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Chemoprotection of lipoic acid against microcystin-induced toxicosis in common carp (*Cyprinus carpio*, Cyprinidae)

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ABSTRACT

This paper evaluated the chemoprotective effect of lipoic acid (LA) against microcystin (MC) toxicity in carp *Cyprinus carpio*. To determine the LA dose and the time necessary for the induction of three different classes (alpha, mu and pi) of glutathione S-transferase (GST) gene transcription, carp were i.p. injected with 40 mg/kg lipoic acid solution. A group was killed 24 h after the first i.p. injection (condition 1); another group received two i.p. injections with a 24 h of interval between each one and was killed 48 h after the first injection (condition 2) and a third group received one i.p. injection and was killed 48 h latter (condition 3). Results showed that LA was effective in promoting an increase in GSTs gene transcription in liver only in the condition 2. A second experiment was done, where carp pre-treated with LA (condition 2) were gavaged twice with a 24 h interval with 50 μg MC/kg. Ninety-six hours after experiment beginning, carp were killed, and organs were dissected. Results of GST activity in liver and brain suggest that LA can be a useful chemoprotection agent against MC induced toxicity, stimulating detoxification through the increment of GST activity (brain) or through reversion of GST inhibition (liver).

1. Introduction

Lipoic acid (LA) is well-known as a cofactor of Krebs cycle dehydrogenases (Trattner et al. 2007). Some of their antioxidants properties (metal chelator, free radical interception, control of antioxidant genes expression) were recognized long time ago (Packer et al. 1997), leading to the study of its protective effects in different fields of science like toxicology and cancer prevention (Shila et al. 2005; Wang et al. 2008), aging (Suh et al. 2004), aquaculture (Terjesen et al. 2004; Trattner et al. 2007) and fish biochemistry (Monserrat et al. 2008). The use of LA as chemo and neuroprotective molecule has been proposed (Packer et al. 1997; Suh et al. 2004; Wang et al. 2008) in virtue of the control that LA exerts on genes of antioxidant defense like the rate-limiting enzyme of glutathione synthesis (glutamate cysteine ligase or GCL; EC 6.3.2.2) and glutathione-S-transferase (GST; EC 2.5.1.18) forms that confer to cells and organs a better antioxidant competence and also better abilities to detoxify through phase II reactions catalyzed by GST (Lee and Surh 2005; Monserrat et al. 2008).

Treatment with LA can be considered a potential preventive procedure to be applied in aquaculture, since LA posses the ability to cross the brain blood barrier (Packer et al. 1997), and also confers protection to cells infected by different strains of the bacteria *Pseudomona aeruginosa* (de Assis et al. 2004). The use of open ponds to rear aquatic organisms is common in Brazil (Poersch et al., 2006), an un-expensive practice that depends on water quality where organisms are reared. The confinement is a problem in subtropical environments, because of the occurrence of cyanobacterial blooms (Magalhães et al. 2001) that frequently releases cyanotoxins like microcystins (MC) exerting their primary toxic effect through inhibition of phosphatases (Dawson 1998). It is known that microcystins affect various wild fish (Qiu et al. 2007) as well as human health through food chain (Chen et al. 2009). However, well documented evidences in the last years point to other toxic mechanisms after microcystins exposure in several aquatic organisms: oxidative stress (Jos et al. 2005; Wiegand and Pflugmacher 2005) and alteration of glutathione-S-transferase (GST) activity, even augmenting (Cazenave et al. 2008) or lowering (Cazenave et al. 2006) this enzyme activity.

The family of GST is a well-known group of enzymes that catalyzes phase II reactions, conjugating glutathione (GSH) with a broad range of hydrophobic and electrophilic compounds, chemically non-related, including microcystins (Pflugmacher et al. 1998; Huang et al. 2008). Interestingly, recent reports indicate that fish species exposed to MC

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altered the transcriptional levels of several GST forms. For example, Li et al. (2008) found that microcystin exposure of goldfish (*Carassius auratus*) suppressed transcripts of GST alpha in liver and intestine, contrary to result obtained in kidney. A dose of 50 μg of MC/kg reduced transcript levels of GST theta in the three organs cited above. Other GST forms, like mu and pi decreased in at least one of the analyzed organs of the same fish species when exposed to crude extracts of MC (Hao et al. 2008). In common carp *Cyprinus carpio*, a decrease in transcription levels of GST pi was observed in liver after 24 h of exposure to 100 μg of MC/kg and also a transient augmented expression of GST alpha after 6 h exposure. A transient lowered expression (12 h) was registered for liver GST mu (Fu and Xie 2006).

It is also important to consider that other important findings about the subject of this study show that: MC are cyanotoxins that elicit oxidative stress and can be conjugated with glutathione, a reaction catalyzed by GST that lowers microcystins toxicity (Metcalf et al. 2000; Jos et al. 2005); conspicuous fish inter-organ differences exist in terms of antioxidant defenses (Amado et al. 2009), as well as their ability to up or down regulate genes coding for phase II reactions as GST, showing differences in the activity of this enzyme (Li et al. 2008; Monserrat et al. 2008); LA possess not only the ability to act as a direct antioxidant (Packer et al. 1995) but also the capacity of activate genes associated to antioxidant defense as well as phase II reactions (Suh et al. 2004), resulting in higher activity of brain and liver GCL and brain GST in fish fed with an LA enriched ration (Monserrat et al. 2008).

Taking into account the information cited above, the present study analyzed possible inter-organ differences of the common carp *C. carpio* after MC exposure to evaluate the role of LA as a chemoprotective molecule. The few studies that analyzed biochemical responses in fish treated with LA performed protocols where the antioxidant was added to the food (Terjesen et al. 2004; Trattner et al. 2007; Monserrat et al. 2008). However, in this kind of treatment the nominal dose always differs from the actual dose, as different fish will eat different amounts of food. In the present study, we preferred to use i.p. injection as the route for LA administration. Gavage was chosen as the exposure route for MC, as a way to represent the actual via of MC ingestion and also to control the dose of toxin given to exposed organisms. In order to verify the effects of the different treatments detailed below, we analyzed the following parameters: expression of different forms of glutathione S-transferase – GST (alpha, mu and pi); concentration of GST mu and pi; GST activity; antioxidant competence against peroxyl radicals (ANCOMROS); reduced glutathione (GSH) concentration and glutamate cysteine ligase (GCL) activity.

2. Materials and methods

2.1. Fish

Common carp (*C. carpio*, Teleostei, Cyprinidae), an omnivorous fish that can have a significant portion of its diet constituted of Microcystis cells during bloom periods (Li et al., 2004), was the biological model of this study. Forty-eight carp, with mean mass of 27.9 ± 7.7 g (mean ± SEM) were obtained from local suppliers and acclimated in a 300 L aerated freshwater tank equipped with a filtering system (pH 7.0; 7.20 mg O_2/L, 20 °C), for at least two weeks prior experiments. Feeding of food pellets at a rate of 1% of the body mass per day was terminated 1 day before beginning of experiments. No food was supplied to fish during experimental periods.

2.2. Preparation of lipoic acid solution

Lipoic acid (DL-α-lipoic acid, Fluka, BioChemika) solutions were prepared according to Suh et al. (2004) at a concentration of 4 mg/mL, employing an alkaline solution (2 mM NaOH and 154 mM NaCl) as solvent. After dissolution, the pH was adjusted to 7.40 and the final volume of the solution was obtained by employing 2.154 M NaCl. A similar solution without lipoic acid was prepared in order to inject in control fish (see 2.4.1).

Each fish was weighed prior to the i.p. injection and the volume injected in each animal was adjusted to achieve a dose of 40 mg/kg (around 280 μL per fish). This dose is known to induce Nrf-2 migration to nuclei in rats i.p. injected up to 48 h, leading to an increase in expression of genes regulated by this transcription factor, such as glutamate-cysteine ligase and phase II enzymes, improving antioxidant defenses and detoxification processes (Suh et al. 2004).

2.3. Microcystin solution

Cells of *M. aeruginosa* strain RST 9501 were cultured in BG11 (8.82 mM of $NaNO_3$) medium at 25 ± 1 °C and employed as toxin source. Toxin identification was performed through high performance liquid chromatography (Shimadzu SCL-10Avp HPLC) and mass spectrophotmetry (Lawton et al. 1995). Lyophilized *M. aeruginosa* cells were re-suspended in MilliQ water to reach a toxin concentration of 5 μg/mL (~5 mg/mL of lyophilized cells), frozen and thawed three times and centrifuged (12,000 *g*; 4 °C) for 10 min. Each fish was weighed prior to the gavage and the volume of microcystin solution injected was adjusted to achieve a dose of 50 μg/kg. Previously it was showed that strain RST 9501 altered the antioxidant status in liver, gills and brain of the fish *Jenynsia multidentata* (Anaplebidae) (Amado et al. 2009).

2.4. Experimental protocols

2.4.1. Determination of lipoic acid dose and exposure time

Two experiments were done to determine the dose and the time of lipoic acid exposure needed to induce transcriptional responses of GST. Firstly, sixteen carp were equally distributed in four aquaria. Fish of two aquaria received saline i.p. injection (control group; CTR) and fish of the other two aquaria were i.p. injected with lipoic acid solution (lipoic acid group; LA), prepared as described in 2.2. Twenty-four hours after the first injection, four fish from each group (CTR and LA) were killed (condition 1) and the remaining fish received another i.p. injection (CTR, saline; LA, lipoic acid). Twenty-four hours after the second injection, these fish were also killed (condition 2). Carp were cryoanesthetized, killed by spinal section and liver, brain, gills and muscle were dissected and stored at −80 °C. In the second experiment, 8 carp were equally distributed in two aquaria. Fish of one aquarium received saline i.p injection (CTR) and the others received lipoic acid i.p. injection (LA). Forty-eight hours after injection fish were cryoanesthetized, killed and dissected as described above (condition 3).

2.4.2. Determination of lipoic acid antioxidant and detoxification potential against microcystin intoxication

Twenty-four carp were equally distributed in four aquaria, each aquaria corresponding to a different treatment: (1) control group (CTR); (2) lipoic acid group (LA), (3) microcystin group (MC); and (4) lipoic acid + microcystin group (LA + MC). In the first two days of experiment, CTR and MC groups received saline i.p. injection whereas LA and LA + MC groups received lipoic acid i.p. injection, according to the results obtained in the first set of experiments described in 2.4.1 (condition 2). In the following two days CTR and LA groups were gavaged with Milli Q water whereas MC and LA + MC groups were gavaged with a microcystin solution prepared as described in Section 2.3. The gavages were performed twice with a 24 h interval between each one. Ninety-six hours after experiment beginning all carp were cryoanesthetized, killed by spinal section and dissected. Liver, brain, gills and muscle were stored in −80 °C until analyses.

2.5. Analysis of GST forms expression

2.5.1. Sequences analysis and primers design

Carp sequences encoding to GST alpha, GST mu and GST pi were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among the sequences were used for searching specific primers, which were designed using the program Oligos 9.6. In order to confirm the primers specificity, each primer was compared with carp genome using NCBI BLAST searches of GenBank (nucleotide database) and it was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The *β-actin* primers were designed as described previously for zebrafish (Chen et al. 2004) and the optimal PCR conditions were determined (Table 1).

2.5.2. Semi-quantitative reverse transcription – polymerase chain reaction (RT-PCR) analysis

Carp brain, gills and liver were dissected and immediately total RNA was isolated from each organ using TRIzol® Reagent (Invitrogen) in accordance with manufacturer instructions, adapted as follow: tissues were homogenized in 500 μL of TRIzol® Reagent and 100 μL chloroform was added and then vortexed and centrifuged at 10,600 g for 15 min at 5 °C. The transparent aqueous phase was transferred to 250 μL of isopropyl alcohol for RNA precipitation through centrifugation (10,600 g for 10 min at 5 °C). RNA pellets were washed with 500 μL of 75% cold ethanol and centrifuged at 6800×*g* for 5 min at 5 °C. Supernatant was dispensed and RNA re-suspended in 15 μL of RNase-free water plus 0.4 μL of RNAse OUT Ribonuclease Inhibitor Recombinant (Invitrogen). RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/μL. cDNA species were synthesized with SuperScript™ First-Strand (Synthesis System for RT-PCR) from Invitrogen, following the manufacturer instructions. Each RNA sample (2 μg/mL) was mixed with 1 μL of 50 μM oligo(dT) and 1 μL of annealing buffer up to a final volume of 8 μL. Samples were incubated at 65 °C for 5 min in a thermal cycler, following a 1 min on ice step, when 10 μL of 2 X First-Strand Reaction Mix and 2 μL of SuperScript™ III/RNaseOUT™ Enzyme Mix were added. Products were incubated by 50 min at 50 °C and next 85 °C for 5 min. The cDNA products were used as a template for each PCR amplification. PCR parameters were first optimized and reactions were performed allowing product detection within the linear phase of mRNA transcripts amplification for each primer pair (Table 1). The amplified products were visualized on 1.0% agarose gel with GelRed® under ultraviolet light. Low DNA Mass Ladder (Invitrogen) was used as molecular marker. The relative mRNA abundance of each GST versus β-actin was determined by optical densitometry using ImageJ1.37 freeware. Each experiment was repeated at least four times, using RNA isolated from independent extractions.

2.6. Organs samples preparation

Organs samples (liver, brain, gills and muscle) were homogenized (1:5 – w/v) in Tris–HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) (Amado et al., 2009). Samples were centrifuged at 10,000×*g* during 20 min at 4 °C and the supernatant were employed for all measurements described below. Previously, total protein content was determined through Biuret method (λ = 550 nm), in triplicate, using a microplate reader (BioTek LX 800).

2.7. Antioxidant competence against peroxyl radicals (ANCOMROS)

Total antioxidant competence against peroxyl radicals was analyzed through ROS determination in organs samples incubated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (35 °C) decomposition of 2, 2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich). For ROS determination it was employed the fluorogenic compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) at a final concentration of 40 μM, according to the methodology described by Amado et al. (2009). H_2DCF-DA passively diffuses through cellular membranes and once inside the acetates are cleaved by intracellular esterases. Thereafter, the non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound DCF. The readings were carried through in a fluorescence microplate reader (Victor 2, Perkin Elmer), in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$, 40 μM DCF-DA and 166 μg of proteins of tissues samples. Background fluorescence was determined before the addition of DCF-DA. Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were expressed as area difference of FU × min in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The relative difference between ROS area with and without ABAP was considered a measure of antioxidant capacity, with high area difference meaning low antioxidant capacity, since high fluorescence levels were obtaining after adding ABAP, meaning low competence to neutralize peroxyl radicals.

2.8. Glutathione (GSH) concentration and glutamate cysteine ligase (GCL) activity

Activity of GCL (EC 6.3.2.2) and GSH levels were determined according White et al. (2003). This method is based in the reaction of naphthalene dicarboxialdehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC) to form cyclic products that are highly fluorescent. On a 96-well round-bottom reaction plate, aliquots (25 μL) of GCL reaction cocktail (400 mM Tris, 40 mM ATP, 20 mM L-glutamic acid, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine and 40 mM $MgCl_2$) were added into each well. For assays, aliquots of 25 μL of sample were pipetted into a pre-warmed (25 °C) reaction plate at 15 s time intervals. After 5 min of pre-incubation, the GCL reaction was initiated by adding 25 μL of 2 mM cysteine dissolved in buffer solution (100 mM Tris–HCl, 2 mM EDTA and 5 mM $MgCl_2.6H_2O$, pH 7.75). In order to measure GCL activity, cysteine was not added to the GSH-baseline wells at this time. After 10 min, the GCL reaction was stopped by adding 25 μL of 200 mM sulfosalicylic acid to all wells, and then 25 μL of 2 mM cysteine was added to the GSH-baseline. An aliquot (20 μL) was mixed with 180 μL of NDA derivatization solution (50 mM Tris, pH 12.5; 0.5 N NaOH; and 10 mM NDA in dimethyl sulfoxide, 1.4/0.2/0.2 v/v/v) in 96 wells plate. The plate was covered to protect the wells from ambient light and was allowed

Table 1
PCR conditions used in this study.

Enzymes	Primers sequences (5′→3′)	Annealing temperature (°C)	PCR product (bp)	GenBank accession number (mRNA)
GST-alpha	F-GGTGGAAATAGACGGGATGCAGCTCG R-GGCCTTCATCTTCTCTTGAAACGCCTG	60	394	DQ411310
GST-Mu	F-TCTGTGGGGAAACTGATGAAGCGCAG R-TTTGGCCATCTTGTTGTTCACGGGTG	60	383	DQ411312
GST-Pi	F-CTATGTTAAGGCATTTGGGTCGCAAAC R-ATCCACATAGCTCTTGAGAGTTGGGAAGG	60	338	DQ411313

to incubate at room temperature for 30 min. Following incubation, NDA-γ-GC or NDA-GSH fluorescence intensity was measured (472 nm excitation/528 nm emission) in a fluorescence microplate reader (Víctor 2, Perkin Elmer).

2.9. Determination of glutathione S-transferase (GST) activity

The activity of the enzyme GST (EC 2.5.1.18) was determined following the conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, as described by Habig and Jakoby (1981).

2.10. Western blot of GST forms

Liver, brain and gills samples (4 mg/mL) were submitted to SDS-PAGE and electroblotted to PVDF membranes. Immune reactions for GST forms content were performed with antibodies against GST pi and mu (AbCam) after SDS-PAGE and Western transfer. Bands were visualized using a chromogenic immunodetection kit (Invitrogen). After scanning the PVDF membrane, bands intensity was analyzed in each treatment and organ using the ImageJ1.37 freeware.

2.11. Statistical analysis

All variables were analyzed by means of ANOVA (Zar, 2010). Previously, normality and variance homogeneity were verified and mathematical transformation applied if at least one assumption was violated. Means comparisons were performed with the Newman–Keuls method. In all cases, the significance level was fixed at 0.05.

3. Results

A single lipoic acid i.p. injection (40 mg/kg) 24 h or 48 h (one injection) before carp were killed did not induce the expression of any GST isoform ($p>0.05$; Fig. 1). However, two i.p injections of lipoic acid with an interval of 24 h from each other, lead to a significant ($p<0.05$) increment of all GST isoforms expression, as shown in Fig. 1.

The expression of the three isoforms was suppressed in liver of carp that were treated both with i.p. LA injection and MC gavage. It was not observed the induction of any isoform in this organ 96 h after the last LA i.p. injection (Fig. 2a). In brain, although GST mu and pi did not varied among treatments, GST alpha had a significant decrease of its expression ($p<0.05$) both in carp that received only MC and in carp that were pre-treated with LA (Fig. 2b). In gills, the expression of all GST isoforms was significantly higher ($p<0.05$) in carp treated with LA than in control carp. The expression of GST alpha was also induced in the other treatments, but it was not significant different from the

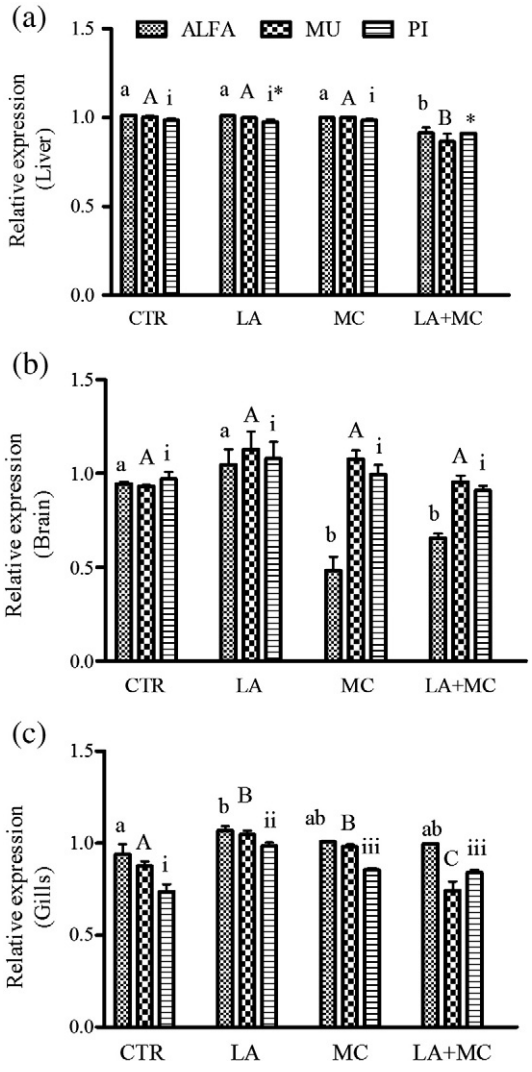

Fig. 2. Gene expression patterns of GST alpha, mu and pi in the liver (a), brain (b) and gills (c) samples from common carp *Cyprinus carpio*. CTR: control group; LA: lipoic acid group (i.p. injection 40 mg/kg); MC: group gavaged with microcystin (50 μg/kg); LA + MC: group pre-treated with LA (i.p. injection 40 mg/kg) and subsequently gavaged with MC (50 μg/kg). Data are expressed as relative area mean ± 1 SE ($n=4$). Different letters or asterisks indicate significant differences ($p<0.05$) between groups for GST alpha (lower case letters); GST mu (capital letters) and GST pi (asterisk is different of i which is different of ii and all of them are different of iii).

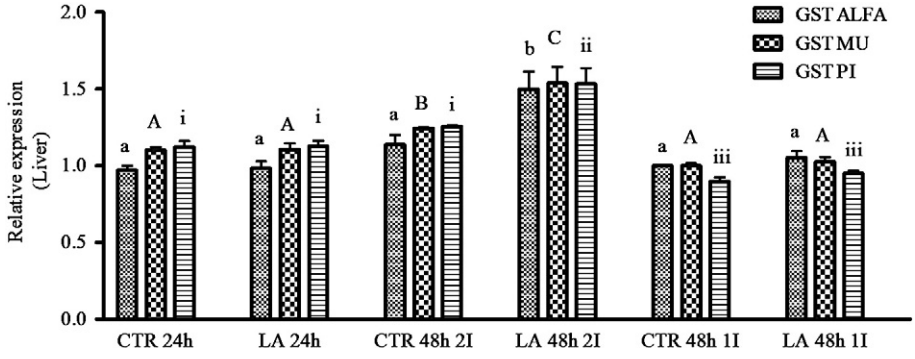

Fig. 1. Gene expression patterns of GST alpha, mu and pi in the liver of common carp *Cyprinus carpio* injected (i.p.) with lipoic acid (40 mg/kg). CTR: control group; LA: lipoic acid group; 2I: two injections; 1I: one injection. Data are expressed as relative area mean ± 1 SE ($n=4$). Different letters indicate significant differences ($p<0.05$) between groups for GST alpha (lower case letters); GST mu (capital letters) and GST pi (i is different of ii and both are different of iii).

control. GST mu expression was increased both in carp treated only with LA and only with MC, but the co-administration of LA and MC lead to a significant decrease in the expression of this isoform. The expression of GST pi was higher in all treatments than in the control (Fig. 2c).

In liver, total antioxidant capacity was higher in carp treated both with MC and with MC plus LA than in the control carp ($p<0.05$). LA treatment alone did not lead to an increase in ANCOMROS (Fig. 3a). In brain (Fig. 3b) and in muscle (Fig. 3d) there were no significant differences among treated and control fish ($p>0.05$), whereas in gills, all treatments improved total antioxidant capacity compared to the control (Fig. 3c).

GSH concentration was higher ($p<0.05$) in carp brain from all treatments than in control carp. In the other analyzed organs the GSH concentration remained the same as in control ($p>0.05$) (Fig. 4). GCL activity in all analyzed organs was not altered by the treatments ($p>0.05$) (data not shown).

Each organ presented a different pattern of GST activity according to treatment. In liver, GST activity was suppressed in carp exposed to MC, however the pre-treatment with LA prevented this effect as animals treated with LA plus MC showed the same activity as controls and LA treated carp (Fig. 5a). In brain, GST activity was higher in carp treated with LA plus MC than in carp from other treatments (Fig. 5b). In gills, GST activity was higher in all treatments respect to the control (Fig.5c). In muscle, GST activity was not altered by the treatments (Fig. 5d).

We performed the western blot assays using three antibodies, against three different GST isoforms (alpha, mu and pi). As polyclonal antibodies used in this study were raised against rabbit GSTs, the bands that appeared in western blot membranes are referred to as GST immune reactive bands. Rabbit polyclonal antibody against GST alpha showed many cross reactions in the three analyzed organs and could not be applied in this study. The same occurred with GST pi in brain samples. Rabbit polyclonal antibody against GST mu showed 2 immune reactive bands in liver samples. These bands had very close molecular masses (between 82.2 and 64.2 kDa). In brain samples, we also observed 2 GST mu immune reactive bands, and both showed molecular masses close to 64.2 kDa. The *C. carpio* GST mu amino acid sequence (NCBI, protein database, http://www.ncbi.nlm.nih.gov) deduced from the mRNA sequence (Fu and Xie, 2006) was used to calculate the expected molecular mass (25.98 kDa). Taking into account these results, only data of GST pi isoform in brain and liver were considered, since the molecular masses of immune reactive band ranged between 37.1 and 25.9 kDa, close to 23.57 kDa of carp GST pi primary sequence deduced from the mRNA sequence (Fu and Xie, 2006).

Rabbit polyclonal antibody against GST pi in liver and brain showed an immune reactive band in a molecular mass between 37.1 and 25.9 kDa. The analysis of the bands intensity showed no difference among treatments (data not shown). In gills the concentration of the isoforms mu and pi was very low, as showed by weak bands in western blot analysis. This finding is consistent with the lower GST activity ($p<0.05$) observed in this organ respect to liver and brain.

4. Discussion

The obtained results confirmed previous studies that reported [D-Leu1] microcystin-LR as the most abundant MC produced by strain RST 9501, a variant with a similar potency in terms of phosphatases inhibition respect the common [D-Ala1]microcystin-LR (Matthiensen et al., 2000).

As oxidative stress is a well-known toxic effect of MC exposure (Ding and Ong 2003), different kinds of antioxidants have been used prior to MC exposure, trying to identify possible protective effects against MC intoxication. Vitamin E, for example, was used in several studies, showing its protective effects in crabs (Pinho et al. 2005) and fish (Prieto et al. 2008). In fish, selenium proved to have chemoprotective roles (Atencio et al. 2009) against microcystin-induced toxicosis.

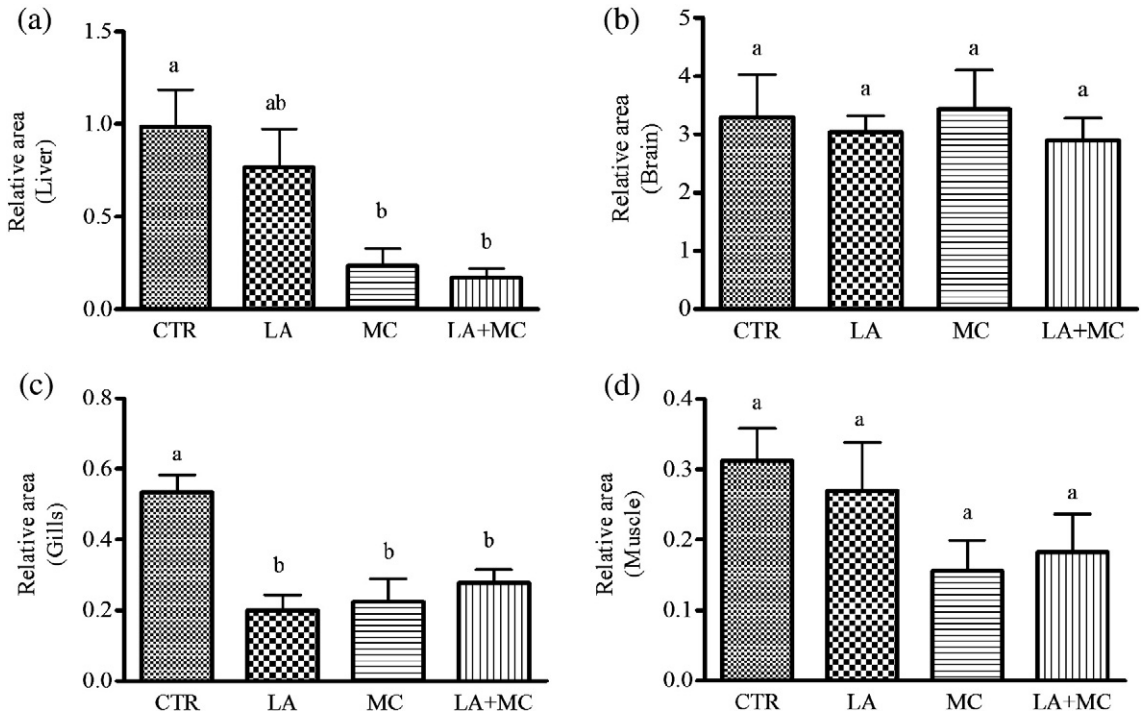

Fig. 3. Total antioxidant capacity against peroxyl radicals in liver (a), brain (b), gills (c) and muscle (d) samples from common carp (*Cyprinus carpio*). CTR: control group; LA: lipoic acid group (i.p. injection 40 mg/kg); MC: group gavaged with microcystin (50 μg/kg); LA + MC: group pre-treated with LA (i.p. injection 40 mg/kg) and subsequently gavaged with MIC (50 μg/kg). Data are expressed as relative area mean ± 1 SE ($n=4$–5). Relative area was calculated dividing area difference (with and without ABAP – 2, 2′-azobis 2 methylpropionamidine dihydrochloride) by area without ABAP (background area). Different letters indicate significant differences ($p<0.05$) between groups.

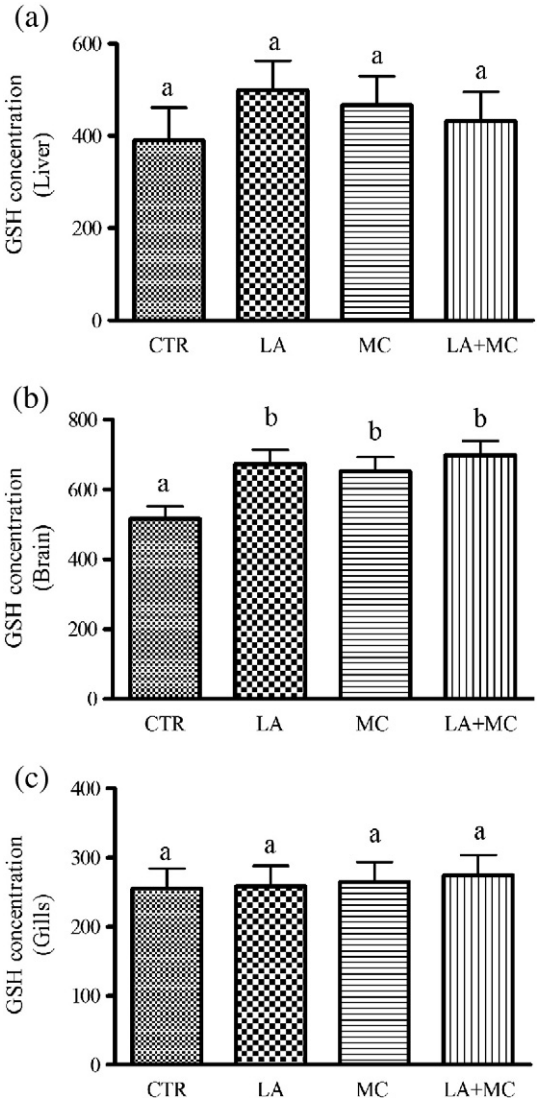

Fig. 4. Reduced glutathione (GSH) concentration (in nmol GSH/mg of proteins) in liver (a), brain (b) and gills (c) samples from common carp (*Cyprinus carpio*). CTR: control group; LA: lipoic acid group (i.p. injection 40 mg/kg); MC: group gavaged with microcystin (50 μg/kg); LA + MC: group pre-treated with LA (i.p. injection 40 mg/kg) and subsequently gavaged with MC (50 μg/kg). Data are expressed as relative area mean ± 1 SE ($n = 5$). Different letters indicate significant differences ($p < 0.05$) between groups.

Because it is known that MC exposure can affect the expression of different classes of GST (Fu and Xie 2006; Hao et al. 2008), the enzyme involved in MC detoxification, a chemical inducing the expression of these enzymes could have important roles in protection against MC toxicity. The present study shows, for the first time, the effects of lipoic acid administrated i.p. on different GST classes transcripts, correlating expression, enzyme activity and protein concentration in order to analyze the role of its pre-treatment in MC induced toxicity.

As mentioned in the Introduction, lipoic acid could have positive effects against MC toxicity because it can act both as a direct antioxidant molecule, chelating metals and intercepting free radicals (Packer et al. 1995; Packer et al. 1997) and also controlling expression of genes involved on antioxidant defense like GCL and GST (Lee and Surh 2005). In this study, the experiments performed to determine the time and the dose of lipoic acid needed to alter positively GSTs classes expression, revealed that, at least in liver, it was necessary two i.p. injections of 40 mg/kg lipoic acid in a 48 h period to induce a significant increment of GST alpha, mu and pi classes. Based on these data, a second experiment was done; where after treatment with lipoic acid i.p. injections in the conditions determined by the first experiment, carp were gavaged with microcystin (50 μg/kg) twice with a 24 h interval between each gavage. The objective of this experimental design was to determine whether a previous lipoic acid treatment that was able to promote an increase in expression of different GST genes involved in MC detoxification, could protect against MC toxic effects.

Similar to previous studies (Cazenave et al. 2006), we observed the inhibition of GST activity in liver of carp exposed to MC. However, carp pre-treated with LA i.p. injections showed a recovery of liver GST activity, returning to the levels of control and LA groups. Atencio et al. (2009) showed similar results in terms of recovery of GST inhibition induced by MC exposure. They analyzed the role of selenium dietary supplementation (1.5; 3.0 and 6.0 μg Se/g diet) during seven days in tilapia (*Oreochromis niloticus*) subsequently exposed to MC-LR (120 μg MC-LR/fish) for 24 h. Authors showed that the GST inhibition was reverted in fish fed with the highest Se dose, returning GST activity to control levels.

Results of GST forms mRNA levels in liver were not similar to those of GST activity. We observed that the increase in GST forms expression, verified in carp treated with two lipoic acid i.p. injections in a 48 h period (Fig. 1), was not maintained 96 h after the experiment beginning. Also, the co-administration of LA and MC led to a reduction in transcription of all GST forms after 96 h (Fig. 2a), a result not observed when GST activity was analyzed in liver (Fig. 5a).

Some studies have demonstrated that, in terms of antioxidant defense, there is a lack of correlation between mRNA transcription levels and enzymes activities. For example, Henrik-Hansen et al. (2007) demonstrated that the mRNA levels of oxidative stress related proteins in gills of brown trout (*Salmo trutta*) exposed to waterborne Cd/Zn did not correlated with enzymes activities. They found no induction in catalase mRNA in gills, but enzyme activity measurements showed that there was an initial non-significant decrease two days after exposure, with a subsequent increase above control levels after 15 days.

There are at least three possible explanations for the lack of similarity between mRNA levels and GST activity observed in this study:

(1) the classes of GST involved in the recovery of GST activity were not analyzed in terms of mRNA levels, as we only studied the alpha, mu and pi forms and there are at least 6 classes more;
(2) there is a time window between transcription and translation of GST proteins. Thus, the observed increase in the expression of the three GST forms after 48 h of LA injection led to the recovery of GST activity demonstrated after 96 h, when expression of GST forms in LA group have returned to control levels.
(3) GSTs are subjected to post-transcriptional regulation. Some (and few) experimental evidences have shown that GST can have its activity increased when phosphorylated (Lo et al. 2004) which is very likely after MC exposure, because of their well-known inhibitory effects on phosphatase activity (Dawson, 1998). However, we cannot discard the effect of LA in the reversion of inhibition, as carp exposed only to MC showed lower GST activity than the other treatment groups.

We also observed in carp liver an increase of antioxidant capacity against peroxyl radicals in both groups that were gavaged with MC (MC and LA + MC). As GCL activity and GSH concentration in these groups were not significantly different from the control group, it is possible that other antioxidant defenses have been induced by MC exposure. Li et al. (2003) showed in *C. carpio* hepatocytes a reduction in GSH concentration accompanied by an increase in the activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, suggesting that these enzymes can have significant roles

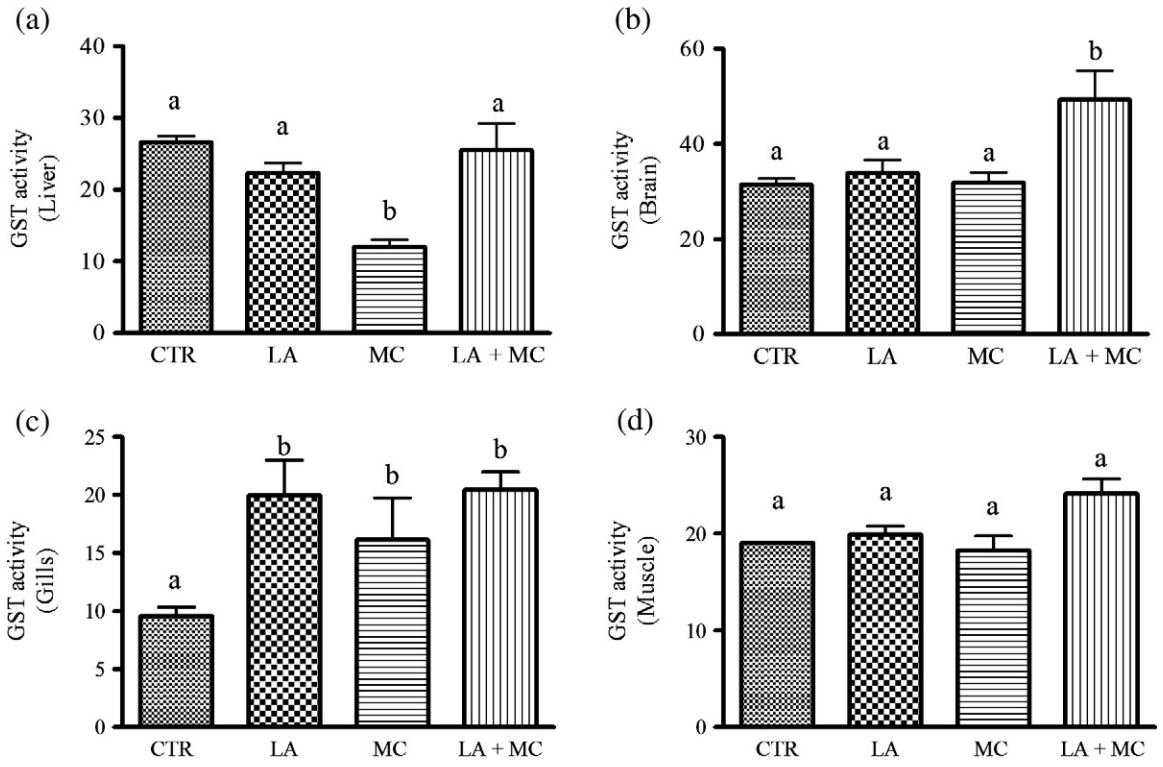

Fig. 5. Glutathione-S-transferase (GST) activity (in nmol CDNB–GSH conjugate/min/mg of proteins) in liver (a), brain (b), gills (c) and muscle (d) samples from common carp (*Cyprinus carpio*). CTR: control group; LA: lipoic acid group (i.p. injection 40 mg/kg); MC: group gavaged with microcystin (50 μg/kg); LA + MC: group pre-treated with LA (i.p. injection 40 mg/kg) and subsequently gavaged with MC (50 μg/kg). Data are expressed as mean ± 1 SE ($n = 5$). Different letters indicate significant differences ($p < 0.05$) between groups. CDNB: 1-chloro-2,4-dinitrobenzene.

in protecting cells from oxidative stress induced by MC. Studies with other fish species exposed to MC also showed increase in antioxidant competence, demonstrated by the increment in the activity of different antioxidant enzymes (Jos et al. 2005) or liver total antioxidant competence (Amado et al. 2009). In this context it is important to cite the study of Qiu et al. (2007), where they found that the phytoplanktivorous fishes possessed higher basal GSH concentrations and better correlations between the major antioxidant enzymes in liver, which might be responsible for their powerful resistance to MC.

Fischer et al. (2005) provided evidences on the ability of MC to cross through the blood-brain barrier and raised questions on the probable neurotoxicity of MC, especially considering that fish exposed to MC-LR evidenced behavioral changes (Cazenave et al. 2008). In the present study, the co-exposure with MC and LA led to an increase in GST activity in brain tissue (Fig. 5b), suggesting that LA can have an important role in neuroprotection against MC intoxication.

Besides the increase of the total GST activity in brain samples of LA + MC group, the mRNA levels of GST mu as well as GST pi was not affected by the treatments and GST alpha expression was lower in both groups that received MC (MC and LA + MC) than in CTR and LA groups (Fig. 2b). Again, a lack of parallelism was observed between GST expression and activity, as previously observed in liver.

In gill samples, treatments induced different patterns of GST forms expression. LA significantly induced the three GST forms analyzed. Treatment with MC was also efficient in inducing the expression of two GST forms (mu and pi). The co-administration of LA and MC affected differently the three forms: GST mu had decreased expression, GST pi showed augmented transcription and GST alpha was unaffected, respect to the CTR group (Fig. 2c). As gills are not the preferential targets of MC and LA, the effects observed in this organ could occur latter than in liver and brain. So, in this organ it was possible to verify the modulation of GST forms induced both by MC and LA in a latter time window, whereas in liver and brain these effects probably had occurred earlier. Note also that the toxin was administered through gavaging, a route that also should aid to get a latter time window in this organ.

GST activity in gills samples was higher in all treatments respect the CTR group, probably as a result of the transcriptional induction. Also, the total antioxidant activity against peroxyl radicals was higher in all treatments when compared with CTR group, perhaps a consequence of the increased detoxification capability. The results in MC-exposed carp gills are in complete agreement with Vinagre et al. (2003), which found both increased GST activity and total antioxidant capacity in gills of the crab *Chasmagnathus granulata* (now *Neohelice granulata*) injected at every 24 h intervals during 48 h with an aqueous extract of *Microcystis aeruginosa* from the same strain used in the present study.

In muscle, treatments did not affect analyzed parameters. Several laboratory and field studies have shown that MC is accumulated in muscle of different fish species, although the concentration of MC in muscle is usually much lower than that in other tissues (Magalhães et al. 2001). So, the lack of effects on analyzed parameters in carp muscle exposed to MC is considered a consequence of the lower toxin concentration in muscle than in other organs. Cazenave et al. (2006) also observed differential responses in fish organs exposed to MC-RR and suggested that it might be related to the uptake route as well as biotransformation and bioaccumulation capabilities of the different studied organs.

Regarding to the lack of LA effects in muscle, our results are in agreement with Monserrat et al. (2008), that reported no difference in glutathione concentration and GCL, GST and glutathione reductase (GR) activities between muscles of *Corydoras paleatus* fed during four weeks with a LA enriched diet.

In conclusion, the results obtained in liver and brain suggest that LA can be a useful chemoprotective agent against MC induced

toxicosis, stimulating detoxification through the increment of GST activity (brain) or through reversion of GST inhibition (liver). Also, i.p. injection can be an interesting route of LA administration in aquaculture systems, leading to fish protection against MC toxicity more efficiently than food supplementation due to its faster mode of action (within 48 h). Besides that this kind of treatment assures the desired dose, i.p. injection is a common practice in aquaculture drug administration (Stoskopf, 1993; Prieto and Romano, 1998).

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