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RESEARCH ARTICLE

Environmental Toxicology

Effect of cylindrospermopsin on the hepatotoxicity in wistar rats

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Abstract: A naturally-derived cyanotoxin, cylindrospermopsin (CYN), present in freshwater systems, poses a threat to human health due to potential hepatotoxicity. The present study aimed to determine the hepatotoxic effects of CYN on male Wistar rats. Following ethical clearance, 35 rats were divided into five groups. Test groups were administered with pre-prepared solutions to provide three doses of CYN: 0.175 µg/kg, 0.140 µg/kg, and 0.105 µg/kg. Well-water collected from Padaviya (0.161 µg/kg of CYN) was given to the environmental-exposure group (EN) and distilled water was administered to the control. The total duration of exposure was 90 days. Blood samples were collected at 0, 7, 14, 28, 42, 60, 90 days. Aspartate amino-transferase (AST), aspartate alanine-transferase (ALT), and full blood count (FBC) were analyzed. At 90 days, hepatic tissue samples were taken for histology. The mean body weight of the treated and control groups of rats gradually increased until the 90th day. A statistically significant reduction ($p < 0.05$) in increment of body weights was observed in CYN-treated rats, compared to the control, at 12th and 13th weeks. Relative weights of the livers of treated groups were significantly lower ($p < 0.05$) than those of the control group. The highest AST and ALT concentrations were recorded in rats given a CYN dose of 0.175 µg/kg. Haematology revealed a significant ($p < 0.05$) reduction in monocyte and lymphocyte counts in the EN group given well-water compared to the control. Ballooning degeneration, Kupffer cell hyperplasia, lobular haemorrhage and necrosis, perivenular inflammation, and sinusoidal congestion were observed in the livers of treated groups. The findings show that prolonged exposure to CYN contaminated water leads to hepatotoxicity in Wistar rats.

Keywords: Cylindrospermopsin (CYN), hepatotoxicity, histopathology, Wistar rats.

INTRODUCTION

Cyanobacteria are a group of over 2000 prokaryotic organisms commonly named 'blue-green algae' (Woese, 2002). Several genera of cyanobacteria produce two major toxin groups, the microcystins (MCs) and cylindrospermopsin (CYN). The *Microcystis* spp. and *Cylindrospermopsis* spp. of cyanobacteria grow well in a wide range of fresh, brackish, and marine habitats (Sethunga & Manage, 2010; Kulasooriya, 2011), dug wells (Abeysiri *et al*., 2018a, 2018b; Abeysiri & Manage, 2018), terrestrial ecosystems (Codd *et al*., 2005), extreme habitats of hypersaline localities (Chatchawan *et al*., 2011), hot springs (Wijesekara & Manage, 2017; Sadeepa *et al*., 2019a; 2019b) and arid deserts (Kulasooriya, 2011). Approximately 40 cyanobacteria species have been recorded as potential producers of cyanotoxins (Merel *et al*., 2013; Paerl *et al*., 2013). Cyanotoxins are secondary metabolites categorized chemically as cyclic peptides and alkaloids with a stable chemical structure (Zanchett & Oliveira-Filho, 2013). These are recognized as a potential hazard in drinking water worldwide, resulting in organ toxicity in humans and animals (Falconer & Humpage, 2005).

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CYN, a cyanotoxin contaminant of water supplies, is produced by freshwater, non-bloom-forming filamentous cyanobacteria; *Cylindrospermosis raciborskii, Anabaena bergii, Anabaena lapponica, Lyngbya wollei, Aphanizomenon flosa-aquae, Aphanizomenon ovalisporum, Umezaka natans,* and *Raphidiopsis curvata* (Falconer & Humpage, 2005; Sethunga & Manage, 2010; Mazmouz *et al*., 2011). *C. raciborskii* and *Anabaena* sp. are abundantly present in lakes, reservoirs, ponds, and rivers in many tropical and subtropical areas globally, with increased colonization recorded in temperate regions of Australia, Europe, Japan, India, Sri Lanka, and South America (Sethunga & Manage, 2010; Piyathilaka *et al*., 2015; Wijewickrama & Manage, 2019). CYN is a water-soluble, stable, tricyclic guanidine combined with uracil by the hydroxyl bridge (Runnegar *et al*., 2002) (Figure 1).

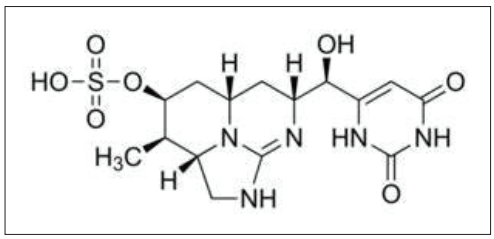

Figure 1: Chemical structure of cylindrospermopsin

The toxicity of CYN to the humans was first recorded by the "Palm Island Mystery Disease" of 1979, in Queensland, Australia, which affected 148 children and 10 adults, causing severe hepatotoxicity with renal tubular damage (Byth, 1980; Hawkins *et al*., 1985; Ohtani *et al*., 1992). Fatalities in haemodialysis patients due to the reverse osmosis unit of the dialysis water supply has been reported (Carmichael, 2001). *In vitro* animal experimental studies in rodents fed with MCs contaminated environmental water samples and extracts, or purified toxins of CYN have shown toxic effects on the vascular system (Hawkins *et al*., 1985), lymphatic system (Hawkins *et al*., 1985), reproductive system (Chen *et al*., 2011), immune system (Lankoff *et al*., 2004), and other organ damage: liver (Hawkins *et al*., 1985; Manage *et al.*, 2009c; Drobac *et al*., 2011), kidney (Hawkins *et al*., 1985; Piyathilaka *et al*., 2015; Abeysiri *et al*., 2018a; Abeysiri & Manage, 2018a), lungs, heart, stomach, and adrenal glands (Hawkins *et al*., 1985).

Thus, considering the toxic nature of CYN on human health, the World Health Organization (WHO) established a provisional guideline limit for CYN of 2 µg/L in drinking water (WHO, 2003; SLSI, 2013) and 0.02 µg/kg of body weight/day as the Tolerable Daily Intake (TDI) for humans (Guzman-Guillen *et al*., 2014). Recently, there have been increasing reports on the number and the intensity of blooms in reservoirs in the North Central, North East, and Uva Provinces of Sri Lanka (Madhushankha *et al*., 2013; Yatigammana & Perera, 2017; Manage, 2019). Further, scientific research has revealed contamination of the majority of drinking water reservoirs by toxin-producing cyanobacterium *M. aeruginosa*, *Cylindrospermopsis* sp., *Anabaena* sp. and a significant correlation between cyanobacterial cell density and the cyanotoxins in drinking water sources including dug wells (Hettiarachchi & Manage, 2014; Piyathilaka *et al*., 2016; Manage, 2019). Therefore, the present study was aimed at evaluating the effects of subchronic exposure to different doses of purified CYN and CYN contaminated well water on the liver of male Wistar rats through oral gavage over 90 days.

MATERIALS AND METHODS

Ethical approval was obtained from the Ethics Review Committee of the Faculty of Medical Sciences (An.E.17/18), University of Sri Jayewardenepura. The study was carried out following the relevant guidelines and regulations given by the Ethics Review Committee.

Animals

Eight-week-old male Wistar Rats (183.2 ± 0.2 g) purchased from the Medical Research Institute (MRI) of Sri Lanka were acclimatized for one week at the Animal House, Faculty of Medical Sciences, University of Sri Jayewardenepura. The rats were provided with food and water *ad libitum* unless otherwise stated, and maintained on a 12 hour light/dark cycle at 28 ± 2ºC. Water and food consumption were measured, and the behaviour of the animals was observed throughout the study.

Compound

CYN used in these investigations was Lot # BCBS9482v supplied by Sigma-Aldrich (USA). The purified toxin ($C_{15}H_{21}N_5O_7S$; product no: 32087) had an estimated purity of >95%.

Experimental design

In this study, three CYN doses were prepared based on the WHO standard recommendation of CYN in drinking water: higher than the WHO standard (HW) 2.5 µg/L, equal to the WHO standard (W) 2.0 µg/L and lower than the WHO standard (LW) 1.5 µg/L. The pure CYN doses, according to the mean initial body weights of the rats fed with the above concentrations, were HW = 0.175 µg/kg/day, W = 0.140 µg/kg/day, and LW = 0.105 µg/kg/day. A single well water sample collected from the Padaviya area of the North Central Province of Sri Lanka contained 2.3 µg/L CYN, which was above the WHO recommended level. This was used as the environmental sample. Accordingly, the environmental exposure group (EN) received 0.161 µg/kg/day of CYN. Distilled water was given to the control group. The CYN solutions, well water, and distilled water were fed for 90 days. The mouse groups were labeled as HW, W, LW, EN, and control according to the solutions received by them. Rats behaviour, and water and food consumption were recorded at intervals of every two days throughout the experiment. Nutritionally complete solid food (Saboudry, 1988) was supplied *ad libitum* to avoid contaminated urine samples with food dust. The solutions of CYN were formulated biweekly and adjusted to reflect weight changes in the treated groups before administration. After 90 days of dosing, the animals were euthanized, weighed (g), and the liver was resected out with minimum trauma.

Collection of blood

Venous blood samples were drawn from the lateral tail vein at 0, 7, 14, 28, 42, and 60 days for clinical chemistry. At the end of the experiment (90 days), when the rats were under anaesthesia prior to euthanization, blood was collected by cardiac puncture for haematological and chemical analysis. Blood for haematological analysis was transferred to a 1 mL tube containing EDTA to prevent coagulation.

Clinical chemistry

Serum concentrations of aspartate amino-transferase (AST) and aspartate alanine-transferase (ALT) were analyzed using Bialabo diagnostic kits (France) and a fully automated analyzer (Thermo Fisher Scientific, INDIKO, Finland). All animals were used to measure AST and ALT at 0, 7, 14, 28, 42, 60, and 90 days.

Haematology

Full blood count (haemoglobin (Hb), red cell indices, platelet count, total and differential white blood cell counts) and reticulocyte count were analyzed using a fully automated 3 part haematology analyzer (BCC-3000, China).

Histopathological evaluation

At necropsy, the liver was observed for gross pathological changes, and the weight was measured. The liver was serially cut, and the cut surfaces were examined for any difference in colour, areas of necrosis or fibrosis, and photographed with the reference number. Two random slices were selected and immediately fixed in ten times the volume of the slices in 10% formalin for routine histological assessment. If any abnormal areas were present, additional slices were fixed similarly for the histological evaluation. The changes observed were recorded in the datasheet. Following processing and dehydration, prepared slides were transferred to xylene for 5 min., absolute alcohol for 5 min., 90% alcohol for 2 min., 80% alcohol for 2 min., 70% alcohol for 2 min., and 60% alcohol for 2 min., and stained with hematoxylin and eosin; special stains used included periodic acid – Schiff stain (PAS) and silver special stains. Masson trichrome to assess fibrosis was not used due to the short duration of the experimental period.

For histological changes, the prepared sections were examined under the light microscope (Olympus CX31, magnification ×40, ×100, and ×400). The scoring system consisted of evaluating the tissues and assigning a semi-quantitative score scheme, from 0 = no lesion to 3 = severe lesions, according to the changes observed in the liver architecture, hepatocytes, and the presence of inflammation. After an initial review, selected tissues were re-assessed.

Statistical evaluation

One-way ANOVA was used to statistically evaluate significant differences, by using MINITAB version 17 statistical software (MINITAB, State College, PA, USA), and differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Clinical findings

The pure CYN doses received by the rats in different groups according to the mean initial body weights were HW = 0.175 µg/kg, W = 0.140 µg/kg, LW = 0.105 µg/kg and EN = 0.161 µg/kg.

The external appearance and signs of toxicity were not observed in CYN-treated animals during the study. Similar findings were reported by Diez-Quijada *et al.* (2021); clinical and visual observations during the study period did not show abnormalities in the groups studied. In general, there were no changes in behaviour or locomotor activity during the study period due to repeated exposure to CYN. Gradual mean body weight increments were noted in both treated (190.4 ± 3.8 – 300.3 ± 2.2 g) and control (198.4 ± 1.6 – 317.7 ± 3.5 g) groups of animals until the thirteenth week (Figure 2). However, there was a statistically significant reduction ($p < 0.05$) in body weights of rats in the W, HW, and EN groups given CYN when compared to the control group at weeks 12 and 13. Comparable results were reported by Chernoff *et al*. (2018) after the 90-day gavage of CYN in a mice experiment. Increased body weight with low toxin doses (30 and 60 µg/kg/day) and decrease of body weight at high doses (432 and 657 µg/kg/day) of CYN in mice had been reported in a previous study (Humpage and Falconer, 2003). In another study, no statistically significant differences were observed in body weight between treated and control groups in males (Diez-Quijada *et al.,* 2021). CYN is known to affect both hepatic and renal systems (Seawright *et al*., 1999; Humpage & Falconer, 2003; Bazin *et al*., 2012), and data presented here specify hepatic toxicity at four dose levels.

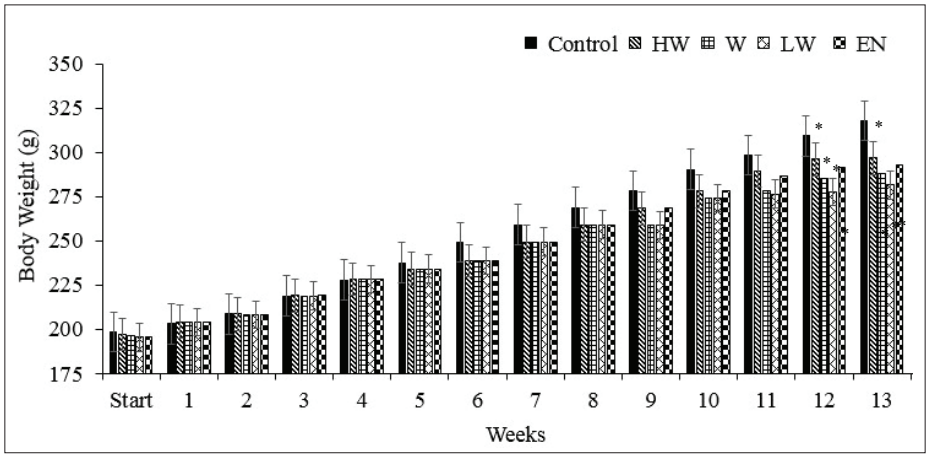

Figure 2: Mean body weight changes of Wistar rats in different treatment groups (HW = 0.175 µg/kg, W = 0.140 µg/kg, LW = 0.105 µg/kg, EN = 0.161 µg/kg)
*$p < 0.05$

Table 1: Bodyweight (g) and relative percentages of the liver weight of male Wistar rats exposed to different doses of CYN for 90 days.
Values are mean ± SD for 7 rats/group. Statistics ANOVA test; *$p < 0.05$

Parameters	Control	Pure CYN dose (µg/kg)			CYN environmental dose (µg/kg)
	0 N = 7	0.105 (LW) N = 7	0.140 (W) N = 7	0.175 (HW) N = 7	0.161(EN) N = 7
Body weight (g)	318 ± 0.98	284 ± 1.03 *	289 ± 1.26 *	300 ± 2.42 *	296 ± 1.33 *
Absolute weight (g)	9.02 ± 0.63	8.62 ± 0.56	10.41 ± 0.70*	9.27 ± 0.94	9.27 ± 1.30*
% Liver	3.19 ± 0.13	2.87 ± 0.03*	2.87 ± 0.05*	2.75 ± 0.02*	2.78 ± 0.01*

There were no gross pathological changes observed in the livers. The liver weights as a percentage of the body weight showed a significant decrease in treatments groups: 284 ± 1g in LW ($p < 0.05$), 289 ± 1g in W ($p < 0.05$), 300 ± 2g in HW ($p < 0.05$), and 296 ± 1g in EN ($p < 0.05$) in comparison to the control group (Table 1). No significant differences were found in the absolute and relative weights of males at any toxin concentration in the study by Diez-Quijada *et al.* (2021). However, Reisner *et al.,* 2004 found an increase in the relative weights of the livers in male mice exposed to CYN.

The AST level gradually increased up to the endpoint of the study and the differences were statistically significant in HW ($p < 0.05$), W ($p < 0.05$), LW ($p < 0.05$), and EN ($p < 0.05$) groups compared to the control at day 7, 14, 28, 42, 60, and 90 (Figure 3a). Significant differences of the ALT activity was found in HW ($p < 0.05$), W ($p < 0.05$), LW ($p < 0.05$) and EN ($p < 0.05$) groups compared to the control at day 7, 14, 28, 42, 60, and 90 (Figure 3b). Increased serum levels of AST and ALT activity indicated hepatic toxicity, which was compatible with changes observed on histology. Elevated ALT levels in mice with a high dose of CYN exposure were reported by other investigators (Chernoff *et al*., 2011; 2014). The AST activity was also elevated after oral exposure at the intermediate and highest dose groups (37.5 and 75 µg/kg CYN) to the control group ($p < 0.001$) (Diez-Quijada *et al.,* 2021). The data obtained from Dordevic *et al.* (2017) showed that the alterations in serum biochemical parameters did not produce significant liver damage in rats. Moreover, increased AST activity was found in females at all doses assayed (Chernoff *et al.,* 2018).

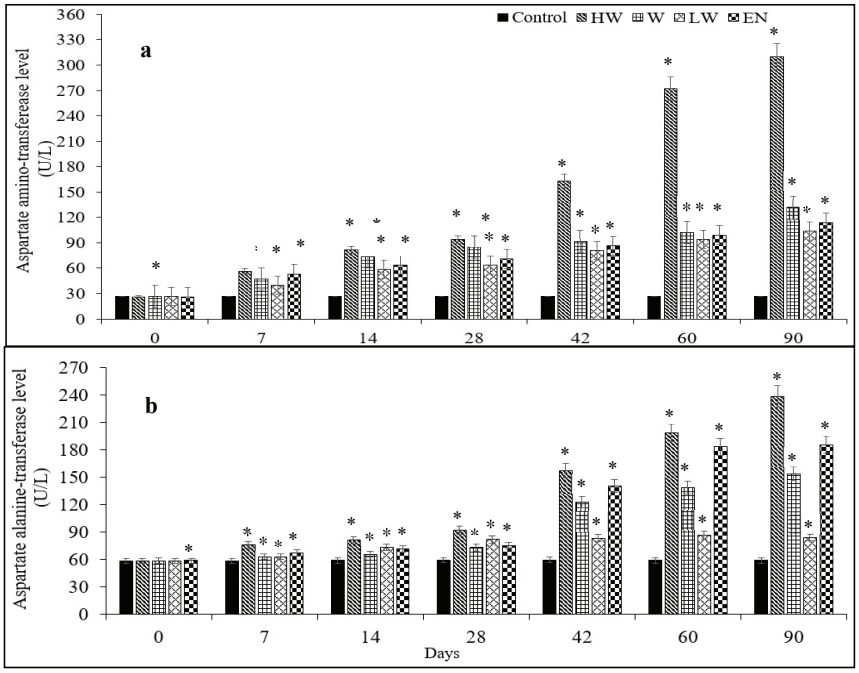

Figure 3: (a) Mean serum aspartate amino-transferase (U/L), (b) Mean serum as partate alanine-transferase (U/L) concentrations changes of male Wistar Rats in different treatment groups (HW= 0.175 µg/kg, W= 0.140 µg/kg, LW= 0.105 µg/kg, EN=0.161 µg/kg)
*$p < 0.05$

Haematology

Haematological parameters measured at day 90 are summarized in Table 2.

Non-significant reductions in both haematocrit and haemoglobin levels were reported in the HW, W, and EN groups. In the EN group, a significant decrease in lymphocyte count ($p < 0.05$) and monocyte count

($p < 0.05$) were observed in comparison to the control group. The absence of similar changes in the groups treated with pure CYN extracts makes it unlikely that the haematological changes can be attributed to the toxic effects of CYN. However, the rats in the EN group received the highest dose of CYN. Augmented haematocrit levels were reported as a response to exposure to low levels of CYN and morphological changes in the RBC of mice, which gradually developed into an acanthocyte-like form, cytoplasmic irregularly spaced projections, etc. (Reisner *et al.,* 2004). Similarly, elevated haematocrit levels in male and female mice after 16 weeks of exposure to variable concentrations of CYN in their drinking water (10–55 µg/kg/day during 42 weeks) were also found as the most critical effect observed (Sukenik *et al.,* 2006). By contrast, all the values remained within the normal range for the strain (Lira *et al.,* 2020). There were no significant alterations in the differential white blood cell counts of rats exposed to CYN in comparison to the untreated groups (Diez-Quijada *et al.,* 2021).

Table 2: Selected haematology parameters of Wistar Rats on the 90th day of the experiment
Statistics ANOVA test; *$p < 0.05$

Parameters	HW (0.175 µg/kg)	W (0.140 µg/kg)	LW (0.105 µg/kg)	EN (0.161 µg/kg)	Control
Haematocrit (%)	23.59 ± 1.18	22.27 ± 0.71	24.58 ± 0.96	22.93 ± 1.20	24.00 ± 1.14
Haemoglobin (g/dL)	16.23 ± 1.02	15.68 ± 0.25	17.07 ± 0.68	16.13 ± 0.89	16.63 ± 0.97
Reticulocyte count (10^{12}/L)	8.85 ± 0.35	8.19 ± 0.29	9.06 ± 0.23	8.55 ± 0.56	8.83 ± 0.50
Mean cell volume (fL)	106.54 ± 1.96	108.77 ± 3.09	108.47 ± 4.07	107.07 ± 1.41	108.93 ± 3.74
MCHC (g/dL)	69.11 ± 0.74	70.46 ± 1.38	69.43 ± 0.41	70.53 ± 0.61	69.27 ± 1.00
PLT (10^9/L)	689.1 ± 112.3	682.9 ± 82.6	754.3 ± 118.4	682.7 ± 97.2	622.3 ± 184.3
Lymphocyte count (10^9/L)	6.80 ± 2.58	6.06 ± 1.49	9.33 ± 2.81	3.50 ± 1.51*	7.03 ± 2.71
Monocyte count (10^9/L)	1.31 ± 1.65	0.74 ± 0.22	0.77 ± 0.27	0.40 ± 0.18*	0.67 ± 0.24
Granulocyte count (10^9/L)	1.51 ± 2.00	0.57 ± 0.21	0.83 ± 0.29	0.37 ± 0.15	0.47 ± 0.24

Table 3: Incidence and severity of histopathologic liver lesions in Wistar rats on the day 90 of exposure to cylindrospermopsin
Lesion severity was based on a 0 – 3 grading scale

Histological features	HW (0.175 µg/kg)	W (0.140 µg/kg)	LW (0.105 µg/kg)	EN (0.161 µg/kg)	Control
Hepatocytes	Ballooning (3)	Ballooning (2)	Ballooning (1)	Ballooning (3)	Normal (0)
Inflammation-lobular	Mild/spotty inflammation < 5/lpf (1)	Mild/spotty inflammation < 5/lpf (1)	Mild/spotty inflammation < 5/lpf (1)	Mild/spotty inflammation < 5/lpf (1)	Normal (0)
Hepatocyte cell death	Lobular/spotty (2)	Lobular/spotty (2)	Lobular/spotty (2)	Lobular/spotty (2)	Normal (0)
Hepatocyte regeneration	Bi-nucleation (2)	Normal (0)	Normal (0)	Bi-nucleation (2)	Normal (0)
Kupffer cells	Prominent (3)	Mild (1)	Mild (1)	Prominent (3)	Normal (0)
Nuclear pyknosis	Mild (1)	Mild (1)	Mild (1)	Mild (1)	Normal (0)
Haemorrhage	Mild (1)	Normal (0)	Normal (0)	Mild (1)	Normal (0)

Histopathology

Histopathological changes of the liver associated with CYN exposure are summarized in Table 3. These changes include hepatocyte ballooning, focal inflammation nuclear pyknosis, hepatocyte cell death/necrosis, features of hepatocyte regeneration, Kupffer cell hyperplasia, and haemorrhages (Table 3).

The hepatocytes showed ballooning degeneration (Figure 4b), Kupffer cell hyperplasia (Figure 4c), lobular haemorrhage and necrosis (Figure 4d) in EN (0.161 µg/kg). Lobular haemorrhage and necrosis (Figure 5b), lobular inflammation (Figure 5c), lobular haemorrhage (Figure 5d), sinusoidal congestion (Figure 5e), and centrilobular haemorrhage and necrosis (Figure 5f) were prominent in HW (0.175 µg/kg) group. Lobular inflammation and necrosis were observed in W (0.140 µg/kg) (Figure 6b). Lobular inflammation (Figure 7b) was significant in the LW (0.105 µg/kg) group. All histological features were typical in the control group (Figure 4a, 5a, 6a, 7a). In this study, hepatic lesions were seen in all treated groups. The effects were more severe in the HW and EN groups. The well water fed to the EN group contained CYN well above the safe level recommended by the WHO. These results were similar to the findings in 50 µg/kg doses of CYN administered for 5 days which produced a widespread disruption of hepatic cords, hepatocellular degeneration, necrosis, and centrilobular inflammation, as reported by Chernoff (Chernoff *et al*., 2011; 2014). In CYN-exposed cells in a culture, prominent features included necrosis and/or apoptosis of hepatocytes possibly resulting from a disruption of the cytoskeleton (Fessard & Bernard, 2003). Rats exposed by gavage to pure CYN (7.5–75.0 µg/kg b.w.) at 0, 24, and 45 h, showed irritation in the stomach and changes in hepatocytes (Diez-Quijada *et al.,* 2019).

Thus, Bernard *et al.* (2003) tested *in vivo* the toxicity of cell extracts of different *C. raciborskii* strains in mice and found fatal acute neurotoxicity, hepatotoxicity, and the absence of toxicity, depending on the toxins and the toxicological activity of unknown compounds detected. In the 2021 study by Leticia *et al.*, at a dose of 75.0 µg/kg b.w, only scarce microscopic changes were observed in the livers of rats. Livers displayed a very mild hepatic sinusoidal ectasia and small numbers of hepatocytes with apoptotic features or spotty necrosis, mainly localized in the surroundings of the centrilobular vein. Occasionally, hepatocytes presented eosinophilic cytoplasmic inclusions (Leticia *et al*., 2021). The present study further confirmed the signs of hepatic injuries due to CYN. Liver/body weight ratios, the elevation of AST and ALT levels, and histopathological changes were important parameters of CYN-induced liver toxicity.

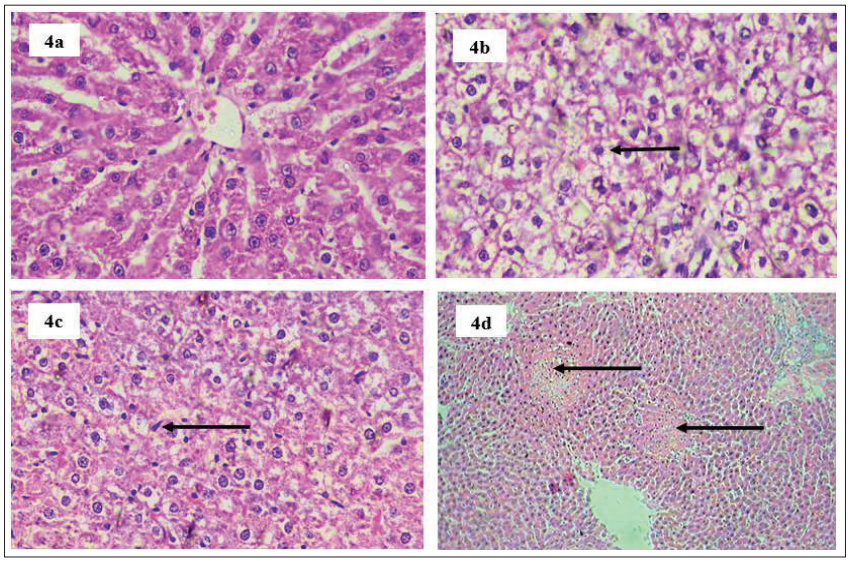

Figure 4: Environmental exposure (EN) Haematoxylin and eosin stain.
Figure 4a - Control ×400; Figure 4b - Ballooning degeneration of hepatocytes ×400; Figure 4c - Kupffer cell hyperplasia (arrow) ×400; Figure 4d - Lobular haemorrhage and necrosis (arrows) ×100

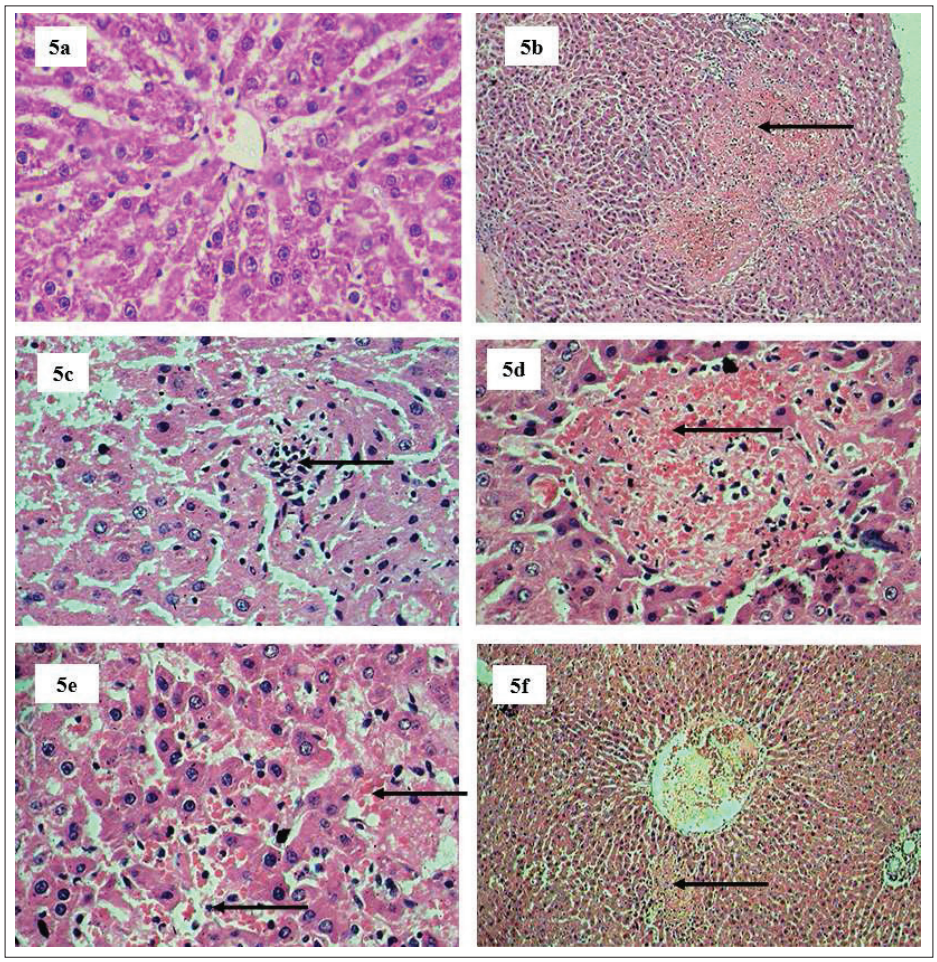

Figure 5: Above WHO level (HW) Haematoxylin and eosin stain
Figure 5a - Control ×400; Figure 5b - Lobular haemorrhage and necrosis ×100; Figure 5c - Lobular inflammation (arrow) ×400; Figure 5d - Focus of lobular haemorrhage ×400; Figure 5e - Sinusoidal congestion ×400; Figure 5f - Centrilobular haemorrhage and necrosis shown by the arrow ×100

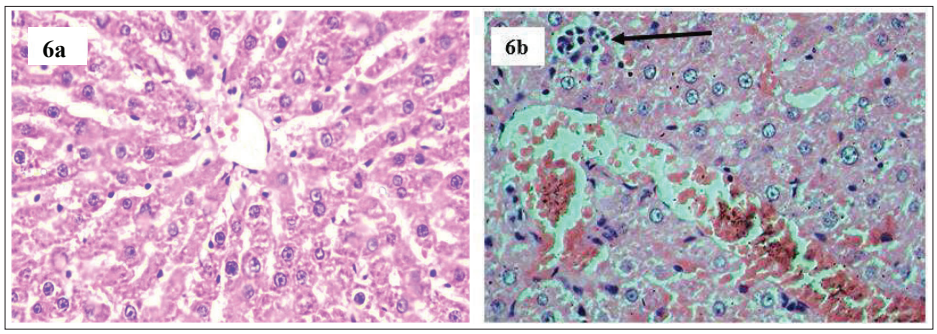

Figure 6: WHO level (W) Haematoxylin and eosin stain
Figure 6a Control ×400; Figure 6b - Lobular inflammation and necrosis (Arrow) ×400

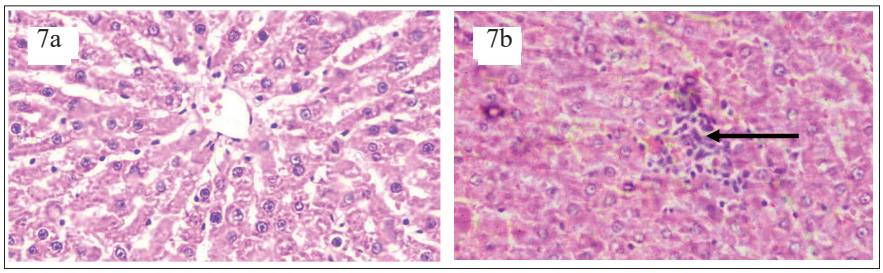

Figure 7: Below WHO level (LW). Haematoxylin and eosin stain
Figure 7a - Control ×400; Figure 7b - LW - Lobular Inflammation (Arrow) ×400

CONCLUSION

The study results showed that the differences in liver toxicity were associated with the administered CYN dose. It provides substantial evidence that CYN contaminated water for drinking purposes may lead to mammalian liver cell injury.

Conflict of interest statement

The authors declare that they have no competing interests

Acknowledgments

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