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Hepatic and intestine alterations in mice after prolonged exposure to low oral doses of Microcystin-LR

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ABSTRACT

Oral intake of Microcystin-LR (MC-LR) is the principal route of exposure to this toxin, with prolonged exposure leading to liver damage of unspecific symptomatology. The aim of the present paper was therefore to investigate the liver and intestine damage generated by prolonged oral exposure to low MC-LR doses (50 and 100 μg MC-LR/kg body weight, administrated every 48 h during a month) in a murine model. We found alterations in TBARS, SOD activity and glutathione content in liver and intestine of mice exposed to both doses of MC-LR. Furthermore, the presence of MC-LR was detected in both organs. We also found hepatic steatosis (3.6 ± 0.6% and 15.3 ± 1.6%) and a decrease in intraepithelial lymphocytes (28.7 ± 5.0% and 44.2 ± 8.7%) in intestine of 50- and 100-μg MC-LR/kg treated animals, respectively. This result could have important implications for mucosal immunity, since intraepithelial lymphocytes are the principal effectors of this system. Our results indicate that prolonged oral exposure at 50 μg MC-LR/kg every 48 h generates significant damage not only in liver but also in intestine. This finding calls for a re-appraisal of the currently accepted NOAEL (No Observed Adverse Effect Level), 40 μg MC-LR/kg body weight, used to derive the guideline value for MC-LR in drinking water.

1. Introduction

Cyanobacteria, also known as blue–green algae, are prokaryotic and photosynthetic organisms living in a variety of environments like ponds, rivers and lagoons.

Numerous species of cyanobacteria, such as *Microcystis aeruginosa*, *Anabaena* sp., and *Oscillatoria* sp., are toxin-producers (Sivonen and Jones, 1999).

Within hepatotoxins, microcystins (MCs) are widely distributed and Microcystin-LR (MC-LR) is one of the most frequently found in water bodies (Ueno et al., 1998). MC-LR is a cyclic heptapeptide with a general structure of cyclo-(D-alanine1-X2-D-MeAsp3-Y4-Adda 5-D-glutamate 6-Mdha7) with leucine and arginine at positions 2 and 4, respectively (Rinehart et al., 1988). It was reported that protein phosphatase (PP1 and PP2A) inhibition and increased production of reactive oxygen species are the main deleterious effects caused by MC-LR (Yoshizawa et al., 1990; Ding et al., 1998). The hyperphosphorylation and pro-oxidative state induced by MC-LR leads to alterations in the cytoskeleton (Solter et al., 1998; Codd, 1996; Milutinovic et al., 2003), in mitogen-activated protein kinase (MAPK) activity (Pahan et al., 1998), glycogen storage (Guzman and Solter, 2002), and in mitochondrial structure and function (Ding and Ong, 2003), all damages that severely affect crucial cellular processes.

Although the liver is the main target organ of MC-LR, the toxin also causes damage to other organs such as kidney, gastrointestinal tract, brain, lung, heart and thymus, and immune system (Bell and Codd., 1994; Takahashi and Kaya, 1993; Chen et al., 2004; Zhao et al., 2008; Soares et al., 2007). The damage generated and the organs affected in mammalians exposed to microcystins depend

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largely on the animal model employed and the characteristics of MC-LR exposure, mainly in terms of dose, route and time of exposure (Ohta et al., 1994; Falconer and Humpage, 1996; Ito et al., 1997; Zhang et al., 2002, 2007; Moreno et al., 2005; Zhao et al., 2008, 2009). As expected, the toxic effect caused by acute MC-LR exposure is different from that produced under chronic intoxication. The principal characteristics of acute MC-LR exposure consisted of loss of liver architecture and intrahepatic hemorrhage, finally leading to death within a few hours (Carmichael, 1994). This type of exposure also resulted in biochemical alterations such as a decrease in antioxidant enzyme activities and increased lipid peroxidation in liver and kidney (Moreno et al., 2005). Chronic MC-LR exposure on the other hand is characterized by a completely different damage pattern involving hepatocyte alterations such as single-cell necrosis, cytosolic vacuolation, apoptosis, fibrosis, and tumor promotion (Ito et al., 1997; Solter et al., 1998; Guzman and Solter, 1999; Andrinolo et al., 2008; Lezcano et al., 2012). In addition, sub-chronic exposure to MC-LR induces liver and renal lipid peroxidation, alteration in enzymatic and non-enzymatic components of the antioxidant system, and modifications of the liver lipid profile (Andrinolo et al., 2008; Sedan et al., 2010).

Cyanobacterial blooms favored by environmental conditions and the eutrophication of water bodies are common occurrences worldwide (Hallegraeff, 1992; Paerl and Millie, 1996). Furthermore, the water bodies where blooms occur are frequently used as recreational places or as drinking water sources, thus exposing large numbers of people and animals to cyanobacteria and their toxins. Numerous cases of human intoxication as a result of acute exposures have been reported, causing severe symptoms and in some cases leading to death (Tisdale, 1931; Zilberg, 1966; Turner et al., 1990; Teixera et al., 1993; Azevedo et al., 2002; Giannuzzi et al., 2011).

Prolonged oral exposure to low doses of MC-LR is of particular concern since it can arise from recreational activities, the intake of toxin-contaminated water, the ingestion of fish from contaminated water bodies, or the ingestion of toxin-contaminated dietary supplements (Dietrich et al., 2007; Chen et al., 2009) but often goes undiagnosed because this type of intoxication has no specific symptoms.

The aim of this work was to study the toxic effect(s) of prolonged oral MC-LR exposure at low doses in N:NIH-Swiss mice. We focused on the effects of MC-LR on liver (the main target for MC-LR) and intestine (directly involved in the uptake of the toxin).

2. Materials and methods

2.1. Microcystin-LR purification

MC-LR was purified from natural blooms of *Microcystis aeruginosa* collected at the Río de la Plata basin by established methods (Barco et al., 2002) with slight modifications. Firstly, cells were broken by sonication (Omni-Ruptor 400, 15 min) and the extract was cut with chloroform/methanol (50:50; v:v). The aqueous fraction was concentrated in a rotavapor (Decalab, R-23, Buenos Aires, Argentina). Purification was performed with semipreparative high-performance liquid chromatography. We used a Shimadzu 20A HPLC apparatus with a degassed module and a diode array detector system set at 238 nm. The preparative column used was TERMO Hyperprep HS C18 (250 × 10 mm) and the mobile phase was deionized water (TFA 0.05%) with 35% acetonitrile (TFA 0.05%) run in gradient conditions at 5 mL/min. The peak corresponding to MC-LR was collected separately and concentrated with a previously activated C18 cartridge. Pure MC-LR was eluted with a solution of methanol:water (90:10, v:v) after which the methanol was evaporated. The toxin purity and structure were tested by the HPLC-MS method (Barco et al., 2002). Final identification and concentration of MC-LR were achieved by comparison with a Sigma Chemical Inc. toxin standard (St. Louis, MO).

2.2. Mice and treatment protocol

Twelve male N:NIH-S (20–22 g) mice with specific pathogen-free certified status were obtained from the Animal Care Facility Unit of the Veterinary Medicine School of La Plata National University. They were housed in plastic cages (four animals per cage) and fed *ad libitum* on balanced feed (Alimentos Ganave, Rosario, Argentina) and water. Animals were maintained on a 12 h-light/darkness cycle and allowed to acclimatize to their surrounding conditions (well-ventilated room maintained at 23 ± 1 °C) for 1 week before experiments started. Studies were conducted in accordance with international protocols for laboratory animal care (National Research Council, 1985). Experimental designs were also approved by the local body for protecting animal welfare.

2.3. Experimental design

2.3.1. Prolonged oral MC-LR exposure

Mice were divided at random into three groups. Two groups were treated with 50 μg MC-LR/kg body weight (group 2) or 100 μg MC-LR/kg body weight (group 3) by gavage (300 μl) every 48 h during 1 month (15 total administration). These doses are close to the NOAEL (40 μg/kg) currently accepted (Fawell et al., 1999). The toxin was prepared freshly by dilution of the stock preparation with saline solution (0.9%; w/v). Corresponding control groups of animals were treated with equivalent volumes of saline solution (group 1). Mice were evaluated and weighed before each toxin administration. Exposed and control mice were sacrificed 24 h after the last administration. To minimize diurnal variations, animals were routinely killed between 12:00 and 13:00 h. Blood samples were obtained from the animals by intracardiac puncture. Liver and small intestine tissues were dissected and washed with cold PBS. The liver was weighed. Tissue samples were placed on ice-cold buffered solution (14 mM sodium phosphate, 0.1 mM EDTA, pH 7.40) (Marra et al., 1986) and homogenized by means of a glass-teflon homogenizer (Kontes Glass Company, Vineland, NJ). Cell debris and nuclei were removed by centrifugation at 10,000 × g (20 min at 1–2 °C) in a Sorvall RC5C Dupont centrifuge (Newtown, CT). Supernatants were aliquoted and frozen in hermetic polypropylene vials at −70 °C under N_2 atmosphere until used for experimental measurements. Appropriate aliquots of samples were treated immediately with 40 mM N-ethylmaleimide and precipitated with 15% (W/V) trichloroacetic (TCA) acid solution for glutathione determinations as described in the analytical section.

2.4. Analytical determinations

2.4.1. Histological studies

Liver and small intestine sections were fixed in 25 volumes of 10% formalin in phosphate-buffered saline (PBS), pH 7. Sections (4–5 mm thick) were stained with Hematoxylin & Eosin (H&E) (Culling, 1975), Oil Red (Cholewiak et al., 1968), Trichrome (Gomori, 1950) and Periodic Acid staining methods (PAS) (Mc Manus, 1948; Bancroft and Cook, 1994). Specific staining techniques (Oil Red, Tricrhome and PAS) were performed in order to demonstrate the presence of lipids (Oil Red), connective tissue (Trichrome) and glycogen (PAS). The slices were analyzed under optical microscope (Olympus Binocular Microscope) and pictures were taken for the morphometric analysis. ImageJ software (free access software) was used to carry out the morphometric analysis. In order to quantify hepatic steatosis, we determinated the area occupied by vacuoles

and the total area of tissue in each photograph (4 per mice). The ratio vacuoles area/total area was expressed as percentage.

To determine the percentage of intraepithelial lymphocytes, enterocytes and intraepithelial lymphocytes were counted in 30 representative *villi* per animal. The ratio intraepithelial lymphocytes/enterocytes was expressed as percentage.

2.4.2. Biochemical markers

Blood was collected in heparinized tubes and immediately centrifuged at 5000 g for 5 min. Plasma fractions were separated and analyzed for alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT) levels following the instructions of the manufacturer of the commercial kits (Wiener Labs., Rosario, Argentina). Total lipids were extracted from supernatants of liver homogenates by means of the Folch procedure (Folch et al., 1957). The total amount of lipids was gravimetrically determined up to constant weight under nitrogen atmosphere (Marra and Alaniz, 1989) using a Mettler H6T digital balance (Zürich, Switzerland).

2.4.3. Oxidative stress biomarkers

To determine lipid peroxidation, samples from intestinal and hepatic homogenates were processed for thiobarbituric acid reactive substances (TBARS) and measure as malondialdehyde (MDA). The colorimetric method described by Okawa et al. (1979) was used. Oxidized glutathione content (GSSG) was determined in liver and small intestine samples following the method of Rahman et al. (2006) and reduced glutathione (GSH) was measured following the glutathione-S-transferase assay described by Brigelius et al. (1983). Samples for glutathione analyses were obtained in the presence of N-ethylmaleimide and deproteinized (1:9, by vol.) using trichloroacetic acid (15% final concentration) as recommended by Asensi et al. (1994). Superoxide dismutase activities (SOD) were assayed as described by Marklund and Marklund (1974). Protein content was determined by the micromethod of Bradford (1976) with crystalline bovine serum albumin (BSA) as standard.

2.4.4. MC-LR determination

MC-LR was determined in liver and small intestine. MC-LR extraction from samples was carried out following the technique described by Soares et al. (2006). Briefly tissue homogenates samples were extracted twice with methanol centrifuged 7000 × g and mixed with equal volume of hexane. After that, hexane fraction was discarded, methanol was dried and the obtained extract was resuspended in deionized water. MC-LR analyses were performed by inhibition phosphatase assay (MacKintosh, 1993). Briefly Protein phospatase-1 catalytic subunit (Sigma) and an aliquot of aqueous extract previously obtained, were incubated for 10 min at 30° in reaction buffer pH 7.4. Then *p*-nitrophenyl phosphate (*p*NPP) 40 mM was added as the substrate and the mix were re-incubated by 30 min. The reaction was stopped by addition of 1N NaOH. The amount of *p*-nitrophenol was determined by the absorbance at 405 nm. A calibration curve was carried out with MC-LR standard (Sigma) between 1 and 10 ppb.

2.5. Statistical treatment

All results were subjected to one-way analysis of variance (ANOVA) with the aid of Systat (version 12.0 for Windows) from SPSS Science (Chicago, IL), and represent the means ± SD of 4 animals per group. Differences in mean value between groups were assessed by the two-tailed Student's t-test and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Weight and appearance

No death or symptoms were observed in mice from group 2 (50 μg MC-LR/kg) or group 3 (100 μg MC-LR/kg). The body weight gain in exposed groups (group 2: 3.37 ± 1.43 g; group 3: 2.74 ± 0.59 g) did not differ significantly from that of the control groups (3.77 ± 0.95 g), nor did the physical appearance and size of the livers of treated mice.

3.2. Histological studies and biochemical markers

Histopathological studies showed that doses as low as 50 μg MC-LR/kg were able to produce significant histological alterations in liver and small intestine.

At the end of treatment, control liver had a normal appearance with a clear cell radial arrangement surrounding the central vein (Fig. 1-A). However, oil red-stained liver sections from treated animals (50 μg MC-LR/kg; Fig. 1-B or 100 μg MC-LR/kg; Fig. 1-C) showed cytoplasmatic vacuolation in centrilobular zones, suggesting lipid accumulation. The livers of animals treated with the higher dose (group 3, Fig. 1-C) showed extensive lesions, whereas the injuries of those treated with a lower dose (group 2, Fig. 1-B) were mostly focalized within specific areas (arrows). These alterations are consistent with 3.6 ± 0.6% and 15.3 ± 1.6% of hepatic steatosis for 50 (group 2)- and 100 μg MC-LR/kg (group 3)-treated animals, respectively (Fig. 1-D). Furthermore, we observed several lipid vacuoles affecting the cytoplasm in the case of 100 μg MC-LR/kg treated animals (Fig. 2-E). In the case of the 50 μg MC-LR/kg-treated mice (Fig. 2-C) there were fewer and smaller vacuoles affecting only a small proportion of the cytoplasm. In agreement with histopatological data, the total lipid content in exposed animals increased from 0.40 ± 0.04 mg/mg protein in control groups up to 0.69 ± 0.02 and 0.58 ± 0.03 mg/mg protein in 100 μg MC-LR/kg- and 50 μg MC-LR/kg-treated mice, respectively. PAS-stained liver slices (Fig. 2-D and -F) showed unstained vacuoles indicating the absence of glycogen or glycogen-derived substances and thus supporting the lipid nature of the observed deposits. No changes were observed in the activities of transaminases (ALT, AST) and alkaline phosphatase (ALP) in plasma from 50 μg- (ALT: 17 ± 4 U/L, AST: 25 ± 5 U/L, ALP: 149 ± 21 U/L) or 100 μg-MC-LR/kg (ALT: 17 ± 3 U/L, AST: 30 ± 9 U/L, ALP: 164 ± 28 U/L) treated mice with respect to controls (ALT: 21 ± 2 U/L, AST: 39 ± 9 U/L, ALP: 174 ± 20 U/L).

Small intestine also showed histological alterations. Though intestinal *villi* remained unaltered with a normal vascularized connective axis and normal cellularity, there was a decrease in the number of intraepithelial lymphocytes (determined as a percentage of the number of enterocytes per *villi*) in mice treated with 50 μg MC-LR/kg (13.6 ± 0.9%) or 100 μg MC-LR/kg (10.7 ± 1.6%) compared to controls (19.2 ± 0.2%) (Fig. 3). These alterations are consistent with a decrease in intraepithelial lymphocytes of 28.7 ± 5.0% (group 2) and 44.2 ± 8.7% (group 3).

3.3. MC-LR content

Liver and small intestine MC-LR levels were measured by inhibition phosphatase assay. Significant differences in MC-LR levels in liver of 50- and 100 μg-MC-LR/kg treated animals were found. The increase in MC-LR content in liver of mice treated with 100 μg MC-LR/kg was 17-fold higher (358.7 ± 59.6 ng/g liver) than that of mice treated with 50 μg MC-LR/kg (20.8 ± 2.4 ng/g liver). An average of 1.4% of the total dose of MC-LR administrated to each mouse was

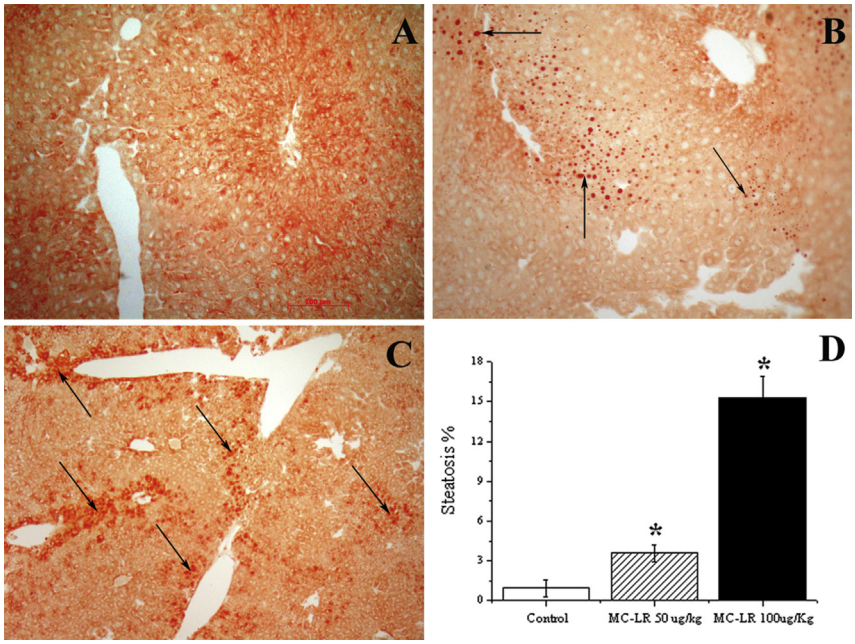

Fig. 1. Representative slices oil red-stained (100×) of liver from (A) control, (B) 50 μg MC-LR/kg and (C) 100 μg MC-LR/kg. Black arrows indicate intracytoplasmic vacuoles in liver of mice treated with MC-LR, which represent a 3.6 ± 0.6% and 15.3 ± 1.6% of steatosis (D) for 50 and 100 μg MC-LR/kg treatment respectively (mean ± SD, N = 4). * Represents significant difference from control values according to ANOVA test of two independent populations ($p < 0.05$).

found in the liver of mice treated with 100 μg MC-LR/kg and 0.2% for those treated with 50 μg MC-LR/kg. Our results indicate a higher toxin uptake in livers of mice treated with the highest dose (Fig. 4). Small intestine samples showed lower levels of MC-LR with respect to those found in liver samples, and there were no significant differences between MC-LR levels in intestine from 50 to 100 μg MC-LR/kg treated mice, respectively.

3.4. Lipid peroxidation and redox homeostasis

Lipid peroxidation induced by MC-LR treatment was estimated by measuring the thiobarbituric acid-reactive substances (TBARS) in hepatic and intestinal homogenates. Increased lipid peroxidation was found in liver and small intestine of mice treated with both doses of toxin (Fig. 5-A and -C). However, we did not observe a dose-dependent accumulation of TBARS in any of the tissues examined.

As one of the main components of the enzyme antioxidant system, superoxide dismutase (SOD) activity was also determined. We observed lower SOD activity in liver of treated mice at both doses tested, compared to control data (Fig. 5-B). In the case of the small intestine, SOD activity was lower in both 50 μg- and 100 μg-MC-LR/kg treated animals with respect to controls (Fig. 5-D).

Oxidized (GSSG), reduced (GSH), and total glutathione were measured in liver and small intestine homogenates. Liver GSSG levels were significantly higher in 50 and 100 μg MC-LR/kg treated mice and the corresponding GSH/GSSG ratios therefore lower than in control data (Fig. 6-A). No statistical differences were observed as a function of the dose. Small intestine levels of GSH and GSSG were also severely affected by MC-LR administration (Fig. 6-B). GSH was lower in small intestine of mice treated with either dose than in control data. A depletion of total glutathione levels was also found. Even though the lower GSH could contribute to the observed decline in the GSH/GSSG ratio, the higher GSSG content is likely to the major determinant of this parameter. There were no statistically significant differences between doses.

4. Discussion

The oral administration in mice of 50 μg MC-LR/kg or 100 μg MC-LR/kg every 48 h was studied during one month with the aim of elucidating possible damage produced by this toxin under prolonged exposure. Such exposure was found not to cause death or symptoms of clinical illness in mice. Moreover, the body and liver weights of treated mice did not differ significantly from control data. These results are coincident with previous studies performed with i.p. sub-lethal doses (45 μg MC-LR/kg) in a rat model (Guzman and Solter, 2002) and are also congruent with data reported on a 13-week MC-LR oral treatment (at 40, 200 and 1000 μg MC-LR/kg) carried out in mice (Fawell et al., 1999). These earlier experiments also showed that no symptoms were found under MC-LR administration.

A dose-dependent histological liver injury was observed at both doses tested in the current work. The treatment with 100 μg MC-LR/kg induced a 15.3% hepatic steatosis and the administration of 50 μg MC-LR/kg resulted in 3.6%. In line with this finding, higher liver lipid content was found in treated mice with respect to controls. No alterations were detected in biomarker enzyme activities such as ALT, AST, and ALP measured in peripheral plasma. In this connection, Fawell et al. (1999) observed hepatocyte vacuolization in mice exposed to prolonged daily oral doses (40 μg MC-LR/kg during 13 weeks) without plasma alterations of liver damage biomarkers. These authors have associated hepatocyte degeneration, vacuolization, and chronic liver inflammation with ALT and AST increased plasma levels only in male mice treated orally daily doses of 200 μg MC-LR/kg. Thus, our results indicate that prolonged and intermittent oral exposure at doses as low as 50 μg MC-LR/kg are capable of generating liver injury even when peripheral liver damage biomarkers remain unaltered. An important implication of these results from the clinical point of view, is that the measurement of plasma ALT, AST, and ALP is not the best choice of technique for detecting MC-LR exposure in a low-dose oral exposure scenario (Solter et al., 1998; Andrinolo et al., 2008). The experimental evidence supports the hypothesis that it is necessary to determine a

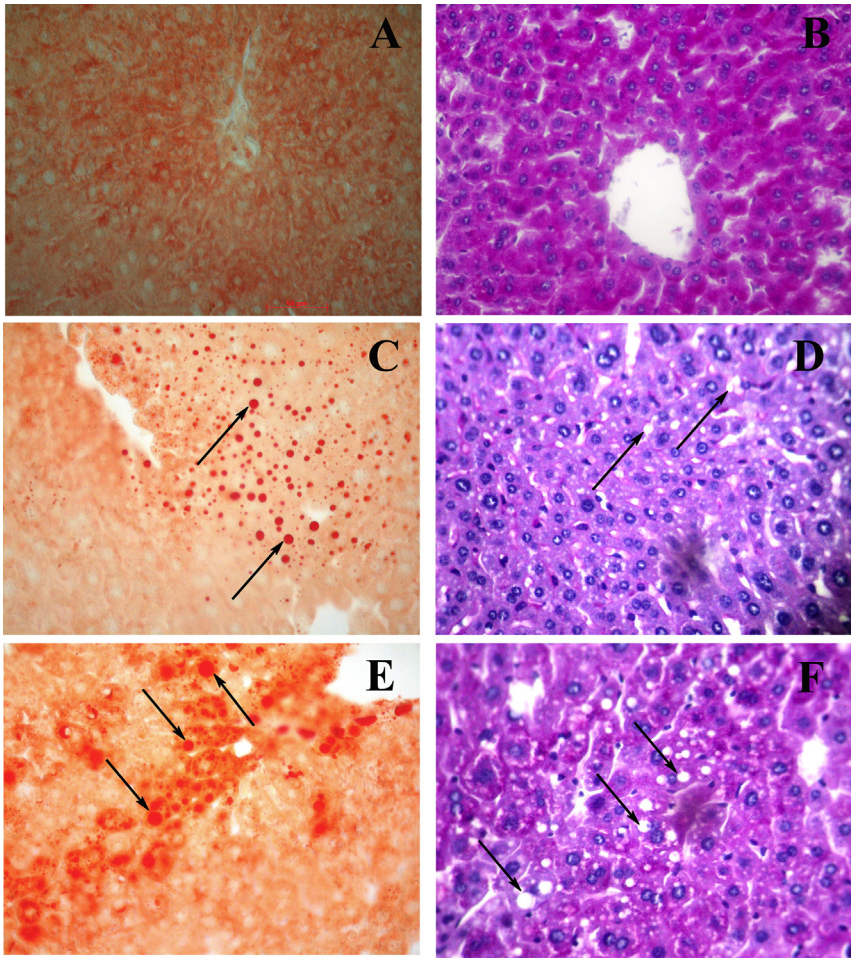

Fig. 2. Representative liver slices oil red-stained (A: control, C: 50 μg MC-LR/kg, E: 100 μg MC-LR/kg) and PAS-stained (B: control, D: 50 μg MC-LR/kg, F: 100 μg MC-LR/kg) 400×. Black arrows indicate intracytoplasmic vacuoles stained in red in oil-red-stained slices and unstained in PAS-stained slices of MC-LR treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

range of parameters in addition to MC-LR plasma/urine quantification in order to more accurately evaluate microcystin exposure (Sedan et al., 2013). The lack of alteration in these biomarkers is evidently not a reliable indication of liver integrity.

Little is known to date about the impact of MC-LR on the intestine, especially under prolonged exposure. Rocha et al. (2000) reported that the supernatants of macrophages stimulated with MC-LR (0.1, 0.3 and 1.0 μg/ml) caused a time-dependent electrogenic intestinal effect and the release of interleukin-1β and tumor necrosis factor-α in *in vitro* studies performed in rabbit ileum. Norbe et al. (2004) demonstrated that Microcystin-LR and supernatants of macrophages stimulated by this toxin were capable of provoking intestinal secretion of water and electrolytes (sodium, potassium and chloride) *in vivo* using perfused rat ileal segment and ligated intestinal loop models. The results of Moreno et al. (2003) suggest that exposure to a single intraperitoneal injection of 100 μg MC-LR/Kg in rats alters intestinal peroxidation levels as well as the activity of cellular membrane enzymes (sucrase, acid phosphatase and succinate dehydrogenase).

In line with this we found dose-dependent histological alterations in small intestine of mice under prolonged exposure to 50 μg- and 100 μg-MC-LR/kg in the form of a dose-dependent decrease in intraepithelial lymphocytes. To the best of our knowledge, this is the first report of mammalian intestine alteration produced by oral MC-LR intoxication with doses as low as 50 μg MC-LR/kg every 2 days.

In previous works, Ito et al. (1997, 2000) studied MC-LR distribution and damage generated by the oral administration of 500 μg MC-LR/kg in mice. These authors demonstrated the presence of MC-LR in the gastrointestinal tract and found alterations in small intestine characterized by a decrease in *villi* density, surface erosion, and alterations in lamina propria. In our work, performed with doses 10 times lower than those used by Ito et al. (1997, 2000), we found alterations in intraepithelial lymphocytes but not in *villi* structure or lamina propria. Also, we detected the presence of MC-LR in small intestine of treated mice. Since our results suggest that toxin does not accumulate in the intestine, this alteration could result from the *direct* (or local) action of MC-LR on the tissue, or from the toxin affecting the systems regulating the lymphocytes production (thymus and/or bone marrow), or both. These results could have important implications in terms of the effect MC-LR could have on mucosal immunity, a system in which intraepithelial lymphocytes are major effectors. This acquires particular importance when one takes into account the probable concomitant exposure to MC-LR and bacteria or parasites, organisms that are often present in water sources together with toxic bloom.

Furthermore, over time, a dose of 50 μg MC-LR/kg administrated every 48 h could be considered as similar (though not exactly equivalent) to a 25 μg MC-LR/kg dose every 24 h. With this in mind, our results suggest that adverse effects possibly occur not only in liver but also in intestine at lower doses than 40 μg MC-LR/kg. This is the dose currently accepted as the NOAEL obtained from the

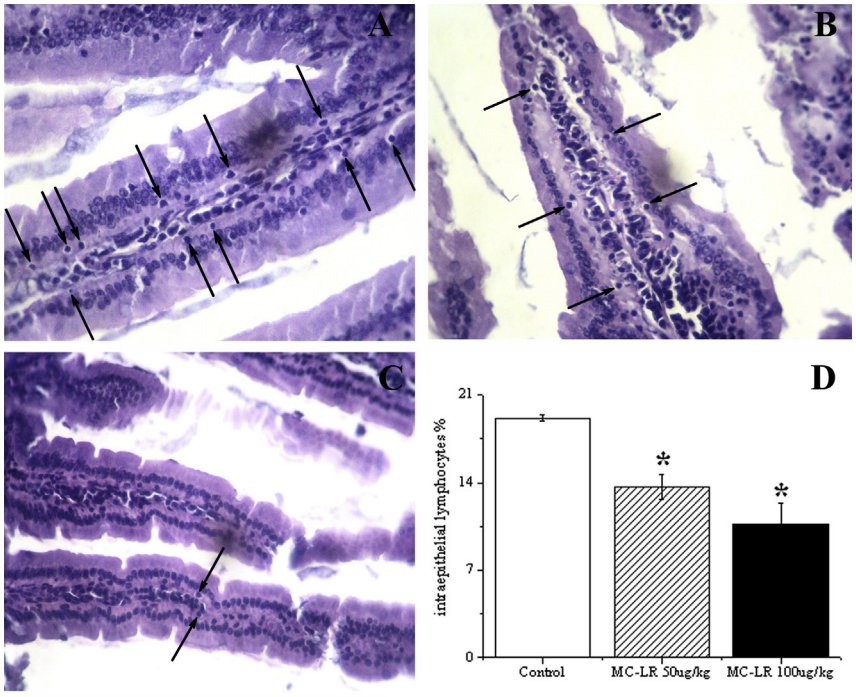

Fig. 3. Representative slices H&E-stained (400×) of small intestine from (A) control, (B) 50 μg MC-LR/kg and (C) 100 μg MC-LR/kg. Black arrows indicate intraepithelial lymphocytes. The percentage of intraepithelial lymphocytes (expressed as % intraepithelial lymphocytes/enterocytes per *villi*) were decrease in 28.7 ± 5.0% and 44.2 ± 8.7% for 50 and 100 μg MC-LR/kg treated mice respectively (D) (mean ± SD, N = 4). * Represents significant difference from control values according to ANOVA test of two independent populations ($p < 0.05$).

experimental data of Fawell et al. (1999) with daily oral administration of MC-LR in mice (Chorus and Bartrman, 1999; WHO, 2003).

We also demonstrated an oxidative stress condition induced by MC-LR exposure. In liver, lipid peroxidation increased whereas SOD activity, GSH levels and the GSH/GSSG ratio decreased. All these findings are in agreement with an earlier *in vivo* and *in vitro* report (Runnegar et al., 1987; Ding and Ong, 2003, Moreno et al., 2005; Sedan et al., 2010). Redox unbalance and lipid peroxidation were also observed in small intestine. Moreno et al. (2003) reported increased lipid peroxidation in intestine of rats treated with a single intraperitoneal injection of 100 μg MC-LR/kg. These authors suggested that the alterations found were due to *indirect* MC-LR action and reflect an excess production of oxygen free radicals induced by this cyanobacterial toxin. In our work we also found lower SOD activity, GSH levels and GSH/GSSG ratio in this tissue that surely has a crucial impact on the redox system. In the case of intestine, the loss of GSH is of major importance since this tissue does not have a highly active glutathione metabolism. In fact, most of the GSH is biosynthesized in liver and taken up for the intestine from the blood stream.

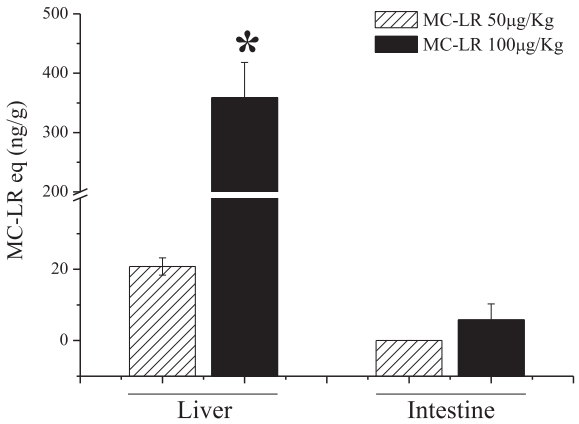

Fig. 4. Liver and small intestine levels of MC-LR in mice exposed to 50 and 100 μg MC-LR/kg, performed by inhibition Phosphatase test (mean ± SD, N = 4). The results are expressed as ng MC-LReq/g tissue. * Represents significant difference according to ANOVA test of two independent populations ($p < 0.05$).

Our results indicate that the mechanisms involved and the damage generated by MC-LR are essentially similar in oral and i.p. administration, even when there are differences in the level of concentration in the tissue generated by these different ways of exposure.

In agreement with previous reports (Solter et al., 1998; Ito et al., 2000; Sedan et al., 2013) we found higher levels of MC-LR in liver of mice treated with the highest dose.

In this case, the toxin detected in liver was a 1.4% of the total amount administrated in mice exposed to 100 μg MC-LR/kg and 0.2% for those exposed to 50 μg MC-LR/kg. The experimental data indicate that toxin accumulation in liver was 7-fold higher for mice treated with the highest dose (100 μg MC-LR/kg). A previous report (Solter et al., 1998) indicated that prolonged i.p. administration of doses of 16 and 32 μg MC-LR/kg resulted in 2.4% and 4.6% of total toxin administrated being detected in liver, respectively. Under these conditions liver accumulated almost 2-fold more MC-LR with the higher dose than with the lower one. It should be taken into account that the pathways and mechanisms involved in these two routes of exposure – oral or i.p. - are very different and that the toxin could be modified and/or degraded in different ways.

5. Conclusion

Taken together, our results confirm that oral exposure to low doses of MC-LR – even when they are asymptomatic – are able to generate damage not only in liver but also in small intestine.

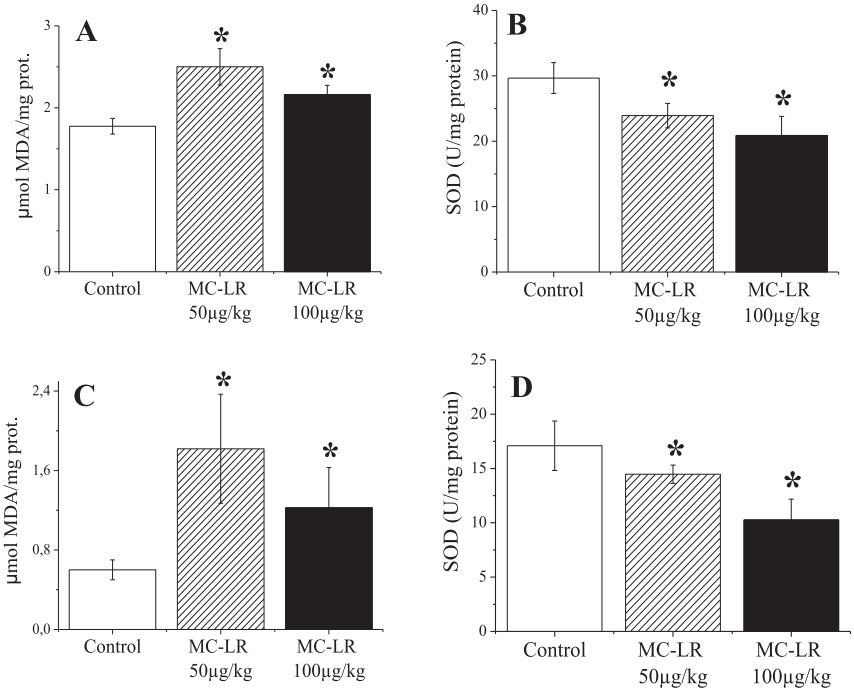

Fig. 5. Liver (A) and small intestine (C) lipoperoxide (LOOHs) levels measured as µmol MDA/mg protein (TBARS assay) in control (white bar), 50 µg MC-LR/kg (dashed bar) and 100 µg MC-LR/kg (black bar) treated mice (mean ± SD, N = 4). Superoxide dismutase (SOD) activity in liver (B) and small intestine (D) from control (white bars), 50 µg MC-LR/kg (dashed bar) and 100 µg MC-LR/kg (black bar) treated mice (mean ± SD, N = 4). * Represents significant difference from control values according to ANOVA test of two independent populations ($p < 0.05$).

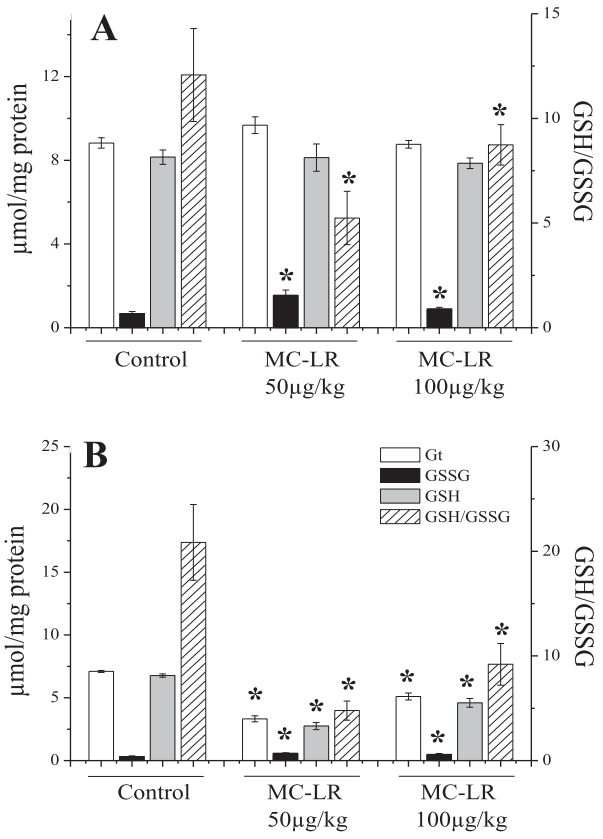

Fig. 6. Total (white bars), reduced (gray bars) and oxidized (black bars) glutathione contents, and GSH/GSSG ratios (dashed bars) in liver (A) and small intestine (B) from control and exposed mice to MC-LR (50 and 100 µg MC-LR/kg body weight) (mean ± SD, N = 4). *: Significantly different from control value ($p < 0.05$).

Prolonged oral MC-LR exposure could affect the immune system, causing a significant decrease in intestinal intraepithelial lymphocytes which are closely involved in mucosal immunity mechanisms. The effects of prolonged oral exposure to low doses of MC-LR should be studied in more depth in order to elucidate (i) the interplay between tissue damage and defense mechanisms, (ii) the involvement of the mechanism of toxicity in the alterations of the antioxidant defense system and its relationship with immune function, and (iii) more reliable NOAEL values for indicating oral MC-LR toxicity.

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