± 53.4	25.7 ± 11.1	224.5 ± 87.8	13.3 ± 5.6	3.2 ± 1.5	2.2 ± 1.0
E	95.0 ± 21.5**	18.2 ± 6.6*	214.6 ± 41.2	9.0 ± 3.8**	2.3 ± 0.8	1.7 ± 0.4
F	102.5 ± 28.1*	20.0 ± 6.1*	213.8 ± 48.2	12.2 ± 5.3	2.0 ± 0.7	1.7 ± 0.7
Large lymphocytes						
B	81.7 ± 16.4	123.3 ± 33.0	120.8 ± 26.1	76.7 ± 22.3	29.7 ± 6.4	15.5 ± 3.3
C	87.0 ± 22.6	115.1 ± 38.2	141.0 ± 46.2	68.6 ± 23.1	28.9 ± 10.7	17.5 ± 7.0
D	79.4 ± 31.3	115.2 ± 47.1	118.2 ± 45.0	68.9 ± 29.0	37.4 ± 15.4	18.7 ± 8.8
E	87.5 ± 18.6	125.9 ± 30.3	119.7 ± 25.4	74.3 ± 17.7	22.9 ± 6.0*	10.9 ± 2.1**
F	79.3 ± 22.0	125.8 ± 32.8	119.9 ± 34.6	75.0 ± 20.8	23.7 ± 6.5*	11.1 ± 3.1**

induced by doses corresponding to those in the present study. As mentioned by many reports however, administration of both cyanobacterial extracts and biomass always produces stronger effects than pure MCs.

5. Conclusions

Overall, the 28 day feeding experiments with rats aimed to simulate a simple food chain transfer of microcystins to fish and then to rat. In comparison to most of the previous studies, we investigated effects of toxins administered by a more realistic exposure scenario using various feed compositions. Although rats consumed considerable doses of microcystins, toxic effects were less pronounced than expected based on previously published studies, which possibly indicate compounds influencing MCs availability in gut or have positive effects on faster elimination of MCs. Due to lack in analysis of microcystin metabolites, we can't confirm any of these theories. For haematological parameters numbers of red blood cells and mean corpuscular volume (MCV) decreased while mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentrations (MCHC) increased in animals exposed to complex samples such as toxic cyanobacterial biomass and fish meat from bloom-contaminated pond. The present study showed that feeding of rats with high concentration of microcystins (group D and E) or with commercial diet with fish meet from locality with heavy cyanobacterial bloom (group F) leaded to changes in proportions of lymphocyte subpopulations. The lymphocyte subpopulations, which were changed in peripheral blood and spleen in groups D and E were mainly NK cells and gamma-delta T cells which count was up-regulated. It cannot be concluded from the present study whether elevation of certain cell subpopulation has positive or negative effect on the immune status of the rat. We could however say that feeding with microcystins influence preferably innate part of the immune system represented by NK cells and by gamma-delta T cells, which are known to play role as a bridge between adaptive and innate immune response. On the other hand feeding of rats with fish meet from locality with heavy cyanobacterial bloom (group F) leaded mainly to changes in proportions of Th, Tc and double-positive T cells which represent cells of adaptive immune system. Despite the fact that the level of microcystins in this type of diet was very low, these effects are probably caused by presence of some other substances contained in the fish meet from the locality with high cyanobacterial bloom.

Ethical statement

The authors hereby declare that this manuscript has never been published/submitted for the publication elsewhere.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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