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LASSBio 596 *per os* avoids pulmonary and hepatic inflammation induced by microcystin-LR

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ABSTRACT

Cyanobacterial blooms that generate microcystins (MCYSTs) are increasingly recognized as an important health problem in aquatic ecosystems. We have previously reported the impairment of pulmonary structure and function by microcystin-LR (MCYST-LR) exposure as well as the pulmonary improvement by intraperitoneally injected (i.p.) LASSBio 596. In the present study, we aimed to evaluate the usefulness of LASSBio 596 *per os* on the treatment of pulmonary and hepatic injuries induced by MCYST-LR. Swiss mice received an intraperitoneal injection of 40 μl of saline (CTRL) or a sub-lethal dose of MCYST-LR (40 μg/kg). After 6 h the animals received either saline (TOX and CTRL groups) or LASSBio 596 (50 mg/kg, LASS group) by gavage. Eight hours after the first instillation, lung impedance (static elastance, elastic component of viscoelasticity and resistive, viscoelastic and total pressures) was determined by the end-inflation occlusion method. Left lung and liver were prepared for histology. In lung and hepatic homogenates MCYST-LR, TNF-α, IL-1β and IL-6 were determined by ELISA. LASSBio 596 *per os* (LASS mice) kept all lung mechanical parameters, polymorphonuclear (PMN) cells, pro-inflammatory mediators, and alveolar collapse similar to control mice (CTRL), whereas in TOX these findings were higher than CTRL. Likewise, liver structural deterioration (hepatocytes inflammation, necrosis and steatosis) and inflammatory process (high levels of pro-inflammatory mediators) were less evident in the LASS than TOX group. LASS and CTRL did not differ in any parameters studied. In conclusion, orally administered LASSBio 596 prevented lung and hepatic inflammation and completely blocked pulmonary functional and morphological changes induced by MCYST-LR.

1. Introduction

Cyanobacterial blooms are an increasing problem worldwide and the massive proliferation of these organisms is an important indicator of eutrophication (Funari and Testai, 2008). Among cyanotoxins, microcystin-LR (MCYST-LR) is considered the most common and dangerous one (Teixeira Mda et al., 1993). MCYST-LR constitutes a potent toxin and tumor promoter, mainly by the inhibition of protein phosphatases types 1 and 2A in hepatocytes (Kiguchi et al., 1992; Nishiwaki-Matsushima et al., 1992; Codd et al., 2005). Additionally, the acute exposure to MCYST-LR causes cytoskeleton disarrangement of hepatocytes. As a result of these actions hepatic failure ensues (Kujibida et al., 2006).

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In humans, exposure to cyanotoxins often occurs through several routes such as oral, dermal, nasal (by the inhalation of contaminated water), and parenteral (by the use of contaminated water in hemodialysis) (Turner et al., 1990; Azevedo et al., 2002; Leal and Soares, 2004; Falconer and Humpage, 2005; Andrinolo et al., 2008; Funari and Testai, 2008). Independently of the exposure route MCYST-LR preferentially reaches the liver and can also be detect in several organs including lungs (Wang et al., 2008).

Recently, our group reported the use of an anti-inflammatory drug candidate, LASSBio 596, to treat the pulmonary damage induced by the acute exposure to MCYST-LR. This compound was designed as an agent that modulates TNF-α and inhibits phosphodiesterases (PDEs) (Lima et al., 2002). Briefly, the intraperitoneal administration of LASSBio 596 avoided most of the pulmonary structural and functional damages, exhibiting a better outcome than dexamethasone. However, both treatments were not effective to avert the liver structural damage (Carvalho et al., 2010).

Even though the pharmacokinetics of LASSBio 596 has been described (Rocco et al., 2010), its therapeutic effects on by oral administration are so far unknown. Considering that the intraperitoneal route is not often used in clinical practice and that the treatments did not show effective for liver changes, an investigation about the therapeutic effects of orally administered LASSBio 596 on pulmonary and hepatic changes seems relevant and could establish the potential of LASSBio 596 as a drug candidate for the treatment of the systemic damage induced by microcystin-LR. Thus, in the present study, we aimed to evaluate the efficacy of LASSBio 596 *per os* in the treatment of pulmonary and hepatic damage in mice acutely exposed to MCYST-LR. For such purpose pulmonary mechanics, morphology and inflammatory cells influx, as well as the levels of pro-inflammatory mediators both in lungs and liver tissues, were assessed.

2. Materials and methods

The present study was approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (Protocol IBCCF 012). All animals received humane care according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences, USA.

2.1. Animals preparation

Twenty-six Swiss mice (35–40 g) were purchased from the animal facilities of the University of Campinas (CMIB/UNICAMP). They were randomly divided into 3 groups: In the control group, 40 μl of sterile saline solution (0.9% NaCl, CTRL, $n = 8$) were intraperitoneally (i.p.) injected, whereas in the other two groups a sub-lethal dose of MCYST-LR (40 μg/kg i.p., purified material kindly provided by Professor Wayne Carmichael, Wright State University, Dayton, OH, USA) was administered. After 6 h, CTRL, TOX ($n = 8$), and LASS ($n = 10$) mice received *per os* 60 μl of a solution composed by 57.5 μl of sterile saline and 2.5 μl of dimethyl sulfoxide (DMSO); in the latter group the solution contained 50 mg/kg of LASSBio 596.

Eight hours after saline or MCYST-LR administration, i.e., 2 h after treatment, the animals were sedated with diazepam (1 mg i.p.), anesthetized with pentobarbital sodium (20 mg kg body weight^{-1} i.p.), tracheotomized, and a snugly fitting cannula (0.8 mm id) was introduced into the trachea. The adequate anesthetic level was assessed by the absence of the palpebral, toe pinching, and corneal reflexes before animal paralysis. Thereafter, animals were paralyzed with pancuronium bromide (0.1 mg/kg i.v.) and mechanically ventilated with a constant-flow ventilator (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with a respiratory frequency of 100 breaths/min, a tidal volume of 0.2 ml, flow of 1 ml/s, and positive end-expiratory pressure of 2 cm H_2O. The anterior chest wall was then surgically removed. Since all measurements took no longer than 30 min and the combination of pentobarbital sodium and diazepam yields a depth and stable anesthetic level for at least 1 h (Fieldi et al., 1993; Green, 1975), the animals were bound to remain under deep anesthesia throughout the experiment.

2.2. Pulmonary mechanics

A pneumotachograph (1.5 mm ID, length = 4.2 cm, distance between side ports = 2.1 cm) (Mortola and Noworaj, 1983) was connected to the tracheal cannula for the measurements of airflow (V'). Lung volume (V_T) was determined by digital integration of the flow signal. Tracheal pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp, Northridge, CA, USA). The flow resistance of the equipment (Req), tracheal cannula included, was constant up to flow rates of 26 mL s^{-1} and amounted to 0.12 cm H_2O mL^{-1} s. Equipment resistive pressure (=Req.V') was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were then passed through 8-pole Bessel low-pass filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, total pressure drop (ΔPtot = ΔP1 + ΔP2), static elastance (Est), and elastic component of viscoelasticity (ΔE) were computed by the end-inflation occlusion method (Bates et al., 1985, 1988). Briefly, ΔP1 selectively reflects airway resistance in normal animals and humans and ΔP2 reflects stress relaxation, or viscoelastic properties of the lung, together with a tiny contribution of time constant inequalities (Bates et al., 1988; Saldiva et al., 1992). Lung static (Est) elastance was calculated by dividing Pel by V_T. ΔE was calculated as the difference between static and dynamic elastances (Bates et al., 1985, 1988). Pulmonary mechanics were measured 10–15 times in each animal. All

data were analyzed using ANADAT data analysis software (RHT-InfoData Inc., Montreal, QC, Canada). The duration of the experiments never surpassed 30 min.

2.3. Histological analysis

A lower mid-line longitudinal laparotomy was done immediately after the determination of pulmonary mechanics, and heparin (1000 IU) was intravenously injected. The trachea was clamped at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly euthanized the animals. Lungs were perfused with an infusion of formaldehyde 10% in Millonig's phosphate buffer (100 ml HCHO, 900 ml H_2O, 18.6 g NaH_2PO_4, 4.2 g NaOH), and, then, removed *en bloc*. After fixation, the tissue was embedded in paraffin. Four-μm-thick slices were obtained by means of a microtome and stained with hematoxylin and eosin (H&E).

Morphometric analysis was performed with an integrating eyepiece with a coherent system with 100 points and 50 lines coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The point-counting technique was used across 10 random non-coincident microscopic fields to evaluate the fraction area of collapsed and normal alveoli (×200), as well as the amount of polymorpho- (PMN) and mononuclear (MN) cells (expressed as cells/pulmonary tissue area) (×1000) (Gundersen et al., 1998). Two investigators, who were unaware of the origin of the coded material, examined the samples microscopically.

The livers were removed immediately after lung excision, fixed in buffered formaldehyde (10%) and embedded in paraffin. Four-μm-thick slices were stained with H&E. A pathologist, who was unaware of the origin of the material, examined the samples at magnifications of×100 and ×400.

2.4. Assay of pro-inflammatory mediators

Another fifteen mice (35–40 g) underwent the same protocol and group assignment as aforementioned. The levels of pro-inflammatory mediators (TNF-α, IL-1β and IL-6) were measured in lung and liver homogenates by ELISA with high sensitivity kits (R&D Systems Inc., Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The detection limit of this method corresponds to 5.1, 3.0 or 1.6 pg mL^{-1} respectively.

2.5. Quantification of microcystin-LR in the lung and liver

The amount of free MCYST-LR in the lung and liver was assessed by a combination of secondary anti–IgG antibodies and primary anti-MCYST-LR rabbit polyclonal antibodies with cross reactivity against several microcystins. Commercial kits for ELISA (Beacon Analytical Systems, Portland, ME, USA) were used according with the manufacturer's instructions. The detection limit of this method corresponds to 0.1 ppb.

2.6. Statistical analysis

SigmaStat 3.11 statistical software (SYSTAT, Chicago, IL, USA) was used. The normality of the data (Kolmogorov–Smirnov test with Lilliefors' correction) and the homogeneity of variances (Levene median test) were tested. Since in all instances both conditions were satisfied, one-way ANOVA followed by Bonferroni test was used to assess differences among groups, when required. The morphometric parameters underwent an arcsine transformation and, then, were compared. In all instances the significance level was set at 5% ($p < 0.05$).

3. Results

The treatment with LASSBio 596 *per os* significantly avoided the influx of PMN cells, airspaces collapse (Table 1), as well as the rising of TNF-α, IL-6 and IL-1β levels in lung and liver tissues (Fig. 1). Additionally, the elevated pulmonary mechanical parameters (Fig. 2), the presence of alveolar collapse, edema and alveolar septum thickness present in TOX (Fig. 3) were not observed in the LASS group. LASS and CTRL did not differ in any parameters studied.

MCYST-LR was not detected in lung tissue, but free MCYST-LR was similarly detected in liver tissue in both LASS and TOX groups (Fig. 4), but not in CTRL.

The disarray in liver architecture expressed by necrosis, inflammation, high degree of binucleated hepatocytes, cytoplasmatic vacuolization, dilated sinusoidal spaces and steatosis were less evident in the LASS than TOX group (Fig. 5).

4. Discussion

The main findings of the present study were: 1) the treatment with LASSBio 596 *per os* avoided lung and liver inflammation and pulmonary mechanical dysfunction found in TOX mice; 2) in addition a qualitative improvement in liver structure was observed.

It is known that MCYST-LR contamination leads to a direct liver insult followed by damage on several organs such as lung, kidney and intestine (Ito et al., 2001). However, acute lung injury related to MCYST-LR exposure is scantly assessed. Our group previously reported that respiratory system can be injured even by sub-lethal doses of MCYST-LR administered by pulmonary or extrapulmonary routes (Picanço et al., 2004; Soares et al., 2007). This suggests that these toxins even when administered at low concentrations may be present in the circulation and directly trigger a network of inflammatory responses mediated by immune

Table 1
Cellularity and morphology in pulmonary parenchyma.

	PMN (cell × 10^{-4} mm^{-2})	MN (cell × 10^{-4} mm^{-2})	Collapsed (%)	Normal (%)
CTRL	6.7 ± 0.5	38.2 ± 2.1	17.5 ± 1.0	82.5 ± 1.0
TOX	22.8 ± 1.9*	23.2 ± 5.6*	33.0 ± 1.2*	67.0 ± 1.2*
LASS	9.9 ± 1.0	40.5 ± 2.5	20.5 ± 1.1	79.5 ± 1.1

PMN and MN, polymorpho- and mono-nuclear cells; collapsed airspaces and normally aerated lungs are expressed as percentage of total microscopic area; CTRL, TOX and LASS, animals that received saline ($n = 8$), microcystin-LR ($n = 8$) and microcystin-LR plus LASSBio 596 ($n = 10$), respectively. Values are mean ± SEM in 8–10 microscopic fields *per* animal. *Significantly different from CTRL ($p < 0.05$). CTRL and LASS did not differ.

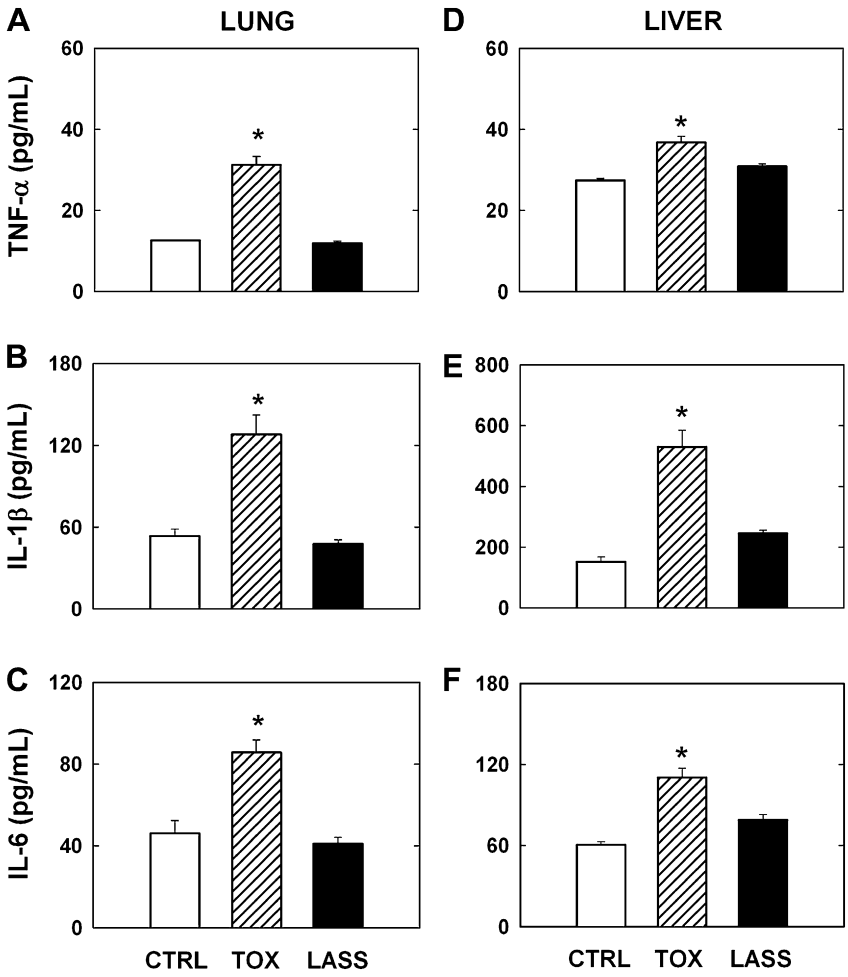

Fig. 1. The levels of pro-inflammatory mediators (TNF-α, IL-1β e IL-6) were measured in lung and liver homogenates by ELISA. Lung (A, B, C, respectively) and liver (D, E, F, respectively). CTRL, TOX and LASS, animals that received saline, microcystin-LR or microcystin-LR plus LASSBio 596, respectively. Values are mean + SEM of 5 animals in each group. *significantly different from CTRL and LASS that did not differ between them ($p < 0.05$).

cells in many organs (Wang et al., 2008). MCYST-LR inhibits PP1 and 2A, yielding an unusual cellular protein phosphorylation, and, thus, possibly activates protein kinase C. The latter activates phospholipase A_2 and cyclooxygenase, triggering inflammation (Nobre et al., 2001, 2003; Kujibida et al., 2006). Moreover, the influx of PMN also yields to the release of pro-inflammatory cytokines and reactive oxygen species (ROS) that adds to the development of tissue injury (Moreno et al., 2005).

When injected intraperitoneally LASSBio 596 seems effective in different models of acute lung injury, such as endotoxin model induced by lipopolysaccharide of *E. coli*, allergic sensitization to ovalbumin, ischemia and reperfusion, and also in acute lung injury induced by MCYST-LR (Rocco et al., 2003; Campos et al., 2006; Morad et al., 2006; Carvalho et al., 2010). In order to circumvent MCYST-LR undesirable effects, we have recently reported a possible treatment of pulmonary damage induced by acute exposure to MCYST-LR by the intraperitoneal administration of LASSBio 596 or dexamethasone (Carvalho et al., 2010). In the present investigation the effectiveness LASSBio 596 was tested for the first time after oral administration.

LASSBio 596, designed as a hybrid of thalidomide and aryl sulfonamide, is a new agent that exhibits weak inhibitory effect on PDE types 4 and 5 that are the main isozymes distributed in the lungs. This compound exhibits important anti-inflammatory and immunomodulatory properties especially by modulating TNF-α levels (Lima et al., 2002; Rocco et al., 2003). However, the precise mechanism whereby LASSBio 596 attenuates lung inflammation is unknown. PDE 4 and 5 inhibition may lead to suppression of chemoattractant and pro-inflammatory cytokine release, as TNF-α and IL-1β, downregulation of cell adhesion molecules, inhibition of leukocyte migration, functional inhibition of various types of cells including lung macrophages, neutrophils, lymphocytes, and monocytes, and increased macrophage anti-inflammatory cytokine production (Turner, 1993; Miotla et al., 1998). Hence, LASSBio 596 contributed significantly to the favorable results observed in our previous study (Carvalho et al., 2010), as well as in the present one.

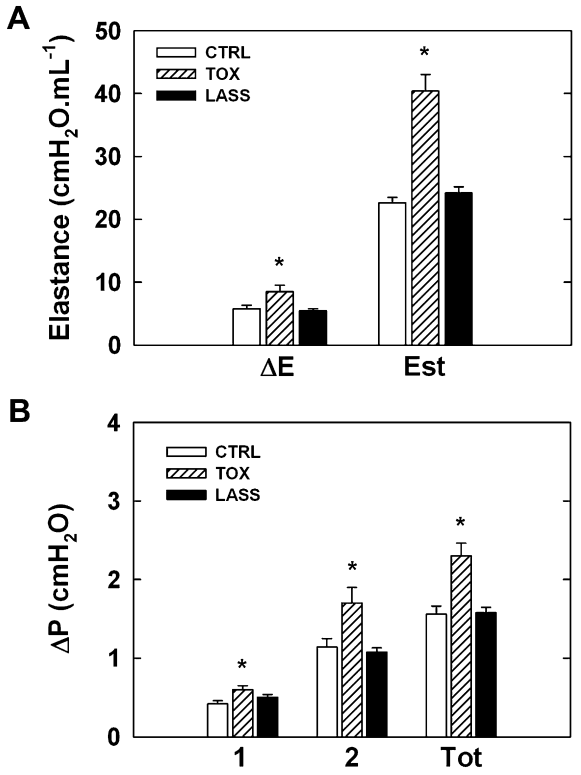

Fig. 2. Pressures used to overcome resistances and elastic components of lung mechanics in mice lung. *A*: Lung static elastance (Est) and viscoelastic component of elastance (ΔE); *B*: resistive (ΔP1), viscoelastic (ΔP2), and total (ΔPtot) pressure variations in mice injected with saline (CTRL), microcystin-LR (TOX) or microcystin-LR plus LASSBio 596 (LASS). Values are mean + SEM of 8–10 animals in each group (10–15 determinations *per* animal). *significantly different from CTRL and LASS that did not differ between them ($p < 0.05$).

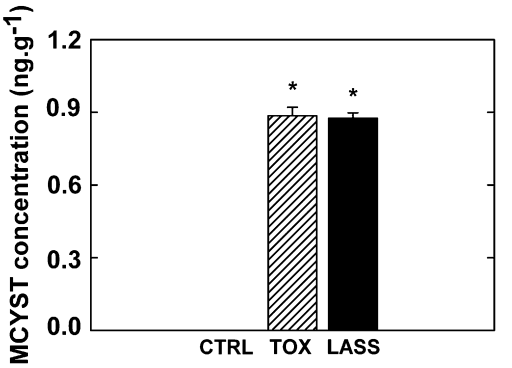

Fig. 4. Free microcystin-LR (MCYST-LR) content in mice liver. CTRL, TOX and LASS representative animals that received saline, microcystin-LR or microcystin-LR plus LASSBio 596, respectively. Values are mean + SEM of 8–10 animals in each group. No significant difference between groups that received microcystin-LR could be detected. *significantly different from CTRL. LASS and TOX did not differ between them ($p < 0.05$).

Despite its favorable lung protection, LASSBio 596 did not attenuate the hepatotoxic effects of MCYST-LR when intraperitoneally administered (Carvalho et al., 2010). In this line, the present study discloses for the first time an improvement in hepatic injuries induced by MCYST-LR after the administration of LASSBio 596 *per os*. Considering the therapeutic potential so far demonstrated by LASSBio 596, we sought to expand the knowledge on the therapeutic use of the compound, approaching its oral administration. The choice of this route of administration stemmed from the fact that although the peritoneal cavity represents a significant absorptive surface and the intraperitoneal injection is a common laboratory procedure, the latter is rarely used in the clinical practice. Therefore, in order to simulate a closer similarity with clinical procedures, we opted for the oral administration of LASSBio 596. Furthermore, it was not known whether this route of administration would result in any significant therapeutic effect. It is noteworthy that the majority of drugs absorbed from the gastrointestinal (GI) tract enters the portal circulation and passes through the liver before being distributed to the organism, whereas the venous drainage of the peritoneal cavity occurs through the inferior vena cava and also by the vena cava. Thus, it is likely that the oral administration of LASSBio 596 rapidly increased its concentration in liver, the target organ of the toxin, with better effects in this organ than when administered intraperitoneally (Brunton et al., 2006).

In this study we used a dose of 50 mg/kg, which represents a 5-fold higher dose than that used intraperitoneally. The selection of the dose was based on a recent report that

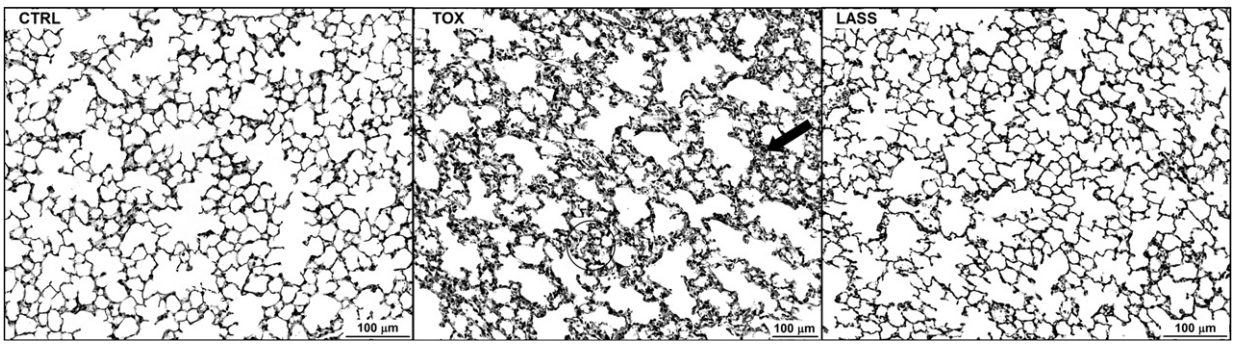

Fig. 3. Photomicrographs of lung parenchyma stained with hematoxylin-eosin. CTRL, TOX and LASS, animals that received saline, microcystin-LR or microcystin-LR plus LASSBio 596, respectively. Original magnification = ×200. It can be seen that LASS was very similar to CTRL, whereas TOX displays a larger amount of alveolar collapse, septal thickening, and edema than the other groups. Circle: alveolar collapse; arrow: septal thickening.

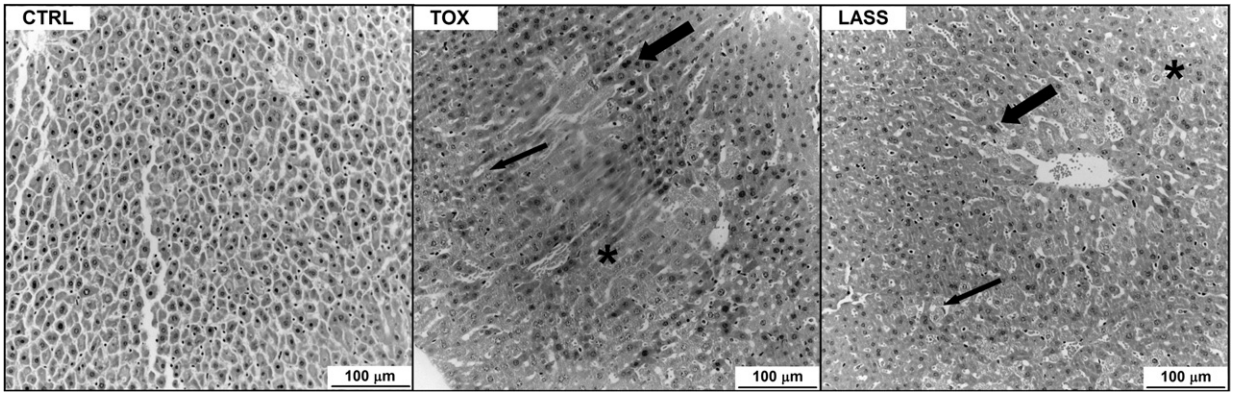

Fig. 5. Photomicrographs of liver parenchyma stained with hematoxylin-eosin. CTRL, TOX and LASS, animals that received saline, microcystin-LR or microcystin-LR plus LASSBio 596, respectively. It can be seen that although lesions in LASS group were less intense than those in TOX. Original magnification = ×400. *cell death; thin arrow: steatosis; thick arrow: binucleated hepatocytes.

describe the lower oral bioavailability of LASSBio 596 when compared to the intraperitoneal bioavailability (Rocco et al., 2010), which could be associated to a possible lower absorption of LASSBio 596 *per os*.

As previously reported by Carvalho et al. (2010), using the intraperitoneal route, treatment with LASSBio 596 *per os* avoided mechanical impairment, i.e., smaller ΔP1, ΔP2, ΔPtot, ΔE and Est in LASS than in TOX (Fig. 2). Part of these findings can be explained by improved structural and functional changes of lung parenchyma as evidenced by morphometric and cellularity analysis (Table 1 and Fig. 3). Indeed, a smaller area of alveolar edema, thinner septa and reduction of collapsed areas were found in LASS group than in TOX. In fact, LASSBio 596 *per os* rendered the results similar to those in CTRL. Accordingly, a significant improvement in the release of pro-inflammatory cytokines in lungs and liver was observed in LASS.

Additionally, the histopathological analysis of the liver showed dilatation and congestion of sinusoids, hepatocellular disarray, loss of hepatic architecture, high level of binucleate or multinucleate hepatocytes, vacuolation and necrosis in TOX group. Indeed, the hepatotoxic effects of MCYST-LR contamination are widely described (Hooser et al., 1989; Fugiki, 1992; Camichael, 1994; Barreto et al., 1996; Azevedo et al., 2002; Andrinolo et al., 2008). The pathological findings in LASS were less evident than in TOX group (Fig. 5). This improvement could probably be explained by the significant less liver inflammation in LASS.

In the present study we could not detect free MCYST-LR in the lungs, but it was present in the animals' livers to a similar extent in both groups that received MCYST-LR (Fig. 4). The liver is the target organ for microcystins, because of the ability of hepatocytes to uptake these toxins through bile acid transporters (organic anion transporting polypeptides) (Camichael, 1994; Feurstein et al., 2009). A damaged liver can release inflammatory mediators causing a secondary lung inflammation (Nobre et al., 2001). Furthermore, inflammatory mediators (TNF-α e IL-1) can be produced by peritoneal macrophages after microcystin injection (Nakano et al., 1991). Thus, even if MCYST-LR did not reach the lungs in our model, probably the acute pulmonary inflammation was started off by cytokines produced by the damaged liver and peritoneal macrophages, which were carried by the blood stream. Another possibility is the direct action of MCYST-LR on lung cells. Thus, it is possible that a recirculation of toxin occurs, increasing MCYST toxicity (Ito et al., 2001; Soares et al., 2007).

In this context, alveolar macrophage stimulated by MCYST-LR can produce prostaglandins F2 and PGE2 as well as thromboxane B2 and arachidonic acid (Naseen et al., 1989). The toxin could also damage type II pneumocytes. Since our method did not allow the determination of bound MCYST-LR, it was not possible to confirm its presence in the lung under this form. Thus, ELISA certainly underestimated the total concentration of MCYST-LR in blood or tissue. To further explain lung lesions in TOX mice, possibly conjugated MCYST-LR reached the lungs and/or free MCYST-LR got to the lungs in very low concentrations that could not be measured.

This study shows that both lung and liver are clearly affected by MCYST-LR, even at sub-lethal doses. The exposure of animals and humans to low doses in water is certainly more frequent than the lethal intoxication (Nobre et al., 2003; Kugibida et al., 2008). Thus, our mice were intraperitoneally exposed to 40 μg/kg of MCYST-LR to mimic a putative human contact with this toxin (Picanço et al., 2004; Soares et al., 2007). This sub-lethal dose already used in previous studies resulted in mechanical and histological impairment as soon as 2 h after intraperitoneal administration of MCYST-LR in Swiss mice; furthermore, these changes persisted for 4 days being the highest percentage of collapsed lung airspaces detected at 8 h after MCYST-LR injection (Soares et al., 2007).

5. Conclusion

We conclude that treatment with LASSBio 596 *per os* was effective to avoid pulmonary functional and structural changes, as well as lung and hepatic inflammation induced by MCYST-LR. A significant attenuation of hepatic injuries was observed for the first time.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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