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PROTECTIVE EFFECT OF RIFAMPICIN ON MICROCYSTIN-LR INDUCED PHYSIOLOGICAL AND HAEMATOLOGICAL CHANGES IN RATS

RAVINDRAN, J., DEO KUMAR AND LAKSHMANA RAO, P. V. ?

Division of Pharmacology and Toxicology, Defence Research and Development Establishment, Jhansi Road, Gwalior-474002. E. mail : pvlrao@rediffmail.com

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Abstract: ***The cyclic peptide toxins microcystins and nodularins are the most common and abundant cyanotoxins present in diverse water systems. They have been the cause of human and animal health hazard and even death. Microcystn-LR (MC-LR) is the most common and toxic among the micrcoystin variants. The physiological effects of MC-LR have not been adequately addressed. We report here the results of our study on acute dose of MC-LR (125 µg/kg; intraperitoneal) on certain haematological and physiological effects in rats. The protective effect of a chemoprotector, rifampicin (25 mg/kg; intraperitoneal; 1 h pre-treatment) on reversal of MC-LR induced haematological and physiological parameters was also evaluated. MC-LR significantly decreased WBC count and mean corpuscular volume and the platelet count increased by 1.7 fold over control. The rifampicin alone did not produce any significant change and prevented all the haematological alterations caused by MC-LR. The physiological end points such as mean arterial pressure, heart rate and respiratory rate showed significant decrease with increase in time in MC-LR treated group. In rifampicin protected group, the body temperature and respiratory rates were found to be significantly lower. The results of this study, especially the increase in platelet count and drastic decrease in blood pressure clearly indicate the inflammation caused by acute toxicity of MC-LR. Rifampicin pretreatment could prevent the MC-LR induced lethality and reverse many of haematoligical and physiological effects.***

Key words: Microcystin-LR, Rifampicin

INTRODUCTION

Toxin producing cyanobacteria pose a world-wide threat to humans and animals due to their wide spread occurrence in both drinking and recreational waters. Primary public exposure to cyanobacterial toxins can come from drinking water, recreational water, dietary supplement and water used for medical treatment like dialysis [1,2]. Human illness attributed to cyanobacterial toxins are categorized into, gastroenteritis and related diseases, allergic and irritation reactions and liver diseases [3]. The most commonly encountered cyanobacterial toxins are the micrcocystins. Variants of microcystin are designated by the two variable aminoacids and vary in their toxicity. Microcystin-LR (MC-LR) is the most common and most toxic variant [4]. Microcystins are also tumor promoting agents [3].

The LD_{50} of MC-LR by i.p or i.v. in mice and rats is in the range of 36-122 µg/kg, while the inhalation toxicity in mice is LCT50 180 mg.min.m3 [5,6]. Symptoms of microcystin intoxication are diarrhea, vomiting, piloerection, weakness and pallor [3]. Acute microcytsin poisoning in mammals is characterized by disruption of hepatic architecture due to phosphorylation of cytoskeletal proteins, massive intrahepatic haemorrhage and death in few hours [5,7]. MC-LR is a potent inhibitor of serine/threonine protein phosphatases 1 and 2A leading to increased protein phosphorylation which is directly related to hepatotoxicity and tumour promotion activity [8].

Apart from the phosphatase inhibitory activity of microcystins, some reports are available on the other toxic manifestation of microcystin variants. Nobre et al. [9] has shown the possibility that chronic intoxication with microcystins may affect the kidneys by directly acting mechanisms. Acute experiments on the isolated perfused rat kidneys have shown that microcystins could act by specific mechanisms unrelated to their systemic effects. MC-LR is also reported to have immunomodulatory effect *in vivo* and *in vitro*. Hernandez et al. [10] found that MC-LR regulated the spontaneous adherence of human polymorphonuclear leukocytes in peripheral blood. Chen et al. [11] study showed the involvement of NO production, iNOS in microcystin-LR induced shock. Reports suggest that microcystin induced toxicosis may be able to modulate the immune system through several mechanisms including the reduction of lymphocyte proliferation, phagocytic activity, modification of natural killer cell activity and disturbance of cytokine synthesis [12,13]. There are very limited studies on physiological effects of microcystin. The emphasis of this study was to characterize haematological and certain physiological effects in rats exposed to lethal dose of MC-LR and protective efficacy of rifampicin in reversal of the effects.

MATERIALS AND METHODS

Chemicals: The toxin microcystin-LR (MC-LR) was obtained from Alexis Biochemicals (Switzerland). All other chemicals were obtained from Sigma Chemical Co. (St.Louis, USA) unless otherwise mentioned.

Animals: Wistar male rats weighing 225 to 300 g body weight, randomly bred and maintained in the Establishment's animal house were used for the study. The animals were housed in polypropylene cages with dust-free rice husk as bedding material and were provided with pellet food (M/s. Ashirwad Industries, Chandhigarh, India) and water *ad libitum*. The experiments were conducted with rats segregated and maintained as separate groups and allowed to acclimatize 7 days prior to dosing. The animals were fasted prior to the treatment. The care and maintenance of the animals were as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. The study has the approval of Institute's ethical committee on animal experimentation.

Experimental Protocol

Acute LD50 determination of microcystin-LR in rats: The acute intraperitoneal LD50 of microcystin-LR was determined in male rats following Dixon's up and down method [14] for small groups. MC-LR was dissolved in minimum amount of DMSO (0.1%) and diluted to required concentration in PBS. The LD50 of microcystin-LR in male rats was found to be 100 µg/kg body weight, while 125 µg/kg dose was found to cause 100% mortality and this dose was used for further studies.

Haematological effects of MC-LR in rats: The animals were injected with 125 µg/kg of microcystin-LR (intraperitoneal). The animals were observed till death and the blood was collected from retro-orbital plexus just before death (~90 min). To study the protective effect of rifampicin on MC-LR toxicity, rifampicin (25 mg/kg) was dissolved in DMSO and given as 1 h pre-treatment, intraperitoneally. After 1 h of rifampicin treatment, the MC-LR (125 µg/kg) was given and the animals were observed for 4 h. The blood was collected from retro-orbital plexus of rats after 3 h and various haematological parameters, viz. white blood cells (WBC), red blood cells (RBC), haemoglobin (Hgb), haemaotocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (Plt) were analysed using Beckman Coulter blood cell counter.

Physiological effects of MC-LR in rats: The physiological parameters such as mean arterial blood pressure (MAP), heart rate (HR), respiratory rate (RR), neuromuscular transmission (NM) and body temperature were recorded using an eight channel polygraph (Grass Instruments, USA Model No 7-16 P-35) as discussed elsewhere [15]. Briefly, the rats were anesthetized with urethane (1.6 g/kg, *i.p.*) and the neck region was dissected and exposed. The trachea was cannulated and connected to a pneumotachograph to record the respiratory rate (RR) through a differential pressure transducer (Hugo Sachs Electronics, Germany). The left carotid artery was cannulated with a thin polypropylene tube connected to a pressure transducer (Statham P23Dc), filled with heparinised normal saline. The pressure transducer was connected to a preamplifier (low-level DC, Grass Instruments, USA) and arterial blood pressure was recorded on the polygraph. Mean arterial blood pressure was calculated from the

Table 1: Effect of rifampicin (25mg/kg) on haematological parameters altered by lethal dose of MC-LR (125 µg/kg)

	Control	MC-LR (125µg/kg, ip)	Rifampicin (25 mg/kg, ip)	Rifampicin +MCLR
WBC ($x10^3$/UL)	21.35 ± 0.35	12.43 ± 1.23*	22.56 ± 0.45	20.45 ± 0.67
RBC ($x10^6$/UL)	6.84 ± 0.73	6.87 ± 0.53	7.12 ± 0.54	6.54 ± 0.79
Hgb (g/dL)	12.35 ± 0.95	11.87 ± 0.74	11.85 ± 0.65	12.83 ± 0.77
Hct (%)	37.58 ± 3.66	34.67 ± 2.5	36.98 ± 2.76	36.76 ± 2.65
MCV (fL)	55.15 ± 0.56	50.47 ± 0.38*	56.35 ± 0.56	55.46 ± 0.35
MCH (pg)	18.28 ± 0.62	17.33 ± 0.26	19.24 ± 0.82	18.39 ± 0.62
MCHC (g/dL)	33.08 ± 0.80	34.30 ± 0.45	34.18 ± 0.78	34.62 ± 0.74
Plt ($x10^3$/UL)	513.75 ± 72.57	652.67 ± 64.43*	524.45 ± 54.56	518.57 ± 62.64

Values are mean ± SE of four replicates per treatment. ***** Significantly different from control at $P \leq 0.05$ by Dunnet's test. WBC – white blood cells; RBC-red blood cells; Hgb- haemoglobin; Hct- haematocrit; MCV- mean corpuscular volume, MCH-mean corpuscular haemoglobin, MCHC-mean corpuscular haemoglobin concentration, Plt-platelets

recorded blood pressure. The pulse signals were also fed in to EKG Tachograph preamplifier (Type 7 P4, Grass Instruments, USA) to record the heart rate (HR). The pneumotachograph was connected to a differential pressure transducer (SWEMA, Germany) and connected to a pre-amplifier (Low level DC, Grass Instruments, USA), and inspiration was recorded as an upward deflection and expiration as a downward deflection to measure the respiratory rate (RR). Neuromuscular transmission (NM) studies were carried out to record the twitch responses. The gastracnemius muscle was opened and the sciatic nerve was stimulated with a supramaximal voltage (1-10V) of 0.2 msec duration at a frequency of 0.2 Hz using a Grass stimulator model S 88. The twitch response of the muscle was recorded using a Force Transducer (model FT0-3). Body temperature (BT) was measured using a rectal probe. The animals were allowed to stabilize for 1 h after the surgical procedures and all the treatments were given intraperitoneally. To study the microcystin induced physiological effects, the animals were injected intraperitoneally with lethal dose of MC-LR (125 µg/kg body weight). The animals were observed till death and the parameters were recorded.

To study the protective effect of rifampicin on MC-LR toxicity, rifampicin (25 mg/kg body weight) was dissolved in minimum amount of DMSO and given as 1 h pre-treatment, intraperitoneally. After 1 h of rifampicin treatment, the MC-LR (125 µg/kg, intraperitoneal) was given and the animals were observed for 4 hrs and the parameters were recorded. All treatments and recording of physiological response was initiated after 1 h stabilization period.

Statistical analysis: Data were expressed as mean ± S.E. from four animals per treatment. Data were analyzed by 't' test or Dunnet's test for comparison between control and treated groups. The level of significance was set at $p \leq 0.05$.

RESULTS

Haematological effects of microcystin-LR: Microcystin-LR induced changes in haematological parameters were analysed after microcystin treatment and after protection with rifampicin (Table 1). The MC-LR treated group showed a significant decrease in WBC, mean corpuscular volume. These changes were restored to control values in rifampicin + MC-LR treated animals. There was no change in other parameters. Only rifampicin treated group did not show significant difference in any of the parameters.

Physiological effects of microcystin-LR: The MAP**,** HR, RR, NM and BT of the experimental rats were recorded using pneumotachograph. After connecting the recorder the animals were allowed to acclimatize for 1 h. During this period the animals showed a steady response. Intraperitoneal administration of MC-LR (125 µg/kg) showed a time dependent decrease in MAP and RR. At time of death (~90 min) significant decrease in respiratory rate - 38.33% (Fig. 1A) and MAP - 39.13 % (Fig. 1B) was observed. A significant decrease in the body temperature (Fig. 1C) and heart rate (Fig. 1D) was also observed at time to death. No significant change in neuromuscular transmission was observed in any of the time points (Fig. 1E). To evaluate the protective

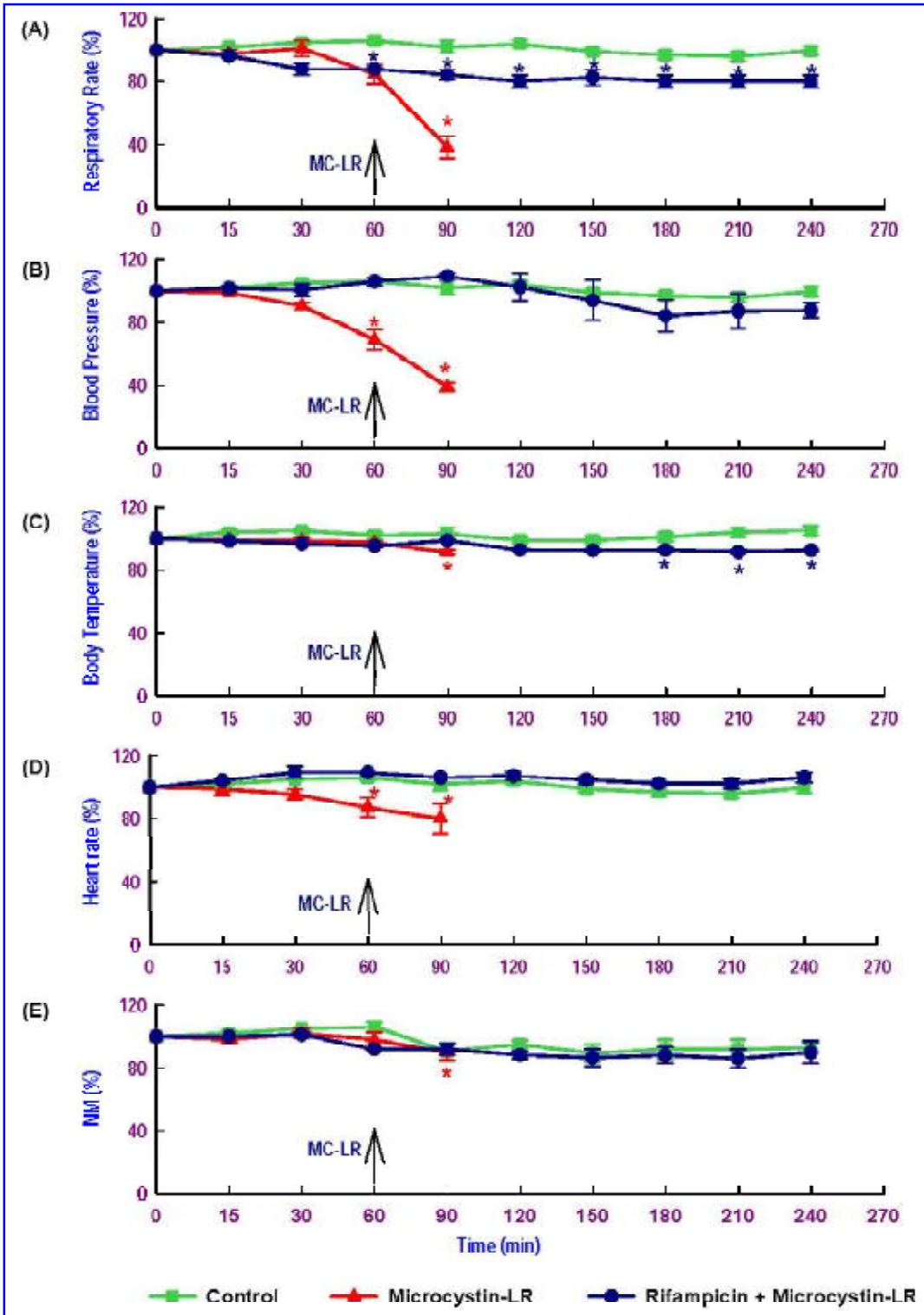

Fig. 1. Effect of intraperitoneal administration of microcystin-LR and Rifampicin + microcystin-LR on respiratory rate (A), mean arterial blood pressure (B), body temperature (C) heart rate (D) and neuromuscular transmission (E) . All treatments were given after 1 h stabilization period subsequent to surgical procedures. Values are mean ± SE of four animals per treatment. * Significantly different from control group at $p < 0.05$ by 't' test.

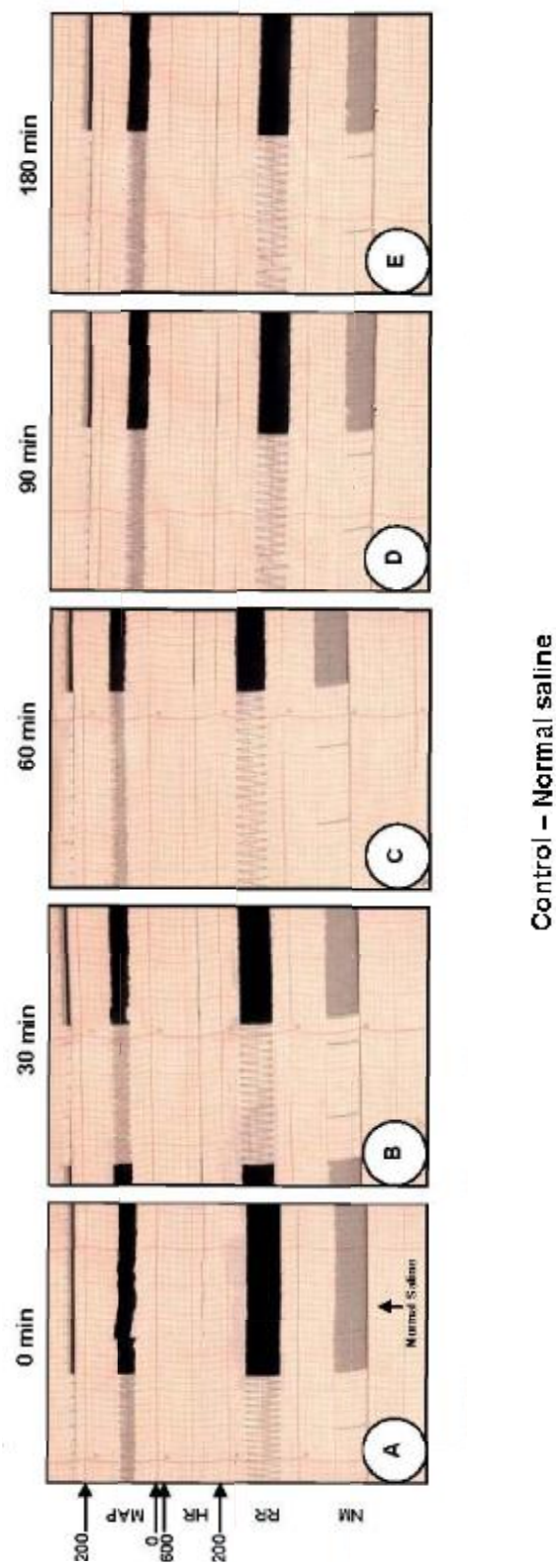

Fig. 2. Physiological effects of microcystin-LR in rats. All treatments were given after 1 h stabilization period subsequent to surgical procedures. Control animals were injected with normal saline ,*i.p*. and observed for 180 min (A-0 min, B-30 min, C-60 min, D-90 min, E-180 min). MAP- mean arterial pressure, HR- heart rate, RR- respiratory rate, NM-neuromuscular transmission. Representative record (up to 180 min) of four animals per treatment

Control – Normal saline

Microcystin-LR (125µg/kg)

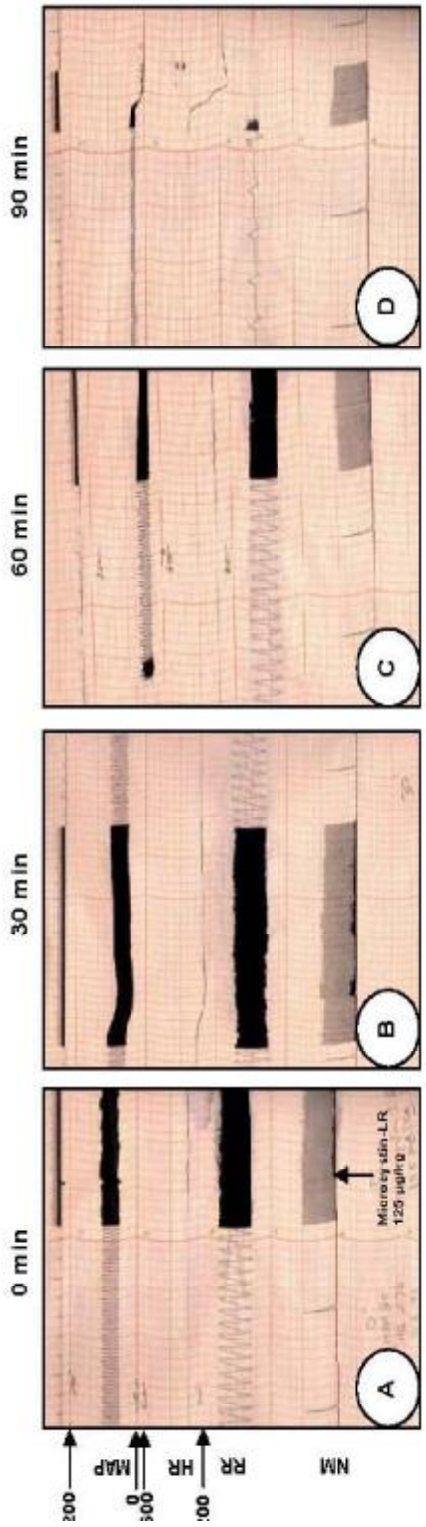

Fig.3. Effects of microcystin LR on physiological parameters in rats. All treatments were given after 1 h stabilization period subsequent to surgical procedures. Animals treated with MC-LR (125 µg/kg, *i.p*) died in 90 min (A- 0 min, B- 30 min,C- 60 min, D-90 min). MAP- mean arterial pressure, HR- heart rate, RR- respiratory rate, NM-neuromuscular transmission. Representative recording of four animals per treatment. MAP-mean arterial pressure, HR- heart rate, RR- respiratory rate, NM-neuromuscular transmission. Representative record of four animals per treatment.

effect of antidotes against microcystin toxicity, the rifampicin was administered intraperitoneally as 1 h pre-treatment. After the administration of rifampicin (25 mg/kg), the animals were observed for 1 h and the animals did not show any significant change in parameters. After 1 h animals were treated with MC-LR (125 µg/kg) and further observed for 4 h. The results show that there was no significant change in any of the parameters measured (Fig. 4). In comparison to MC-LR group, the rifampicin + MC-LR treated animals did not show any changes in physiological parameters (Fig 4). The representative recording (up to 3 h) of control, MC-LR 125 µg/kg, rifampicin alone 25 mg/kg, and rifampicin + microcystin are shown in figures 2 to 4.

DISCUSSION

Microcystins are family of structurally related cyclic peptide toxins that are produced by a variety of cyanobacteria, most often belonging to genus *Microcystis*. Acute illness and deaths in both humans and livestock following exposure of microcystin contaminated water sources have been reported worldwide [16]. The primary target organ for microcystin toxicity is the liver [17], where the toxins cause complete disruption of the liver architecture, leading to rapid death of the animal. The cellular specificity and organotropism of microcystins is due to selective transport system present only in hepatocytes namely the multi-specific bile acid transport system [17].

Acute deaths following microcystin administration have been attributed to haemorrhagic shock related to liver damage and intrahepatic haemorrhage, as well as cardiac failure [18]. Reported circulatory effects of MC-LR, given intravenously in rats include a transient fall in arterial blood pressure followed by a slower, steady decline. Negative chronotropic and moderate negative inotropic effects in the isolated rat heart have been observed previously [19]. Some of the recent studies have shown that even small changes in gene expression can cause significant physiological effect [20,21]. Even small doses of MC-LR are shown to elicit minute structural perturbations of the cell memebrane. The gene expression studies have shown that small but significant changes in gene expression occurred at low dose MC-LR exposure [21,22]. Exposure to environmental agents can cause fundamental changes in basic physiology through alterations in patterns of gene expression, which can lead to increased risk of disease.

The present study was carried out to evaluate the haematological and physiological toxic effects induced by microcystin-LR in rats. Among hematological parameters, a significant decrease in count of white blood cells and a significant increase in the platelets was observed. The increase in platelet count indicates the inflammation induced by MC-LR. Microcystins can promote inflammation, which may contribute to the hepatic shock that leads to death [23]. No prominent changes in RBC, MCV, Hgb, Hct and MCH levels were noted. However, in the present study decrease in MCV was observed. Microcystin induced decrease in PCV and platelets counts [24,25] may be related to activation of clotting cascade as well as to losses associated with intrahepatic hemorrhage and shock.

In our study a time dependent significant decrease in MAP and RR was observed. In MC-LR treated animals the MAP was reduced by 60% at time to death (90 min). The heart rate of MC-LR treated animals showed a significant decrease in 60 min. The reduction in MAP confirms the pooling of blood in liver which is a typical phenomenon observed in animals intoxicated with microcystin. The acute drop in mean blood pressure in toxin group suggests a primary inadequacy in the circulatory system. These results are in tune with the earlier reports of the presence of a cardiogenic component that limits the physiological cardiac reserve, compromising a normal response to circulatory inadequacy [18]. Marked increases in liver weight have been used as typical biomarker for microcystin poisoning . When rats dosed with MC-LR were given blood and blood pressure was resorted, survival rates did not increase [18]. Pathophysiological changes observed in rats after i.v. exposure of MC-LR (50 and 100 µg/kg) include, rapid decline in cardiac output, acute hypotension, decreased heart rate, decline in oxygen consumption, CO_2 production, metabolic rate accompanied by hypothermia. Additionally, failure of bradycardia and low blood pressure to respond to isoproternol or dopamine indicates that MCLR also affects the heart of rats [18]. Any cardiac effect of microcystin is most likely secondary to effects on the liver. Theiss et al (1988) reported decline in aortic blood pressures after 60 min in anesthetized rats after lethal parenteral dose of microcystin [26]. In contrast, Beasley et al (2000) study with swines showed decrease in hepatic perfusion, central venous pressure

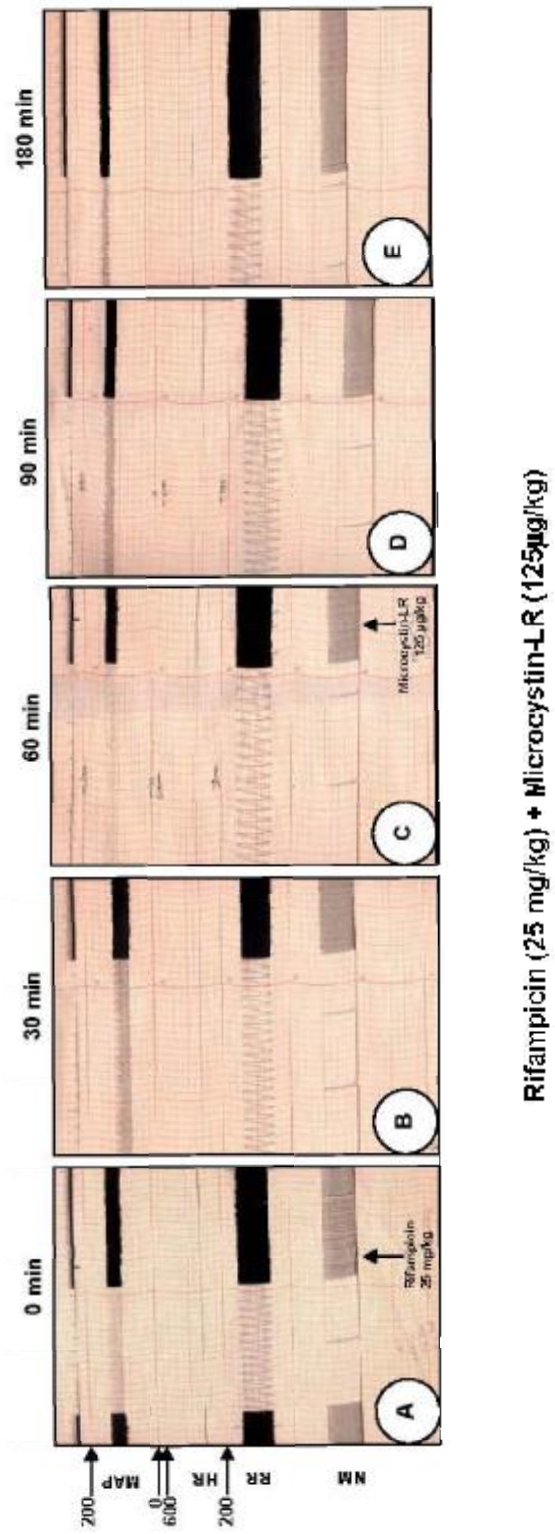

Fig. 4. Effects rifampicin pretreatment on microcystin-LR intoxication. All treatments were given after 1 h stabilization period subsequent to surgical procedures. Rifampicin (25 mg/ kg, *i.p*) is injected to the animals and observed for 60 min (A-0 min, B-30 min, C-60 min) then animals were treated with MC-LR (125 µg/kg, *i.p*) and observed for 240 min (A- 0 min, B- 30 min, C- 60 min, D-90 min, E-180 min). MAP- mean arterial pressure, HR- heart rate, RR- respiratory rate, NM- neuromuscular transmission. Representative record (up to 180 min) of four animals per treatment.

indicating that in pigs, blood flow through the liver rapidly declines in acute MC-LR toxicity [24] .

Studies to find antidotes against microcystin toxicity have shown in vivo protection in mice by a variety of chemically unrelated compounds including antioxidants, enzyme inducers, free radical scavengers, hepatic activity modulators. In our earlier study we have shown that rifampicin, silymarin and cyclosporine could protect animals from MC-LR induced lethality [27,28]. In the present study we evaluated the protective efficacy of rifampicin on reversal of physiological effects. Pretreatment with rifampicin could give 100% protection to the animals. A significant change in body temperature and respiratory rate was observed from 30 min and was persistent till 4 h. In rifampicin protected animals the MAP, HR and NM were found to be normal at all the time points. In conclusion, results of present study and other reports suggest that though the liver is a primary target organ for microcystin and ultimate cause of death has been attributed to hypovolumic shock secondary to hepatic haemorrahge, a potential cardiogenic component is involved in the pathogenesis of shock in microcystin intoxication.

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