Avian high-dose toxicity of cyanobacterial biomass

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Submitted: 2012-09-01 Accepted: 2012-11-15 Published online: 2012-12-26

Key words: ***Coturnix coturnix* japonica, birds, cyanobacteria, microcystins, hepatotoxicity**

Neuroendocrinol Lett 2012; **33**(Suppl.3):161–165 **PMID:** 23353861 NEL330912A24

Abstract

OBJECTIVES: Previous studies using oral administration of environmentally relevant doses of cyanobacterial biomass containing microcystins (MCs) induced only sub-lethal effects in experimental birds. Therefore, the objective of this study was to obtain data on avian high-dose toxicity of MCs and compute LD_{50}, if possible, following the natural oral route of administration.

DESIGN: Responses of birds to single high-dose exposure to MCs were evaluated in fourteen-day old Japanese quail males (*Coturnix coturnix japonica*) with average body weight of 50 g which were randomly divided into five groups. Birds from four experimental groups were administered 7.5 ml of cyanobacterial biomass suspension containing increasing MCs quantities of 2500, 5000, 10000, and 20000 μg/kg using oral gavage. Controls received an equal dose of drinking water instead of the test substance. Birds were observed for clinical signs of acute toxicity. Survivors were killed on day 5 to obtain body and liver weights. A five-grade semi-quantitative system for histopathological liver damage scoring was used to compare cyanobacterial-biomass-exposed birds against controls.

RESULTS: No mortality occurred during the period of five days post exposure in both control and MCs-exposed groups and this high-dose experiment failed to provide data to compute the LD_{50}. Nevertheless, marked sub-lethal effects were recognised in the damage of liver that included dose-dependent changes in the body/liver ratios and morphological changes ranging from mild vacuolar dystrophy to focal liver necroses in the highest exposure group. Hepatic lesions were mainly observed in the pericentral area of the liver.

CONCLUSIONS: Though maximum cyanobacterial biomass dose rates that could be administered to birds of the size were used in the present experiment and more pronounced hepatic lesions than after exposure to environmentally relevant doses

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were observed, birds would probably have survived unless killed for histopathology on day 5 of exposure. These results provide support to previously reported data on sub-lethal effects following exposure to cyanobacterial biomass containing MCs in birds and mortality occurring only in birds under combined action with other stressors.

Abbreviations:

HPLC-DAD	- high performance liquid chromatography with diode array detection
LD50	- median lethal dose
LSD test	- the least significant difference test
MCs	- microcystins
MC-RR	- microcystin RR
MC-YR	- microcystin YR
MC-LR	- microcystin LR

INTRODUCTION

Cyanobacteria and their toxins have been implicated in wild aquatic bird toxicity and mass deaths throughout the world (Alonso-Andicoberry *et al.* 2002; Henriksen *et al.* 1997; Krienitz *et al.* 2002; Matsunaga *et al.* 1999; Onodera *et al.* 1997; Wilde *et al.* 2005). While toxin ingestion and hepatotoxicity represent the natural route of exposure and mechanism of toxicity of microcystins (MCs), respectively (Alonso-Andicoberry *et al.* 2002; Lopez-Rodaz *et al.* 2008), other adverse effects such as testicular toxicity have also been reported in birds (Damkova *et al.* 2011). Interestingly, experiments employing oral administration of environmentally relevant doses of cyanobacterial biomass containing MCs induced only sub-lethal effects in Japanese quails (Paskova *et al.* 2008; Peckova *et al.* 2009; Skocovska *et al.* 2007). Likewise, exposure to crude microcystins via intraperitoneal injection, but not oral gavage, caused hepatotoxicity in ducks (Li *et al.* 2012). Standard toxicological endpoints such as LD_{50} for the oral route of exposure are lacking in birds. The only experimentally-induced mortality associated with microcystin RR was reported following the unnatural intraperitoneal exposure (Takahashi & Kaya 1993). On the other hand, the mammalian oral LD_{50} value is known (Yoshida *et al.* 1997).

Comparing different periods of oral exposure to cyanobacterial biomass in Japanese quails, greater sublethal effects were recognized from day 5 to 15 (Peckova *et al.* 2009; Skocovska *et al.* 2007), while induction of the detoxification and antioxidative systems, biotransformation and elimination of MCs at the longer exposure time of 30 days protected the birds from more serious damage (Paskova *et al.* 2008). Regarding the abovementioned facts on toxicity of microcystins in birds, it seems that the toxic effects and mortality are enhanced by combined exposure to other stressors under natural conditions (Paskova *et al.* 2011; Pikula *et al.* 2010).

Thus, the objective of this study was to obtain data on avian high-dose toxicity of cyanobacterial biomass containing MCs and compute LD_{50}, if possible, following the natural oral route of administration. Responses of birds were evaluated using a semi-quantitative histopathological liver damage score grading.

MATERIAL AND METHODS

Experimental design

The experiment was conducted under approval by the institutional ethics committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. A total of 50 fourteen-day old Japanese quail males (*Coturnix coturnix japonica*) were used for the experiment after one week of acclimatization. Birds with average body weight of 50 g were randomly divided into 4 experimental and 1 control groups. The experiment was started with a single oral dose of cyanobacterial biomass using a crop probe. Control birds were also probed and received an equal dose of drinking water instead of the test substance. Japanese quails were then provided with standard feed mixture and water *ad libitum*. Five days after exposure they were blood sampled, euthanized and necropsied.

Experimental substances

A plankton net (25 μm) was used to collect the cyanobacterial biomass from the Musovska reservoir (Czech Republic) in summer 2007. The biomass included *Microcystis aeruginosa* (90%) and *M. ichthyoblabe* (10%) with 1862.77 μg of total microcystins contained per one gram of dry weight. The biomass was frozen after collection and stored at –20 °C for approximately 1.5 years. It was lyophilized using Christ Gamma 1-16 LSC Freeze dryer (Osterode am Harz, Germany) prior to the experiment. The lyophilized biomass was reconstituted with physiological saline immediately before experimental use of four different doses. The basic dose of microcystins was approximated from the highest mammalian oral LD_{50} of 10 000 μg/kg (Yoshida *et al.* 1997). Microcystin structural variants in the above dose included 4312 μg MC-RR, 1238 μg MC-YR and 4450 μg MC-LR. Experimental groups $¼LD_{50}$, $½LD_{50}$, LD_{50}, and $2LD_{50}$ were thus administered increasing MCs quantities of 2 500, 5 000, 10 000, and 20 000 μg/kg, while the administered suspension amounted to 7.5 ml in each group. The contents of MCs in the biomass and feed were analysed using high performance liquid chromatography with diode array detection (HPLC-DAD; Agilent 1100 Series, Agilent Technologies, Japan) on a Supelcosil ABZ + Plus column, 150 × 4.6 mm, 5 μm, according to the method by Babica *et al.* (2006).

Data collection

Data collected during the study included clinical observations and mortality, body and liver weight measurements using AND GX-400 scales (A&D Instruments Ltd., Japan), and pathological findings on necropsy. The body/liver ratio was computed. Samples of liver were examined histologically.

Histopathology

Liver tissue specimens were collected into 10% buffered formalin during necropsy, 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. A semi-quantitative system for histopathological liver damage score grading was used for comparison of findings in control and cyanobacterial-biomass-exposed birds (Table 1). The grades ranging from normal tissue (0) to massive necroses of hepatocytes (5) were read in five high power fields (objective 40) of each hepatic sample. A total of 50 readings were made for each experimental group.

Statistical analysis

Statistica for Windows® 10 (StatSoft, Inc., Tulsa, OK, USA), the data analysis software, was used to compare different groups using following procedures: one-way analysis of variance (ANOVA), post-hoc analysis of means by the LSD test, Levene's method to test for the homogeneity of variances, log-transformation of non-homogenous parameters prior to analysis and comparison with the non-parametric Kruskal-Wallis test. Considering statistical significance of differences, levels of $p<0.05$ and $p<0.01$ were used.

RESULTS

Clinical observations

There was no case of mortality during five days post exposure in both control and cyanobacterial-biomass-exposed groups. Birds from groups LD_{50} and $2LD_{50}$ had green watery diarrhoea approximately 2 to 3 hours after the single oral dose of cyanobacterial biomass. They did not show, however, any other clinical signs of health deterioration such as lethargy or poor intake of feed and water.

Tab. 1. Characteristics of histopathological liver damage scores.

Score	Definition
0	**Normal** microscopic structure.
1	**Mild damage.** Sporadic hepatocytes with vacuolar dystrophy, mild perivascular infiltrates in portal fields.
2	**Moderate damage.** Focal vacuolar dystrophy of hepatocytes. Moderate grades of perivascular infiltrates in portal fields.
3	**Severe damage.** Marked diffuse vacuolar dystrophy of hepatocytes, sporadic necroses of hepatocytes. Extensive inflammatory perivascular infiltrates.
4	**Severe diffuse damage.** Focal necroses of hepatocytes with infiltration by heterophils, extensive inflammatory perivascular infiltrates.
5	**Massive necroses** of hepatocytes.

Body/liver weight ratio

As shown in Figure 1, apart from the lowest cyanobacterial biomass exposure group the ratio between whole body and liver weights decreased, i.e., the relative weight of liver increased in experimental groups $½LD_{50}$, LD_{50}, and $2LD_{50}$.

Histopathology

Dose-dependent histopathological liver damage score grading results are shown in Figure 2. The range of morphological changes in liver varied from mild

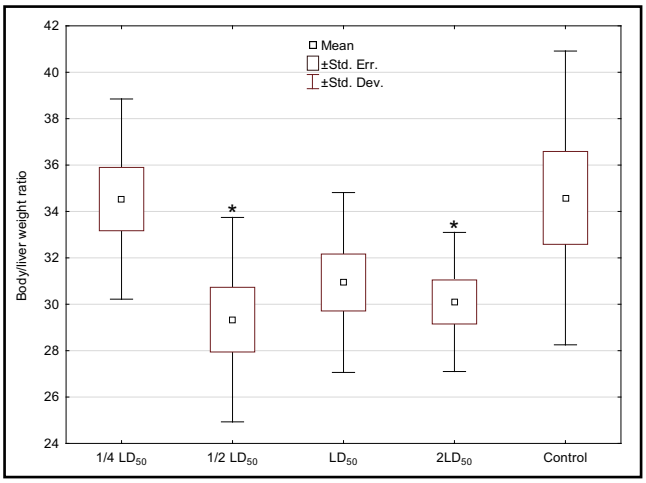

Fig. 1. Body/liver weight ratios in control Japanese quails and birds exposed to four different cyanobacterial doses. LD_{50} (median lethal dose) equal to 10000 µg of microcystins per 1 kg of body weight was approximated from mammals (Yoshida *et al.* 1997). Groups $¼LD_{50}$, $½LD_{50}$, LD_{50}, and $2LD_{50}$ were administered 2500, 5000, 10000, and 20000 µg of MCs/kg, respectively. N=10 randomly selected males in each group, *=$p<0.05$ when compared against the healthy control group.

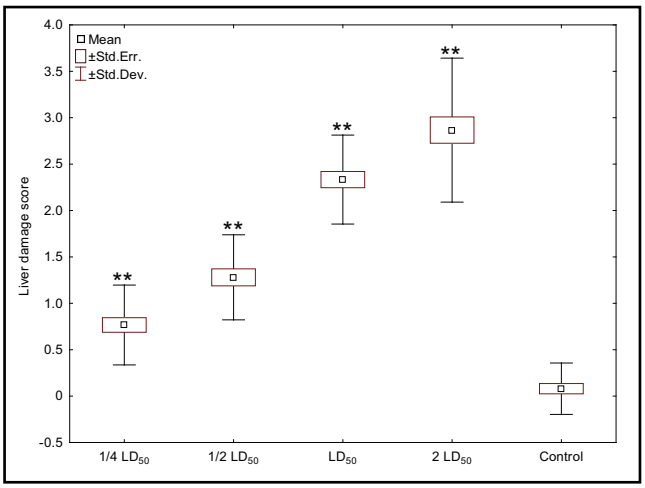

Fig. 2. Histopathological liver damage scores in control Japanese quails and birds exposed to four different cyanobacterial doses. LD_{50} (median lethal dose) equal to 10000 µg of microcystins per 1 kg of body weight was approximated from mammals (Yoshida *et al.* 1997). Groups $¼LD_{50}$, $½LD_{50}$, LD_{50}, and $2LD_{50}$ were administered 2500, 5000, 10000, and 20000 µg of MCs/kg, respectively. N=10 randomly selected males in each group, **=$p<0.01$ when compared against the healthy control group.

vacuolar dystrophy to focal liver necroses. The group treated with mammalian ¼LD_{50} showed mild vacuolar dystrophy of hepatocytes in scattered foci. Multiple vacuoles of circular shape were distributed irregularly in the cytoplasm and ranged in size from 1 to 5 µm. The group dosed with mammalian ½LD_{50} was characterized by mild to moderate vacuolar dystrophy, while the size of vacuoles and severity of damage were most pronounced in the pericentral hepatocytes (cf. Figure 3). Distribution of vacuolar dystrophy of hepatocytes in the group treated with mammalian LD_{50} was diffuse. The most severe damage was observed in the group of birds treated with 2LD_{50} (cf. Figure 4). Diffuse vacuolar dystrophy of hepatocytes and scattered foci of necrotic hepatocytes were observed. Foci of necrotic hepatocytes were surrounded by small numbers of heterophils and lymphocytes that contained shrunken hypereosinophilic hepatocytes and distorted nuclei. The size of necrotic lesions ranged approximately from 100 to 150 µm.

DISCUSSION

A high, environmentally irrelevant, single dose of cyanobacterial biomass containing MCs ranging from 2500 to 20000 µg/kg was used for oral exposure of Japanese quails. Surprisingly, this experiment failed to provide data to compute the LD_{50}. The birds from all exposure groups survived the acute toxicity testing without showing marked clinical signs and were killed on day 5 for histopathology. The high-concentration suspension of the toxic dose was prepared from the lyophilised cyanobacterial biomass so as to obtain the lowest volume of 7.5 ml administered to each bird weighing 50 g, on average. Therefore, higher dose rates would not be feasible for birds of those body weights. It can be stated in exaggeration that, using the present dose rates of MCs in the cyanobacterial biomass available from natural blue-green algal blooms, we were not able to conform to the basic principle of toxicology, i.e, the dose makes the poison.

The present results that demonstrate low sensitivity of birds satiated with cyanobacteria containing MCs and failure to induce mortality following the natural route of oral ingestion are in accordance with other experimental studies (Skocovska *et al.* 2007; Paskova *et al.* 2008; Li *et al.* 2012). The question, therefore, is what drives the reported mass kills in wild aquatic birds associated with natural cyanobacterial blooms (Alonso-Andicoberry *et al.* 2002; Krienitz *et al.* 2002; Lopez-Rodaz *et al.* 2008). Compared with dose rates of the present study, one to two orders of magnitude lower doses of MCs induced mortality after the unnatural route of intraperitoneal administration within a day (Li *et al.* 2012; Takahashi & Kaya 1993). The basic content of MCs in cyanobacteria used for exposure in the present study was approximated from the highest mammalian oral LD_{50} of 10 000 µg/kg. It is clear, therefore, that Japanese quails were much less sensitive than mice (Yoshida *et al.* 1997).

On the other hand, marked sub-lethal effects were recognised in the damage of liver that included dose-dependent changes in the body/liver ratios as well as morphological changes ranging from mild vacuolar dystrophy to focal liver necroses in the highest exposure group. Vacuolar dystrophy of hepatocytes is a non specific morphological finding observed commonly after exposure to many toxic compounds as well as MCs (Gupta *et al.* 2003). Extensive vacuolar dystrophy was previously found in birds exposed to environmentally relevant doses of cyanobacterial biomass (Skocovska *et al.* 2007; Pikula *et al.* 2010). In general, vacuolar dystrophy is characterised by the presence of vacuoles in the cytoplasm of hepatocytes because of the sequestration

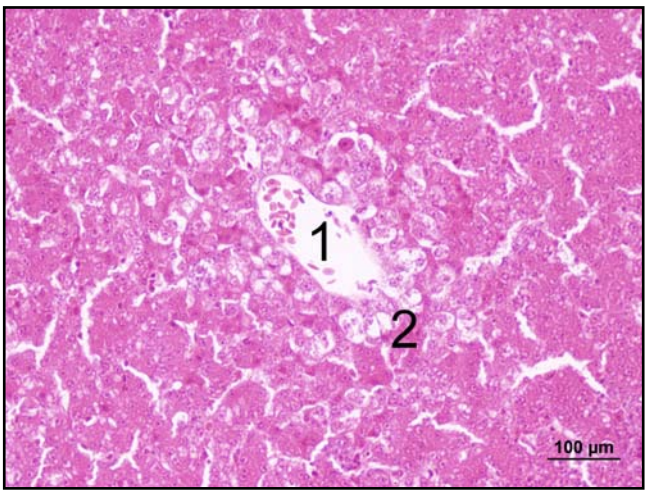

Fig. 3. Mild to moderate vacuolar dystrophy of hepatocytes in small foci in birds from experimental group ½LD_{50} approximated from mammals. 1 = vena centralis, 2 = vacuolar dystrophy of pericentral hepatocytes. H&E stain.

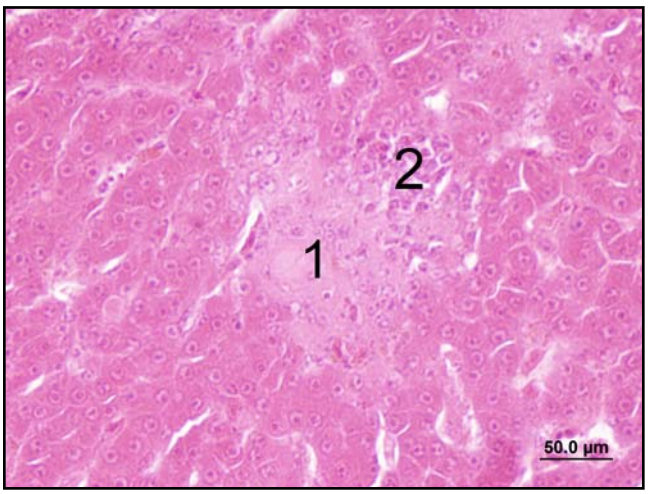

Fig. 4. Severe diffuse vacuolar dystrophy of hepatocytes and foci of necrotic hepatocytes in birds from experimental group 2LD_{50} approximated from mammals. 1 = focal necrosis of hepatocytes, 2 = surrounded by low numbers of inflammatory cells. H&E stain.

of water and proteins in cytoplasmic vacuoles (Cheville 1999). The primary effects of microcystins, which lead to hepatocellular damage, are morphological changes in mitochondria initiated by mitochondrial depolarisation (Ding *et al.* 2000; Ding & Ong 2003). The hallmark of mitochondrial injury in hepatopathology is steatosis, typically microvesicular (Hassanein 2004). This process was most likely responsible for the increase in liver weights compared with control untreated birds in the present experiment. The pericentral distribution of vacuolar dystrophy is suggestive of and characteristic for the action of a hepatotoxic substance absorbed from the digestive tract and brought to the liver via portal vein. New to the toxicity of cyanobacteria containing MCs in avian hepatic tissue, necroses of hepatocytes observed in higher exposure groups of birds were characteristic of early coagulative necrosis with incipient inflammatory response. As shown, excessive doses of cyanobacteria containing MCs induced more pronounced damage in the liver than environmentally relevant ones (Skocovska *et al.* 2007; Pikula *et al.* 2010). However, the damage score 5, i.e., massive necroses of hepatocytes, was not observed in the present study and birds would probably have survived unless killed for histopathology on day 5 of exposure.

CONCLUSION

Results of the present experiment provide support to previously reported data on sub-lethal effects following exposure to cyanobacteria containing MCs in birds and mortality occurring only in birds under combined action with other stressors (Pikula *et al.* 2010; Paskova *et al.* 2011). As sub-lethal effects of toxicants often remain unrecognized, data presented here may be used for avian risk assessment of cyanobacteria containing MCs.

ACKNOWLEDGEMENT

The present study was supported by the Internal Grant Agency of the University of Veterinary and Pharmaceutical Sciences Brno, grant No. 87/2011/FVHE.

Potential Conflicts of Interest: *None disclosed.*

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