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Hepatotoxic Effects of *Microcystis aeruginosa* (PCC 7820) on Wister Rats

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Abstract

The potential toxic effect of Microcystis aeruginosa through oral contamination is becoming important. The present study was carried out to find the possible hepatotoxic effects of toxic M. aeruginosa (PCC 7820) on male Wistar rats as animal model. Hepatotoxicity assessment was done by estimation of serum hepatic enzyme levels of γ-Glutamyl transferase (GGT), Alkaline phosphatase (ALP), Alanine aminotransferase (ALT/GPT), Aspartate aminotransferese (GOT/AST). Rats treated with non toxic (CYA 43) and toxic non homogenized M. aeruginosa (PCC 7820) were normal in appearance where as rats receiving homogenized toxic M. aeruginosa (PCC 7820) were lethargic and gained less weight which indicates a possible toxic effect on the test animals. The absolute and relative (% body weight) mean weight of liver and kidneys of the homogenous toxic M. aeruginosa (PCC 7820) treated animal were lower than those who received non homogenized toxic M. aeruginosa (PCC 7820) and the difference is statistically significant ($p<0.001$). Liver sections of rats receiving fresh toxic M. aeruginosa and the homogenized toxic M. aeruginosa did not show lymphatic infiltration or signs of necrosis. Rats treated with homogenized M. aeruginosa showed lowering of absolute liver weight compared to the fresh M. aeruginosa treated rats. Statisticcally significant ($p<0.005$) increase levels of serum γ-Glutamyl transferase (GGT) was detected in rats 14 days after oral administration with homogenized toxic M. aeruginosa (PCC 7820). There was no significant different found in serum Alanine aminotransferase (ALT/GPT) concentration between treated and control groups. The results indicate that prolonged oral administration of homogenized M. aeruginosa lead to functional hepatotoxicity in male Wister rats without evident histopathological liver necrosis.

Key words : Toxic *M. aeruginosa*, Wistar rats, hepatotoxicity, serum chemistry.

Introduction

Over recent years it has become apparent that toxic cyanobacterial blooms are on the increase, presenting a hazard to animal and human health. Microcystins, produced by *Microcystis sp. Anabaena sp.. Planktothrix sp. and Nostoc sp.* have been most extensively associated with toxicoses and the focus of many studies in recent years (Zurawell et al. 2005).

The dominance of *Microcystis aeruginosa* is not always related to the toxicity as non toxic and toxic species are available (Lawton et al. 1994). In Australian lakes, the incidence of toxicity for natural samples dominated by *M. aeruginosa* was 56% where as in England about two thirds of blooms were found to be toxic out of samples collected from almost 100 water bodies (Codd. et al., 1997). In Japanese lakes and ponds, half of the natural samples of *Microcystis* were toxic (Watanabe, 1996). In Sri Lanka some lakes, ponds and reservoirs are dominated by toxigenic cyanobacteria blooms (*M. aeruginosa*) and some were contaminated with microcystin (13.15 -81.31μgl^{-1}) (Jayathissa et al., 2006). Thus, the potential chronic toxicity from microcystins produced by *M. aeruginosa* led the WHO to establish a guideline value of 1μgl^{-1} as a maximum concentration of microcystin (MC-LR) in drinking water (WHO, 1998). Additional concern regarding the importance of cyanotoxins, is reflected by their inclusion in the US Environmental Protection Agency (USEPA) drinking water contaminant list and in major reviews along with chemical warfare agents (Reemtsma, 2003: Richardson and Ternes, 2005) Furthermore, in the June 2006. MC-LR was classified as a possible human carcinogen (group 2B) (Grosse et al., 2006).

There is a wide spectrum of blue green algal toxins (microcystins, nodularins, and cylindrospermopsin) predominantly affecting the nervous, hepatic and dermatologic systems (neurotoxic, hepatotoxic and dermatotoxic.) These toxins are particularly toxic to the liver in part due to selective transport mechanisms that concentrate from the gut and blood into the liver cells. Microcystin is also beleived to casuse damage to cells DNA by the activation of endonucleases (Jochimsen et al., 1998). The microcystins and the nodularins are protein phosphatase ihibitors, as well as being potent tumor promoters in animals. It has been recorded that microcystins cause liver necrosis leading to death of animal within hours to days (Carmichael, 1994; Chorus, et al,1999; Humpage and Falcorner, 1999; NHMRC,

1994; Ohtani et al., 1992; Repavich et al., 1990; Yu 1995) and there have been frequent reports of thirsty domestic animals and wildlife consuming freshwater contaminated with toxic blue green algal blooms, and dying within minutes to days from acute neurotoxicity and / or hepatotoxicity (Carmichael, 1994; Carbis et al., 1995; Codd et al., 1997; Jochimsen et al., 1998; Mahmood et al., 1988; Negri et al., 1995; Repavich et al., 1990). Mammals and birds appear to be more susceptible to the blue green algal toxins than aquatic invertebrates and fish, with some species variability. In laboratory experimental animals, teratogenic activity has been demonstrated with oral administration of *Microcystis* extracts; approximately 10% of otherwise normal neonatal mice had small brains with extensive hippocampal neuronal damage (Astrachan et al.,1980; Carmichael and Falconer and Falconer, 1993). Studies in cultured cells have also shown tumor promotion, and microcystins are preferentially taken up by hepatic cells, so that hepatic tumor promotion is likely (Carmichael and Falconer, 1993). Carmichael, 1994; Humpage, 1999; Ito, 1997; Sugimara, 1986). There are relatively few case reports and even fewer epidemiologic studies of the human health effects of the blue green algal toxins (Carmichael, 1993; Chorus and Bartram, 1999; Jalaludin and Smith, 1992). Humans can be exposed to the cyanobacteria and their toxins through direct skin contact or by drinking contaminated water (Codd et al.,1997; Chorus and Bartram, 1999) Chorus, 1999; Junshi et al., 1990; Yu et al., (1989a; 1989b; 1995) have studied the possible relationship between the consumption of blue green algal contaminated surface drinking water (pond, ditch, river and well water or deep well) and an increased risk for primary hepatic cancer (as well as chronic gastrointestinal diseases) in China. In addition to liver cancer, recent studies have suggested that a possible role for the blue green algal toxins and colon cancer (Humpage et el., 2000). Although adequate information on hepatotoxic activity of mycrocystin by injecting extracts to the test animals is available the effect of *Microcystis* directly by oral administration is limited. Thus, the potential toxic effect of *M. aeruginosa* through oral contamination becomes important. In view of this, the prestent work was carried out to find the possible hepatotoxic effects of toxic *M. aeruginosa* (PCC 7820) on male Wistar rats as an animal model. Assessment of hepatotoxic effect was carried out by estimation of serum hepatic enzyme levels of γ-Glutamyl transferase (GGT). Alkaline phosphatase (ALP), Alanine aminotransferase (ALT/GPT), Aspartate aminotransferese (GOT/AST) which were used as effective markers for hepatic cell damage or necrosis.

Materials and Methods

Animals

Inbred, genetically homogenous, eight weeks old, weighing on average 167.9g ± 3.11 and twelve weeks old weighing on average 263g ± 2.93 forty male Wistar rats were used. The animals were acclimatized to metalic cages for four days before start of the experiments. Rats were fed fresh self prepared rations and mid-log growth phase culture of toxic *M. aeruginosa* pelet which was re-suspended in distilled water at final concentration of 3-5 x 10^6 cells ml^{-1} for oral feeding at a daily dose of 1 ml/100g body weight, using Sandi needle.

Preparation of toxic *M. aeruginosa* (PCC 7820) culture pellets

An axenic cultures of toxic (PCC 7820) and non toxic (CYA 43) *M. aeruginosa* were provided by Robert Gorden University, Scotland, UK. One liter Erlenmeyer flask containing five hundred milliliters of MA medium (Ichimura, 1978) was autoclaved at 121C^0 for 15 min and *M. aeruginosa* cells were inoculated into the flask at a final concentration of 3 to 5x10^3 cells ml^{-1}. Thus, the treated flask was incubated at 25C^0 ± 2, under light intensity of 2500-3000 LUX with a 12h light 12h dark photo-cycle and the culture was slightly aerated to enchance the growth of algae (Watanabe et al. 1996). Exponentially growing culture was centrifuged and the supernatant was discarded. The pellet was dissolved in distilled water to have cell density around 3-5x10^6 cells ml^{-1} for oral feeding.

Experimental design

Experiment I - The rats (weight 167.9± 3.11) were randomly divided into two groups (n=10) namely experiment group-1 and the control-1. Fresh Toxic *M. aeruginosa* (PCC7820) (3-5x10^6 cells ml^{-1}) to experimental group-1 and non toxic *M. aeruginosa* (CYA 43) (3-5x10^6 cells ml^{-1}) to the control group-1 were administrated orally at daily dose of 1ml/100g body weight for 90 days.

Experiment II - Twenty male Wister rats of twelve weeks old (263± 2.93) were divided randomly into two group (n=10) namely experiment group-II and the control group-II. Homogenized toxic *M. aeruginosa* (PCC 7820) (3-5x10^6) cells ml^{-1}) to experiment group-II and non toxic *M. aeruginosa* (CYA 43) (3 - 5x10^6 cells ml^{-1} to the control group-II were administrated orally at dialy dose of 1 ml/100g body for 30 days.

Serum biochemistry

In both the experiment I and II, the venous blood samples were drawn from the lateral tail vein after anaesthetizing the animals by inhalation of diethyl ether. Final blood samples were taken after sacrificing the animals by performing cardiac punctures. Blood samples were collected for hepatic enzyme analysis at 7 days interval for four weeks and then at 14 days interval for eight weeks from all the animals of experiment-I and 7 days interval for four weeks for the animals of experiment-II. The hepatic enzymes activities of γ-Glutamyl transferase (GPT). Alkaline Phosphatase (ALP). Alanine aminotransferase (GPT/ALT) and Aspartate transferase (GOT/AST). were analyzed by a spectrophotometer (JENWAY 6305 UV/VS) using commercially available enzyme diagnostic kits (RANDOX laboratory UK). The results were expressed as Standard Error of Mean (SEM). The significant difference in the parameters tested between experiment and control groups were analyzed by the students "t" test. The difference was considered as siginificant if $p<0.005$.

Histopathology

The liver samples of the sacrificed rats were removed after observing for gross pathological changes and then fixed, sectioned and stained with haematoxylin and eosin stains following the procedure described by Turkdogan et al (2003). The livers were fixed in 10% formal saline (pH 7.8). Before processing they were left in running water for 24h and then left in distilled water for 3h and were processed. According to the standard procedures they were left in 70%, 80% and 90% alcohol for 2h in each and then in xylene for 3h. They were embedded in wax and 4-5 microns thick sections were made by using the microtome. The prepared sections were examined under light microscope for histopathological changes.

Results

Rats treated with non toxic *M. aeruginosa* (CYA 43) and toxic non homogenized *M. aeruginosa* (PCC 7820) were normal in appearance throughout the study period. The rats in experiment II receiving homogenized toxic *M. aeruginosa* (PCC 7820) were lethargic and gained less weight (Fig. 1 & 2)

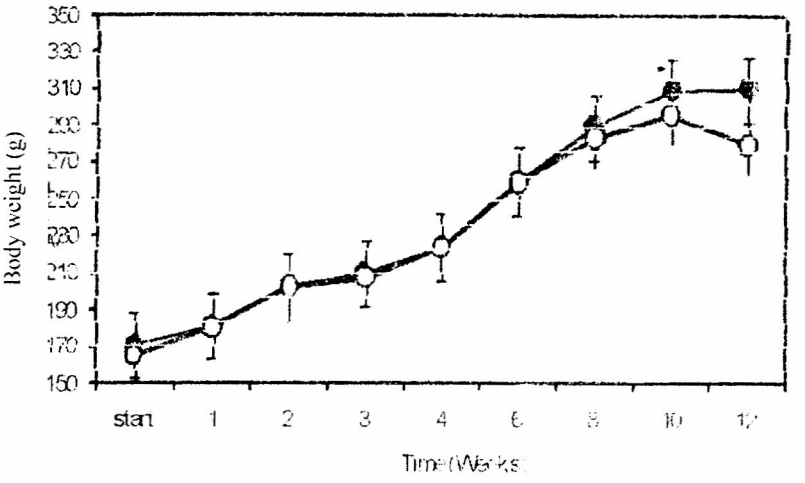

Fig. 1 Effect of oral administration *M. aeruginosa* (PCC 7820) on mean body weight gain of male Wistar rats (n=10) (Open circle-experiment, closed circle-control)

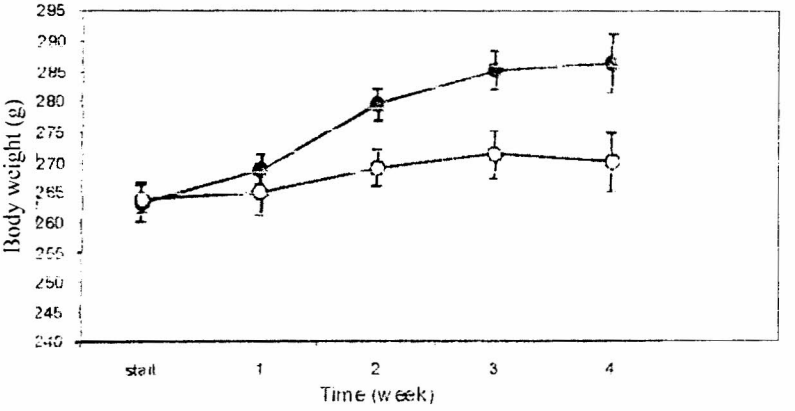

Fig. 2 Effect of oral administration of homogenized *M. aeruginosa* (PCC 7820) on mean body weight gain of male Wistar rats (n=10), (Open circle-experiment, closed circle-control)

The mean body weight of both test and control groups of the experiment I gradually increased until tenth week and thereafter decreased. A significant difference ($P<0.005$) between test and control group (Fig. 1) was not detected. In contrast a statistically significant ($P<0.005$) difference was found between homogenized *M. aeruginosa* (PCC 7820) treated rats and its controls (28g) (Fig.2)

Table 1 Absoulte (g) and relative weights (% body weight) of organs of male Wister rats who received toxic *M. aeruginosa* (PCC 7820) and homogenized toxic *M. aeruginosa* (PCC 7820)

Organ / Weight of rats	Liver		Kidney	
	Test	Control	Test	Control
Treated with toxic *M. aeruginosa* (PCC 7820)				
Absolute weight	11.84 ±0.30	11.23 ±0.31	1.62 ±0.06	1.67 ±0.03
Relative (% weight)	3.94 ±0.12	4.29 ±0.11	0.59 ±0.02	0.61 ±0.02
Treated with homogenized toxic *M. aeruginosa* (PCC 7820)				
Absolute weight	8.78 ±0.40	11.13 ±0.40	1.60 ±0.06	1.68 ±0.03
Relative (% weight)	3.95 ±0.16	3.25 ±0.14	0.58 ±0.02	0.59 ±0.03

The absolute and relative (% body weight) weights of liver and kidneys of the homogenous toxic *M. aeruginosa* (PCC 7820) treated animals were less than those who received non homegenized toxic *M. aeruginosa* (PCC 7820) and the difference was statistically significant ($P<0.001$). The mean absolute weight of kidneys among treated animals in both the experiment I and II were not statistically significant ($P<0.33$) Relative weights of liver and kidneys in both experiment-I and experiment-II did not differ significantly (Table 1)

Table 2. Key hepatic enzyme biochemistry of male rats treated with *M. aeruginosa* (PCC 7820)
Values represent means of the groups (n=10)

Weeks	GOT/AST			GOT/AST			ALP			GGT		
	Test	Control	P	Test	Control	P	Test	Control	P	Test	Control	P
1	67.53 (5.04)	67.13 (4.85)	0.510	19.71 (0.78)	22.14 (1.63)	0.370	485.57 (30.08)	134.24 (32.97)	0.370	1.023 (0.658)	0.954 (0.248)	0.964
2	63.33 (5.15)	65.83 (4.166)	0.477	19.66 (0.83)	17.22 (2.53)	0.091	398.77 (87.39)	329.73 (148.4)	0.317	0.864 (0.558)	0.866 (0.156)	0.998
3	44. (1.69)	45.5 (2.30)	0.70	13.66 (0.83)	14.00 (1.19)	0.849	268.88 (20.23)	211.00 (34.50)	0.094	3.951 (1.51)	0.965 (1.09)	0.064
4	78.83 (3.34)	78.83 (4.15)	0.339	19.22 (2.27)	20.11 (1.03)	0.497	209.76 (17.92)	244.37 (19.09)	0.553	3.769 (0.692)	0.988 (0.207)	0.026
6	60.42 (5.10)	61.44 (5.12)	0.8989	19.00 (1.18)	19.25 (1.03)	0.930	226.05 (11.58)	250.26 (11.93)	0.118	3.394 (1.234)	0.925 (0.283)	0.054
8	55.50 (1.59)	56.37 (2.52)	0.7965	14.37 (0.54)	16.62 (0.09)	0.091	262.10 (87.36)	281.30 (93.77)	0.296	1.534 (0.126)	0.7402 (0.114)	0.002
10	31.83 (2.166)	32.50 (6.85)	0.869	14.50 (1.68)	15.16 (1.01)	0.785	183.34 (17.47)	223.69 (10.19)	0.073	1.9721 (0.218)	0.555 (0.076)	0.003
12	68.66 (7.39)	76.16 (13.6)	0.061	13.5 (0.76)	13..33 (1.05)	0.916	175.35 (20.25)	215.34 (13.77)	0.056	1.455 (0.116)	0.6304 (0.107)	0.002

Blood chemistry - An increase levels of serum γ -Glutamyll transferase (GGT) concentrations were observed with a statistically significant difference ($p<0.005$) in 14 days after oral administration of homogenized toxic *M. aeruginosa* (PCC 7820) (Table 3). and 8 weeks after treatment of non homogenized *M. aeruginosa* (PCC 7820) (Table 2). Although the serum Aspartate transferase (AST) levels increased towards the end of the experiment the difference was not statistically significant. Serum Alanine aminotransferase (ALT) activity was elevated in all treated groups with respect to control after 7 days. However, there was no significant difference found in serum ALT concentration between treated and control groups. The serum Alkaline Phosphatase (ALP) in the test group of the experiment 1 decreased with time and significant difference was found at 12 week (Table 2). No significant difference was detected for homogenized treatment even after 4 week (Table 3).

Table 3. Key hepatic enzyme biochemistry of male rats treated with homogenized *M. aeruginosa* (PCC 7820)
Values represent means of the groups (n=10)

Weeks	GOT/AST			GOT/AST			ALP			GGT		
	Test	Control	P	Test	Control	P	Test	Control	P	Test	Control	P
1	36.51 (1.5)	33.83 (2.53)	0.194	17.66 (0.51)	18.33 (1.12)	0.712	255.76 (12.27)	253.15 (20.83)	0.929	1.234 (0.124)	0.8025 (0.113)	0.110
2	38.16 (2.0)	37.5 (3.19)	0.606	18.17 (1.54)	10.16 (0.79)	0.147	132.48 (7.08)	154.56 (8.74)	0.090	1.358 (0.156)	0.8491 (0.113)	0.004
3	53.16 (2.2)	32.5 (2.73)	0.875	18.83 (3.15)	15.16 (1.01)	0.264	227.08 (16.26)	223.70 (11.16)	0.868	1.4813 (0.165)	0.5582 (0.082)	0.003
4	80.67 (2.5)	36.16 (5.55)	0.351	18.96 (1.64)	13.33 (1.05)	0.016	221.16 (9.07)	242.88 (13.06)	0.277	1.5211 (0.113)	0.5932 (0.078)	0.003

Histopathology

Liver sections of rats receiving fresh toxic *M. aeruginosa* and homogenized toxic *M. aeruginosa* did not lymphatic infiltration or sign of necrosis. Also the hepatic acini maintained normal histological structure without any evidence of necrosis or other toxic effects.

Discussion

The oral administration of homogenized *M. aeruginosa* shows very clear growth retardation which indicates a possible toxic effect on the test animals. Rats treated with homogenized *M. aeruginosa* shows lowering of absolute liver weight compared to the fresh *M. aeruginosa* treated rats. The results indicate that prolonged oral administration of homogenized *M. aeruginosa* lead to functional hepatotoxicity in male Wister rats without evident histopathological liver necrosis. In the present work no evidence of changes in absolute weight of the kidneys were recorded in both the experiment I and II confirm the previous literature (Humpage et al., 1999) of the target hepatotoxic effect of microcystin in liver.

Marked increase of the serum γ-Glutamyll transferase, (GGT) concentration detected in the experiment II was statistically significant ($p<0.005$). Such increase can be due to the cell necrosis, changes in cell membrane permeability or impairment of the biliary excretion (biliary stasis) as γ-Glutamyll transferase (GGT) is a membrane bound enzyme that is released unequally depending on the pathological phenomenon (Szczekil et al., 1961). Although the hepatic biomarkers indicated liver cell necrosis, histological changes were not evident, as histological changes are observed in quite late cell necrosis. Also the activity of serum Alanine aminotransferase (ALP) only with minor variation and the serum ALT activity were elevated towards the end of the experiment II. The elevation of the serum γ-Glutamyll transferase (GGT) detected towards the end of the experiment may further confirm the hepatic injury of the test animals Alanine aminotransferase (ALP) is a cytoplasmic enzyme found in very high concentrations in the liver. Asparate amino transferace (AST) is present in the cytoplasm as well as in the mictrocondria and is less specific than ALT is present in the cytoplasm as well as in the mictrocondria and is less specific than ALT as an indicator of hepatic damage (Wilkinson, 1976). In present study, none of the treatments were observed to

have marked effect on ALP and AST concentrations. Thus, the overall result of the study shows that the hepatotoxic effect of *M. aeruginosa* was more marked in the rats that were exposed to the treatment of homogenized culture and the toxicity of the test algae affected to change the enzyme activity of γ-Glutamyll transferase (GGT). Asparate amino transferace (AST), and Alanine aminotransferase (ALT) which are known bio-markers of hepato-biliary dysfunction.

Mycrocystin is an endotoxin released when the disruption of cell membrane or cell lysis occurred (Ito et al., 1997). Thus, the mycrocystin is released in the homogenized *M. aeruginosa* culture than the fresh toxic culture of *M. aeruginosa.* Therefore in the homogenized *M. aeruginosa* (PCC 7820) culture, the mycrocystin is readily available and it can be absorbed into the blood efficiently than the rats receiving fresh toxic *M. aeruginosa* and it leads to the increase of some hepatic enzymes in the blood by hepatic damage. Humpage (1999) recorded that microcystins are preferentially taken up by hepatic cells, so that hepatic tumor promotion is possible and this may lead to liver cancers. Also, it has been reported that, liver tumors can be detected when animals exposed to chronic low-level non-lethal dose. Thus, the result of the present study clearly indicate that the cell breakage or cell lysis enhance the release of microcystin to water. Release of microcystin is possible when cell lysis or cell brakage takes place during the bloom period and consumption of such contaminated water for drinking purposes may lead to liver injury or liver dysfunction in animals.

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