MODULAÇÃO DA CAPACIDADE ANTIOXIDANTE E DE DETOXIFICAÇÃO NO PEIXE *Cyprinus carpio* (Cyprinidae) APÓS TRATAMENTO COM NANOCÁPSULAS DE ÁCIDO LIPÓICO

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Sumário

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1   **Resumo**

2   O metabolismo aeróbico é muito eficiente no processo de geração de energia, no

3   entanto, é uma fonte de produção de espécies reativas de oxigênio (ERO). Para a

4   prevenção dos efeitos potencialmente danosos dessas ERO, os organismos

5   desenvolveram um sistema de defesa antioxidante (SDA), que inclui compostos

6   enzimáticos e não enzimáticos. O ácido lipóico (AL) é uma molécula lipo e hidro

7   solúvel, com capacidade de atravessar membranas celulares. Ele possui propriedades

8   antioxidantes, auxiliando na eliminação de ERO, induzindo a expressão de genes

9   importantes nas defesas antioxidantes, quelando metais e interagindo com outros

10   antioxidantes. Trabalhos prévios demonstraram que nanocápsulas poliméricas de ácido

11   lipóico favoreceram a proteção deste antioxidante, aumentando sua estabilidade físico-

12   química em comparação com formulações contendo ácido lipóico livre. O objetivo deste

13   estudo foi avaliar e comparar o efeito do AL livre e do AL em nanocápsulas sobre a

14   atividade de enzimas antioxidantes (glutamato-cisteína ligase, GCL e glutationa-S-

15   transferase, GST), a concentração de glutationa reduzida (GSH) e sub-produtos da

16   peroxidação lipídica (malondealdeído, método TBARS) e da expressão de genes que

17   codificam para as diferentes formas da enzima GST (alfa e pi). Para isso o peixe

18   *Cyprinus carpio* (Cyprinidae) foi exposto a uma dose de 40 mg/kg a diferentes formas

19   de AL (livre e em nanocápsulas) por injeção intraperitoneal (duas injeções, sendo a

20   primeira no tempo 0 e a segunda após 24 h), sendo logo sacrificados a diferentes tempos

21   da primeira injeção (48 h, 96 h e uma semana), sendo dissecados o cérebro, fígado e

22   músculo dos peixes de cada tratamento. Os resultados obtidos indicam que os órgãos

23   respondem de forma diferente. A curto prazo, o fígado foi o principal órgão a apresentar

24   respostas antioxidantes após tratamento com AL, enquanto que a longo prazo o cérebro

25   e o músculo se mostraram mais responsivos em termos antioxidantes quando

26 comparado ao fígado. Foi também importante a forma em que o AL é administrado,

27 livre ou em nanocápsulas, sendo observado que um mesmo órgão em um mesmo tempo

28 de exposição pode responder de forma diferente de acordo com o tipo de AL que está

29 sendo utilizado. Além disso, o efeito antioxidante do AL nanoencapsulado parece ser

30 mais efetivo quando utilizado a longo prazo, sugerindo que a forma nanoencapsulada

31 libera o antioxidante em forma mais lenta. Os resultados também indicam que a

32 composição da nanocápsulas deve ser levada em consideração, uma vez que foi

33 observado um efeito antioxidante significativo nos tratamentos que continham apenas a

34 nanocápsulas, sem o AL. Sugere-se que este efeito ocorra devido à produção endógena

35 do próprio antioxidante em questão, favorecida pela composição da própria

36 nanocápsula, que possui ácido octanóico, substrato para a síntese de AL. Também se

37 observou um efeito pró-oxidante em alguns tratamentos onde foi utilizada esta

38 formulação, sugerindo que alguns componentes da nanocápsula, como por exemplo, o

39 surfactante que é utilizado para estabilizar a suspensão, possam aumentar a

40 suscetibilidade dos órgãos ao estresse oxidativo.

41

42 **Palavras chave:** antioxidante, glutationa, espécies reativas de oxigêno, ácido lipóico,

43 nanocápsulas, nanotecnologia.

44

1. Introdução

No metabolismo aeróbio, o processo de fosforilação oxidativa, que inclui o processo de redução do O_2 à água na mitocôndria, gera um gradiente eletroquímico que impulsiona a síntese de ATP, fornecendo energia de uma forma bem mais eficiente do que a oxidação anaeróbia da glicose (Nelson & Cox, 2006). No entanto, esse processo tem o potencial de gerar subprodutos altamente reativos através da redução parcial do O_2, formando as chamadas espécies reativas de oxigênio (ERO), como o ânion superóxido ($O_2^{\cdot-}$), o radical hidroxila ($HO^{\cdot}$) e o peróxido de hidrogênio (H_2O_2) (Halliwell & Gutteridge, 2007). Elas podem ser produzidas no organismo tanto sob condições fisiológicas quanto patológicas (Wajner et al., 2004). Em condições normais, as ERO têm papéis essenciais no crescimento celular (Finkel, 2003), na geração de um ambiente oxidante contra microrganismos estranhos ao organismo (Valko et al., 2006), além de atuarem como mensageiros secundários em muitas vias de sinalização celular (Halliwell & Gutteridge, 2007). No entanto, quando produzidas em excesso, devido a estados patológicos, pela exposição a poluentes e substâncias tóxicas, ou outros fatores ambientais e fisiológicos, as ERO podem oxidar macromoléculas, acarretando danos como oxidação de proteínas; mutações pontuais no DNA; e alterações na fluidez de membranas biológicas pela oxidação de lipídios insaturados (Storey, 1996; Halliwell & Gutteridge, 2007). Numa visão mais moderna, leva-se em consideração que os principais sistemas celulares tiol/dissulfeto, incluindo GSH (glutationa reduzida)/GSSG (glutationa oxidada) não estão em equilíbrio redox e respondem de forma distinta a moléculas tóxicas e estímulos fisiológicos. Assim, de um ponto de vista mecanístico, o estresse oxidativo pode ser melhor definido como uma perturbação da sinalização e do controle redox celular (Jones, 2006).

69   A necessidade de prevenir e/ou interceptar as ERO levou, no decorrer da

70 evolução, ao desenvolvimento de um sistema de defesas antioxidantes (SDA),

71 comumente divididas em enzimáticas e não enzimáticas. Dentre as defesas

72 antioxidantes não-enzimáticas, temos o tripeptídeo glutationa (GSH) que atua como a

73 principal linha de defesa contra a geração de estresse oxidativo, interagindo diretamente

74 com as ERO, reduzindo peróxidos quando utilizada como co-substrato para as reações

75 catalisadas pela glutationa peroxidase (GPx) e sendo conjugada com compostos

76 eletrofílicos via atividade da glutationa-S-transferase (GST). Além disso, o par

77 GSH/GSSG atua na manutenção do ambiente redox da célula (Maher, 2005) e promove

78 a reativação de enzimas antioxidantes, além de ser regenerada por substâncias

79 antioxidantes não-enzimáticas como o ácido lipóico (AL) (Storey, 1996; Valko et al.,

80 2006; Halliwell & Gutteridge, 2007). A síntese de GSH é um processo catalisado por

81 duas enzimas: a glutamato-cisteína ligase (GCL), e a glutamato sintase (GS), utilizando

82 no total 2 moléculas de ATP por cada molécula de GSH sintetizada. A GCL é a enzima

83 limitante para a formação desta (Lu, 2009), sendo que o controle dos genes que

84 codificam para a subunidade catalitica (GCLC) e modulatória (GCLM) ocorre pela rota

85 de sinalização celular Keap1-Nrf-2-ARE. A proteína Nrf-2 ("nuclear factor erythroid-

86 related factor 2") é um fator de transcrição da família "leucine-zipper", que interage

87 como o elemento de resposta antioxidante (ARE), presente na região promotora de

88 genes que codificam enzimas como as citadas acima (Kwak et al., 2004).

89   O AL (Figura 1) é um derivado do ácido octanóico com uma ponte dissulfeto

90 entre os carbonos 6 e 8, e é naturalmente encontrado na mitocôndria participando como

91 co-fator para as enzimas piruvato desidrogenase e α-cetoglutarato desidrogenase

92 (Hagen et al., 1999). É uma molécula lipo e hidro solúvel, podendo atravessar

93 facilmente as membranas celulares, incluindo a barreira hematoencefálica

94   (Muthuswamy et al., 2006). Desde o ponto de vista nutricional, o AL é encontrado no

95   germe de trigo, no levedo de cerveja e na carne vermelha (Morikawa et al., 2001).
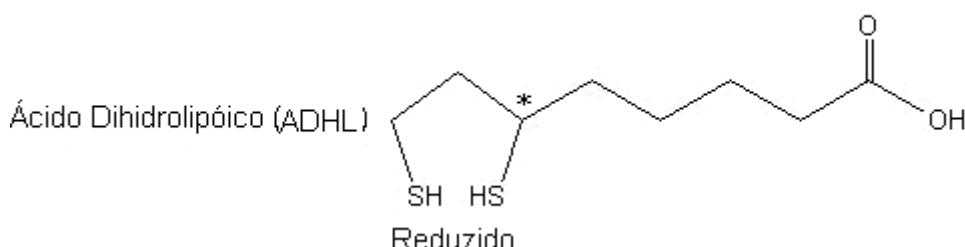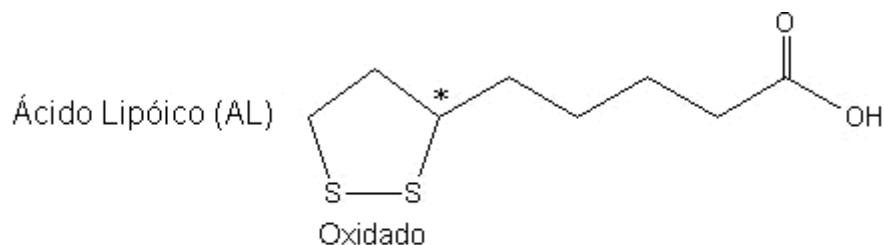
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97   **Figura 1**. O ácido lipóico em sua forma oxidada (AL) e na sua forma reduzida, denominada

98   ácido dihidrolipóico (ADHL). O asterisco indica a presença de um centro quiral.

99

100   O AL e sua forma reduzida, o ácido dihidrolipoico (ADHL) cumprem diversos

101   critérios que caracterizam este par redox como um antioxidante perfeito, apresentando

102   especificidade na eliminação de radicais livres, induzindo a expressão de genes

103   importantes na defesa antioxidante, quelando metais e interagindo com outros

104   antioxidantes, incluindo a GSH (Packer et al., 1995). Suh et al. (2004) demonstraram que

105   em ratos idosos a administração de AL (40 mg/kg) via injeção intraperitoneal, favorece a

106   migração do fator de transcrição Nrf2 ao núcleo, promovendo o aumento da concentração

107   das duas subunidades constitutivas da enzima limitante na síntese de GSH que, como foi

108   dito anteriormente, é a glutamato cisteína ligase (GCL), aumentando consequentemente a

109   atividade desta após 24 h de exposição. O AL também se mostrou efetivo em aumentar a

110   transcrição gênica de diferentes classes de GST (alfa, mu e pi) no fígado de peixes da

espécie *Cyprinus carpio* expostos via i.p. (duas injeções com intervalo de 24 horas entre cada uma) por 48 horas a 40 mg de AL/kg (Amado et al., 2011). Neste mesmo trabalho foi verificado que o pré-tratamento com AL pode ser usado como um agente quimioprotetor contra a toxicidade da cianotoxina microcistina, pois ocorreu um aumento na atividade da GST no cérebro e a reversão da inibição desta mesma enzima no fígado. Em peixes teleósteos (*Corydoras paleatus*), Monserrat et al. (2008) verificaram que o tratamento com AL induziu aumento na atividade da enzima de fase II, glutationa-S-transferase (GST), em cérebro de peixes que receberam suprimento de AL na ração durante um período de 4 semanas, além de um aumento da atividade da GCL em cérebro e fígado. Adicionalmente, estes autores observaram uma queda nos níveis de carbonilação protéica (um dos tipos de dano oxidativo em proteínas) em fígado e músculo dos peixes tratados com AL. Células cardíacas de ratos, expostos a diferentes concentrações de AL aumentaram significativamente a competência antioxidante e as enzimas de fase II de forma tempo e dose dependente, sugerindo que o AL também apresenta um efeito cardioprotetor (Cao et al., 2003).

De fato, nos últimos tempos, o ácido lipóico tem recebido atenção intensa em muitas pesquisas devido a sua função antioxidante que apresentou vários benefícios terapêuticos (Simbula et al., 2007). Este composto é utilizado no tratamento de doenças neurodegenerativas, como Alzheimer, principalmente devido as suas propriedades antioxidantes (Maczurek et al., 2008). O AL é considerado um agente terapêutico contra diabetes, pois ele age através do recrutamento de receptores de glicose para a membrana plasmática da célula, aumentando a captação desta substância pela mesma (Packer et al., 2001). Em ratos foi demonstrado que o AL protege contra isquemia/reperfusão do miocárdio ajudando na inibição desse processo e também por inibir a geração de ERO durante o processo (Wang et al., 2011).

136   No entanto, o AL é uma substância muito instável, que pode ser degradada pela

137   luz e pelo calor, além disso, apresenta uma meia-vida (t/2) muito curta (Kofuji et al.,

138   2009), podendo dificultar a elaboração de uma forma farmacêutica adequada para

139   administração. Por esse motivo alguns autores têm estudado procedimentos que possam

140   resolver este problema. Souto et al. (2005) desenvolveram nanopartículas lipídicas

141   sólidas para liberação tópica do AL, porém as dispersões aquosas destas preparações

142   apresentaram um grande aumento de tamanho quando armazenados por duas semanas à

143   temperatura ambiente. Também foi testada a estabilização do AL por formação de

144   complexo com o polímero catiônico de quitosano (CS), e foi verificado que o CS é capaz

145   de adsorver o AL e de liberá-lo imediatamente por mudança de pH, implicando na total

146   liberação do AL do complexo CS-AL dentro do trato gastrointestinal, quando utilizada

147   via oral para administração do fármaco (Kofuji et al., 2008). Baseada nestes antecedentes,

148   Külkamp et al. (2009) desenvolveram nanocápsulas poliméricas contendo ácido lipóico

149   preparado por deposição interfacial de poli (ε-caprolactona), e foi verificado um efeito

150   protetor que essas nanocápsulas exercem sobre o ácido lipóico, em termos de aumento de

151   sua estabilidade físico-química em comparação com formulações contendo ácido lipóico

152   livre.

153   A estabilidade das nanopartículas é muito importante, pois oferece a

154   possibilidade de utilizá-las como transportadoras de medicamentos, permitindo a sua

155   administração por diferentes vias (oral, i.p., dérmica). Também apresenta a capacidade de

156   atravessar tanto a barreira gastrointestinal quanto a barreira hematoencefálica, carregando

157   fármacos que muitas vezes sozinhos não conseguiriam atingir esses locais. Além disso, as

158   nanocápsulas podem retardar a liberação do fármaco, reduzindo a toxicidade periférica

159   (Lockman et al., 2002). Estudos com melatonina, que tem ação antioxidante direta,

160   agindo na eliminação de radicais livres, protegendo contra a peroxidação lipídica e que

161 tem sido usada clinicamente no tratamento de muitas doenças como câncer e doenças

162 neurodegenerativas, apresentou maior eficácia contra a peroxidação lipídica induzida por

163 radicais ascorbil quando apresentada em forma de nanocápsulas comparada à solução

164 aquosa dessa substância (Schaffazick et al., 2005). A quercitina, um flavonóide

165 polifenólico, conhecido por apresentar propriedades antioxidantes, tem dificuldade de ser

166 aplicada contra danos celulares induzido por tóxicos, devido a sua natureza insolúvel.

167 Com base nisto Ghosh et al. (2009) utilizaram nanocápsulas dessa substância

168 administradas via oral em ratos, 90 min antes da exposição ao arsenito, demonstrando que

169 esta formulação impediu a queda da capacidade antioxidante, provocada pela exposição

170 deste metaloide, no fígado e no cérebro desses animais. Em outro trabalho com

171 administração de quercetina nanoencapsulada, injetada na veia da cauda de ratos expostos

172 anteriormente ao arsênio, foi observada uma proteção no fígado contra a queda dos níveis

173 de antioxidantes induzidos por este, bem como a expressão de genes associados ao

174 estresse oxidativo (Ghosh et al., 2010).

175

176 **2. Hipótese**

177 No trabalho de Monserrat et al. (2008) foi avaliada a administração de AL

178 (70mg/kg) incorporado na ração de peixes (*Corydoras paleatus)* durante 4 semanas,

179 sendo verificada uma diminuição na concentração das espécies reativas de oxigênio,

180 aumento da atividade da GCL e da GST seguidos pela diminuição na oxidação de

181 proteínas nos diferentes órgãos testados. Amado et al. (2011) administrou o AL via

182 injeção i.p. (duas injeções com um intervalo de 24 h entre elas) em uma dose de 40mg/ kg

183 em *Cyprinus carpio*, sendo mortos e dissecados após 48 h da primeira injeção e foi

184 observado um aumento da transcrição gênica de diferentes formas de GST. Além disso,

185 também encontraram que o pré-tratamento com o AL teve a capacidade de proteger

186 contra a toxicidade da microcistina, pois aumentou a atividade da GST no cérebro e

187 reverteu a inibição desta no fígado.

188   Com base nestas informações, a hipótese do presente trabalho é de que a forma

189 nanoencapsulada irá apresentar respostas antioxidantes a maior longo prazo quando

190 comparada com a formulação livre do AL, levando a um efeito a mais longo prazo em

191 termos de respostas antioxidantes.

192

193 **3. Objetivo Geral**

194 **(a)** Avaliar os efeitos antioxidantes do AL na modulação do sistema de defesa

195   antioxidante e na capacidade de detoxificação do peixe *Cyprinus carpio*

196   (Cyprinidae) após o tratamento por injeção intraperitoneal com nanocápsulas

197   contendo ácido lipóico.

198 **(b)** Estudar os efeitos de diferentes tempos de exposição em diferentes órgãos,

199   comparando com os efeitos induzidos pela forma livre do mesmo antioxidante.

200

201 **4. Objetivos Específicos**

202 **(a)** Determinar a atividade da enzima glutamato cisteína ligase (GCL) e

203   concentração de glutationa reduzida (GSH) em diferentes órgãos de peixes

204   (cérebro, fígado e músculo), nos diferentes tratamentos, comparando as formas

205   livre e nanoencapsulada do ácido lipóico;

206 **(b)** Verificar a capacidade de detoxificação através da enzima glutationa-S-

207   transferase (GST), nas mesmas condições experimentais enunciadas no ponto

208   **(a)**;

209 **(c)** Verificar dano oxidativo em termos de peroxidação lipídica nos diferentes

210 órgãos nas mesmas condições experimentais enunciadas no ponto **(a)**;

211 **(d)** Analisar dano oxidativo protéico, através da detecção de grupos carbonila após

212 uma semana de exposição, nas mesmas condições experimentais enunciadas no

213 ponto **(a)**.

214 **(e)** Analisar a expressão de GST alfa e pi no cérebro e no fígado nos dois maiores

215 tempos de exposição, nos diferentes tratamentos, comparando as formas livre e

216 nanoencapsulada do ácido lipóico.

217

218   **5. Manuscrito**

219   Os resultados e discussão serão apresentados na forma de manuscrito que será

220   submetido à revista Free Radical Research e que se intitula:

221   **Modulation of antioxidant and detoxifying capacity in fish *Cyprinus carpio***

222   **(Cyprinidae) after treatment with nanocapsules of lipoic acid**

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241   **Key words**: glutathione, reactive oxygen species, nanotechnology, glutathione-S-

242   transferase, oxidative damage.

15

243 **Abstract**

244 Lipoic acid (LA) is a molecule hydro and lipo-soluble with capacity to pass through cell

245 membranes and with several antioxidant properties. Previous studies have shown that

246 polymeric nanocapsules with LA favor the protection of this antioxidant, increasing

247 their physical and chemical stability compared to formulations containing free LA. The

248 aim of this study was to evaluate and compare the effect of free LA and LA-

249 nanocapsules on antioxidants enzymes, the concentration of reduced glutathione (GSH)

250 and a by-product of lipid peroxidation (malondialdehyde), as well as the expression of

251 genes coding for different forms of GST. For this, the fish *Cyprinus carpio*

252 (Cyprinidae) was exposed to a dose of 40 mg/kg to different forms of LA (free and in

253 nanocapsules) by two intraperitoneal injection, with an interval of 24 h between each,

254 on different times (48 h, 96 h and 1 week). Analyzed organs were brain, liver and

255 muscle. Obtained results indicate that the organs respond differently depending on time

256 and the form which LA is ministered (nanocapsule or free). After 96 h and 1 week of

257 the first injection, a better antioxidant response was found generally in the formulation

258 with nanocapsules. Our results also indicate that the composition of nanocapsules must

259 be taken into account, because in some case there was a antioxidant effect and others a

260 pro-oxidant effect in fish treated only with nanocapsules, suggesting that the

261 components (i.e.: oactanoic acid, polysorbate 80) the nanocapsule are influencing the

262 antioxidant or oxidative damage responses.

263

264 **1. Introduction**

265   Lipoic acid (LA) is a fatty acid derivative, composed of eight carbons with a

266 disulfide bond between carbon six and eight. It is found naturally in mitochondria

267 participating as a cofactor for important enzymes of the Krebs cycle, such as pyruvate

16

268   dehydrogenase and α- ketoglutarate dehydrogenase [1]. It is a hydro and lipo-soluble

269   molecule, capable of crossing cell membranes including the blood brain barrier [2].

270   LA has been extensively studied in lasts years, being used in the treatment of

271   various diseases [3]. It was considered a therapeutic agent for diabetes, because it

272   recruits glucose receptors to the plasma membrane of cells, thereby increasing glucose

273   uptake [4]. Also it was employed for the treatment of neurodegenerative diseases, as

274   Alzheimer, mainly in virtue of their antioxidant properties [5]. In rats it has been

275   reported that LA protects against myocardial ischemia/reperfusion injury, assisting in

276   the inhibition of this process and also by inhibiting ROS generation during myocardial

277   ischemia/reperfusion [6].

278   LA and its reduced form, dihydrolipoic acid (DHLA), posses several properties

279   that allow them to classify as excellent antioxidants, including their capacity to chelate

280   metals, scavenging of reactive oxygen species (ROS), induction of the expression of

281   genes important for the antioxidant defenses system and interaction with other

282   antioxidants [7]. For example, the reduction potential of LA allows the reduction of

283   oxidized glutathione (GSSG) to reduced glutathione (GSH), one of the main

284   intracellular antioxidants [8]. Incubation of rat cardiac cells with low concentrations of

285   LA increased significantly the competence of antioxidant and phase II enzymes [9]. The

286   authors observed an increased activity of catalase, glutathione-S-transferase (GST),

287   glutathione reductase (GR), quinone oxidoreductase-1 and GSH content. Also pre-

288   treatment with LA proved to be protective against exposure to xanthine

289   oxidase/xanthine [9]. In aged rats, i.p administration of LA (40 mg/kg) increased

290   nuclear Nrf2 (nuclear factor erythroid2-related factor 2) levels, increasing proteins

291   levels of catalytic and modulatory sub-units of glutamate cysteine ligase (GCL) and

292   consequently the activity of GCL after 24 h of exposition [10]. The up-regulation of this

enzyme is important in virtue of being the limiting enzyme for the synthesis of one of

the more abundant antioxidants, reduced glutathione (GSH). Amado et al. [11] showed

that fish exposed i.p. for 48 h to 40 mg LA/kg (two injections at intervals of 24 h

between each one) increased gene transcription coding for GST enzymes in liver of

these animals. Moreover, these authors observed that the pre-treatment with LA

increased the GST activity in brain and reversed the inhibition of GST activity in liver

caused by exposure to the cyanotoxin microcystin, suggesting that this antioxidant can

be used as a chemoprotective agent. Fish feed with a LA-enriched diet presented lower

ROS concentration in brain and higher GCL activity in brain and liver. In addition, GST

activity also increased in the brain and lower levels of oxidized proteins (carbonyl

groups) were registered in muscle and liver of LA-treated animals [12].

However, LA is a very unstable substance, which can be degraded by light and

heat and also possess a very short half-life ($t_{1/2}$) [13], all aspects that may hinder the

development of a pharmaceutical form appropriate for administration. For this reason

authors like Souto et al. [14], developed solid lipid nanoparticles containing LA. But,

during storage time (that ranged from 1 day to 3 months), both at 4 °C and 20 °C, the

particle size increased significantly. It has also been tested the stabilization of LA by

complex formation with the cationic polymer chitosan (CS) and it was verified that CS

was able to adsorbs LA and to release it acid pH [15]. Based on this, Külkamp et al.

[16] developed polymeric nanocapsules containing LA prepared by interfacial

deposition of poli (ε-caprolactona) and this formulation has proven to be effective,

increasing physical and chemical stability of this polymeric nanocapsules when

compared to formulations containing only free LA.

Nanomaterials are defined as materials that have at least one dimension up to

100 nm [17]. Nanoparticles are solid colloidal particles that include nanospheres and

318   nanocapsules, with sizes usually in the range of 100-500 nm [18]. These same authors

319   defined nanocapsules as a nano-vesicular system presenting a core-shell structure in

320   which the drug is stored within a cavity surrounded by polymer membrane [18].

321   The stability of polymeric nanocapsules is very important, because this makes it

322   appropriate for use as carriers of drugs, allowing its administration by different routes

323   (oral, i.p., dermal). Another major advantage of using nanocapsules is that they pass the

324   blood-brain-barrier, carrying drugs that alone could not reach the brain. In addition,

325   nanocapsules can delay the release of drugs, thus reducing the peripheral toxicity [19].

326   Nanocapsules containing quercetin showed to ameliorate arsenic toxicity in liver rats,

327   including ROS generation [20]. Schaffazick et al. [21] developed nanocapsules with

328   melatonin aiming to reduce lipid peroxidation induced by ascorbyl free radicals. These

329   authors found a significant decrease in TBARS content in both doses tested (200 and

330   400 µM) of nanoencapsulated melatonin when compared with the control solution.

331   Based on these reports, the aim of this study was to evaluate the antioxidant

332   effects of LA on the modulation of the antioxidant defense system and detoxification

333   capacity of the fish *Cyprinus carpio* (Cyprinidae) after treatment by intraperitoneal

334   injection with polymeric nanocapsules containing lipoic acid. The study aimed to

335   analyze gene expression and biochemical responses comparing two different AL

336   formulations: one employing free LA that showed to be effective in reducing

337   microcystin toxicity [11] and another using nanoencapsulated AL as developed by

338   Külkamp et al. [16] that, at present, it was not evaluated in terms of antioxidant

339   responses in aquatic organisms, but showed good properties in terms of physic-chemical

340   stability. Responses were evaluated at short and long term, taking into account that

341   nanocapsules containing the antioxidant should release AL more slowly. Eventual organ

342   differences were also evaluated, studying gene expression and biochemical responses in

343   liver, brain and muscle.

344

345   **2. Materials and Methods**

346   **2.1. Fish**

347   *Cyprinus carpio* (Teleostei: Cyprinidae) sexually mature (average weight 38.1 g

348    $\pm$ 4.16, n=96) were obtained from local suppliers and acclimated in the laboratory for at

349   least a week before the experiments. Fish were kept in a 300 L aerated freshwater tank

350   equipped with a filtering system, photoperiod fixed in 12h light/12h dark, water pH

351   maintained between 6.8-7.0 and water temperature at 20 °C, at oxygen saturation.

352   During the acclimatation period the animals were fed with commercial feed. The choice

353   of this animal model was based on the study of Amado et al. [11] where it was

354   employed i.p. administration of LA in order to analyze their chemoprotective effects

355   against the toxin microcystin.

356

357   **2.2. Preparation of lipoic acid formulations.**

358   Lipoic acid (LA, Fluka) solutions were prepared at a concentration of 4 mg/ml.

359   For this stock solution, the LA was diluted in a solution of NaOH 2 M and NaCl 154

360   mM (alkaline solution) as describe by Suh et al. [10]. After dissolution, pH was adjusted

361   to 7.40 and the final volume was adjusted with a NaCl 2.154 M solution with their pH

362   previously adjusted to 7.40. This solution was injected in the animals from the LA

363   group and was made a similar solution, but without LA, which was used for the control

364   group. The nanocapsules suspension was made by the technique of interfacial

365   deposition of polymer performs. Lipoic acid (4 mg/ml) was dissolved in the organic

phase composed of triglycerides of caprylic and capric acid (0.33 ml), sorbitan monostearate (76.6 mg), poly (ε-caprolactone) (100 mg), acetone (26.7 ml) and butylated hydroxytoluene (0.01 g). The organic phase was poured over an aqueous phase containing polysorbate 80 (76.6 mg), diazolidinilureia (0.01 g) and Milli-Q water (53.3 ml) through a funnel and maintained under magnetic stirring for 10 min. The formulations were prepared in the dark and evaporated Büchi R-114 (35 °C) until a final volume of 10 ml. It has also prepared a formulation containing all components of the formulations except the lipoic acid, which was used as control of the nanocapsules [16]. The mean diameter and polydispersion of suspended nanocapsules were measured through dynamic light scattering employing the equipment Zetasizer Nano series Nano-Zs (Malvern Instruments). Samples were diluted (1:500 v/v) in Milli-Q water, being analyzed in triplicate the size distribution by intensity.

2.3. Experimental protocol for LA exposure.

All animals were exposed via intraperitoneal injection. The fish were separated in four groups: (1) control group (C) in which the animals were exposed only solution without LA. (2) LA group (LA) in which they were exposed to free LA. (3) control nanocapsules group (CN) animals exposed only the nanocapsules without the presence of LA. (4) LA-nanocapsules group (LAN) fish exposed to LA in nanocapsules. Two experiments were made. In the first, forty-eight carp were divided equally into four groups (as mentioned above) and each group was kept in tanks of 25 L of de-chlorinated tap water. All animals were weighed and the volume to be injected was adjusted in order to obtain a final dose of 40 mg/ kg. Fish received two injections: the first at time 0 and the second after 24 h. Forty-eight hours after the first injection, half (6 carp) of each group (C, LA, CN and LAN) were cryoanesthetized, killed by spinal section and brain,

391 liver and muscle were dissected and processed. The remaining animals (six in each

392 group) were killed and processed after 96 h counting from the first injection. In the

393 second experiment it was considered the same treatments and the same exposure

394 protocol, being fish killed only after one week of the first injection.

395

396 **2.4. Tissue samples preparation.**

397   Tissue samples (brain, liver and muscle) were homogenized (1:5 – w/v) in Tris-

398 HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) [22]. The

399 homogenates were centrifuged at 10,000 x g during 20 min at 4 ^{o}C. After the

400 supernatant is removed and stored at -80 °C for later biochemical analysis indicated

401 below. Previously, total protein content was determined with a commercial kit based on

402 the Biuret method (Doles®), in triplicate, using a microplate reader (Biotek ELX 800®)

403 at a wavelength of 550 nm.

404

405 **2.5. Determination of glutathione-S-transferase (GST) activity.**

406   Activity of the enzyme GST was determined through the conjugation of 1-cloro-

407 2,4 di-nitrobenzene (CDNB, from Sigma) diluted in ethanol 100 % at a final

408 concentration of 1 mM with reduced glutathione (GSH, from Sigma) diluted in

409 phosphate buffer at a final concentration of 1 mM. GST catalyzes the reaction and form

410 a conjugate (GSH-CDNB) which is read at absorbance of 340 nm as described by Habig

411 and Jakoby [23].

412

413 **2.6. Determination of glutathione (GSH) concentration and glutamate cysteine**

414 **ligase (GCL) activity.**

415   The level of reduced glutathione (GSH) and activity of the enzyme glutamate

416   cysteine ligase (GCL) was determined according to White et al. [24], with modifications

417   according Monserrat et al. [12]. The method is based on the detection by reading the

418   fluorescence generated by the complex that is formed by conjugation of naphthalene

419   dicarboxialdehyde (NDA) and GSH or γ-glutamylcysteine and NDA, respectively. In a

420   96-well ELISA reader it was added 25 μl of GCL reaction cocktail (400 mM Tris, 40

421   mM ATP, 20 mM L-glutamic acid, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine

422   and 40 mM $MgCl_2$) to each well. Then, 25 μl of sample (protein concentration fixed in

423   4 mg/mL) was added. After 5 minutes of incubation, the reaction was initiated by

424   adding 25 μl of 2 mM cysteine dissolved in buffer solution (100 mM Tris-HCl, 2 mM

425   EDTA and 5 mM $MgCl_2.6H_2O$, pH 7.75) and incubated during 1 h. The reaction was

426   stopped with 25 μl of 200 mM sulfosalicilic acid (SSA). The plate was incubated during

427   20 min for the precipitation of proteins by SSA and then the plate was centrifuged for 5

428   minutes at 1,500 x g. After, 20 μl of the supernatant was transferred to a white plate

429   where it was added 180 μl of NDA solution (a mix 1.4/0.2/0.2 v/v/v of 50 mM Tris, pH

430   12.5; 0.5 N NaOH; and 10 mM NDA in dimethyl sulfoxide). The plate was incubated

431   for 30 min in the dark at room temperature, followed by fluorescence intensity

432   measurement (485 nm excitation/530 nm emission) in a fluorescence microplate reader

433   (Víctor 2, Perkin Elmer).

434

435   **2.7. Measurement of lipid peroxidation**

436   The lipid peroxidation was measured the according to Oakes and van der Kraak

437   [25]. This method is known as TBARS because malondialdehyde, a by-product of lipid

438   peroxidation, was estimated by measuring thiobarbituric acid-reactive substances. Ten

439   μl sample were mixed, in test tubes with 20 μl of BHT solution (1.407 mM), 150 μl of

440   20 % acetic acid, 150 μl of thiobarbituric acid (0.8 %), 50 μl of Milli-Q water, 20 μl of

441   sodium dodecyl sulfate (SDS, 8.1%). Tubes were then heated at 95 °C during 30 min.

442   After it was added 100 μl of Milli-Q water, 500 μl of n-butanol and centrifuged (3,000 x

443   g for 10 min at 15 °C). It was removed 150 μl of the organic phase and added to the

444   microplate reader and the fluorescence was registered using wavelengths of 520 and 580

445   nm, for excitation and emission, respectively. The concentration of TBARS was

446   calculated employing a standard curve of tetramethoxypropane (TMP, Acros Organics)

447   and expressed as nmols of TMP equivalents per mg of wet tissue.

448

449   **2.8. Detection of protein carbonyl groups**

450   It was employed OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs).

451   Protein samples (fixed at a concentration of 30 μg/ml) are first allowed to adsorb to

452   wells of a 96-well ELISA plate overnight at 4 ºC. Then it was added a

453   dinitrophenylhydrazine (DNPH), in order to derivatize the carbonyl groups to DNP

454   hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated

455   secondary antibody. After several washes, samples were incubated with the HRP

456   substrate 3,3',5,5'-tetramethylbenzidine (TMB) and absorbance at 450 nm was

457   registered. Liquid absorbances of the samples were assumed as a measure of immuno-

458   detection of carbonyl groups present in proteins. The protein carbonyl content in

459   samples was determined employing a standard curve prepared from predetermined

460   reduced and oxidized BSA standards.

461

462   **2.9. Analysis of GST forms expression**

463   Immediately after the removal of organs (liver and brain) of *Cyprinus carpio*,

464   they were immersed in TRIzol® Reagent (Invitrogen) for subsequent isolation of total

465   RNA. The RNA quantification was done by spectrophotometry, making the readings at

466   260 and 280 nm. The cDNA synthesis was performed using the kit SuperScriptTM

467   First-Strand (Synthesis System for RT-PCR, Invitrogen). The cDNA products were

468   used for PCR amplification. Primers for GST alpha and GST pi gene and the optimal

469   PCR conditions were used as described previously [11] (Table 1) The *β-actin* primers

470   were designed as described previously for zebrafish [26]. The amplified products were

471   visualized on 1.0% agarose gel with GelRed® under ultraviolet light. Low DNA Mass

472   Ladder (Invitrogen) was used as a molecular marker. The relative mRNA abundance of

473   each GST versus β-actin was determined by optical densitometry using ImageJ1.37

474   freeware (Table 1).

475

476   **2.10. Statistical analysis**

477   The data from each organ were expressed as mean $\pm$ 1 standard error and analyzed

478   through one-way ANOVA, being the factor the treatments (C, LA, CN and LAN)

479   applied. Assumptions of variance homogeneity and normality were checked and

480   mathematical transformations applied if at least one of them was violated. Mean

481   comparisons of treatments were performed using Newman-Keuls test and orthogonal

482   contrasts. In all cases, type I error (α) was fixed in 0.05.

483

484   **3. Results**

485   As showed in Fig. 1, samples of nanocapsules suspension with lipoic acid

486   showed a symmetric distribution of particles sizes, with a mean size of 232.03 nm.

487   After 48 h of first injection, it was verified an increase ($p<0.05$) in GCL activity

488   (nM GSH/h/mg of proteins) in the liver, being higher in fish that received LA (free or in

489   nanocapsules) compared to the control group. However, no statistical differences

490   (p>0.05) were observed between CN and LAN (Fig 2b). Treatment with LA (free or in

491   nanocapsules) showed no significant differences when compared with their respective

492   control groups (p>0.05) in the brain (Fig 2a) and muscle (Fig 2c).

493   After 96 h of first injection, the activity of GCL in brain (Fig. 2a) increased

494   significantly (p<0.05) in both experimental groups that received nanocapsules (CN and

495   LAN) compared to the group treated with free LA. In the liver (Fig. 2b), the group

496   exposed with nanocapsules of LA presented higher GCL activity than fish exposed to

497   free LA (p<0.05). The muscle (Fig. 2c) showed no significant difference (p>0.05) in the

498   activity of GCL in the experimental groups.

499   After one week no significant differences (p>0.05) in the GCL activity in any of

500   the organs tested and in any treatments was observed (Fig. 2).

501   In the short time (48 h), the treatment with LA (free and in nanocapsules) did not

502   altered GSH content (nM GSH/mg of protein) (p>0.05) in any of the organ tested (brain

503   of C group: 332.8 ± 21; liver of C group: 306.2 ± 59; muscle of C group: 1061.1 ±

504   163.5) (Fig. 3).

505   In 96 h, in the liver the GSH content (nM GSH/mg of protein) was higher in the

506   LAN group compared with C and LA groups (Fig. 3b) (p<0.05). In the brain (Fig. 3a)

507   and muscle (Fig. 3c) no significant difference (p>0.05) was observed.

508   In one week no significant differences (p>0.05) was observed for GSH content

509   in brain (Fig. 3a) and in liver (Fig. 3b). In muscle we observed an increase (p<0.05) in

510   the GSH concentration (nM GSH/mg of proteins) in LAN compared to the others

511   treatments (Fig. 3c).

512   After 48 h, the activity of GST (nmol of GSH-CDNB conjugate/min/mg of

513   proteins) also no showed significant differences (p>0.05) in any of the organs tested

514   (Fig. 4).

In 96 h, brain GST activity (Fig. 4a) showed a decrease in CN compared with C ($p<0.05$). In the liver there was no significant difference ($p>0.05$) (Fig. 4b). However, in muscle, the group LAN showed decreased activity of GST ($p<0.05$) (Fig. 4c).

After 1 week, brain GST activity increased ($p<0.05$), in LAN group compared to its control (CN) (Fig. 4a). In liver no differences ($p>0.05$) between treatments were registered (Fig. 4b). In muscle, it was observed an increase ($p<0.05$) in GST activity in LA group compared with C (Fig. 4c).

After 48 h, the concentration of thiobarbituric reactive substances (TBARS) (nmol of TMP/mg of tissue) presented significant differences ($p<0.05$) in the liver, with CN showing higher concentration of TBARS when compared with the others treatments (Fig. 5b). The others organs tested, brain (Fig. 5a) and muscle (Fig. 5c) showed no significant differences ($p>0.05$).

Also after 96 h, lipid peroxidation in brain (Fig. 5a) showed no significant difference ($p>0.05$) among any of the treatments. In the liver (Fig. 5b) there was a decrease ($p<0.05$) of TBARS levels in treatments with nanocapsules (CN and LAN). Muscle showed no significant difference ($p>0.05$) between treatments (Fig. 5c).

Concentration of TBARS, after one week of the first injection, showed a fall in brain (Fig. 5a) in fish that received nanocapsules formulations ($p<0.05$) (CN and LAN). In the liver (Fig. 5b), an increase ($p<0.05$) of TBARS concentration was observed in CN when compared with the others treatments. In muscle (Fig. 5c) it was registered a similar response as in brain ($p<0.05$).

It was measured the concentration of protein carbonyl groups (nmol/mg of proteins) after 1 week of the first injection. No statistical effects was observed for all organs and treatments ($p>0.05$), although a significant higher content ($p<0.05$) was

539   observed in brain (brain of C group: 2.2 ± 0.6; liver of C group: 0.4 ± 0.2; muscle of C

540   group: 0.4 ± 0.2).

541   Considering the expression (relative expression) of different GST forms, after 96

542   h of first injection, no variation between treatments ($p > 0.05$) was registered for brain

543   GST alpha (Fig. 6a). However, GST pi (Fig. 6b) showed a decrease in the groups

544   treated with nanocapsules (CN and LAN) ($p < 0.05$). The expression of GST alpha in the

545   liver (Fig. 6c) showed a significant decrease ($p < 0.05$) in C group when compared with

546   the other experimental groups. Expression of liver GST pi (Fig. 6d) presented higher

547   expression in LA compared to the other treatments ($p < 0.05$).

548   After 1 week of the first injection it was observed that the expression of GST

549   forms showed significant difference ($p < 0.05$) only for GST alpha in the two organs

550   analyzed (brain, Fig. 6a; and liver, Fig. 6c). In the brain, LAN group showed lower

551   relative expression compared to treatments using the free form of LA and its control

552   (Fig. 6a). In the liver (Fig. 6c), treatment with lipoic acid in the free form (LA) showed

553   increased ($p < 0.05$) relative expression of this form of GST, compared with the other

554   treatments. No differences between treatments ($p > 0.05$) were observed neither for brain

555   (Fig. 6b) nor for liver (Fig. 6d) in pi GST expression.

556

557   **4. Discussion**

558   The use of nanocapsules has presented a wide therapeutic use because of the

559   controlled release of drugs and also because to its low toxicity and high ability to cross

560   the blood brain barrier [27]. The study the Amri et al. [28] demonstrated that substances

561   such as resveratrol, that posses antioxidant activity and cardioprotective, anti-

562   inflammatory and anti-tumor properties, but with low bioavailability in vivo, has been

563   used in formulation with nanocapsules, aiming to increase the biodistribution and

reduce its fast metabolism. It was observed in rats, higher antioxidant concentration in the brain, liver and kidney of these animals when ministered resveratrol nanocapsules, compared to the use of the free form after oral and i.p. administration.

The study aimed to compare two formulations of LA prepared under different conditions: a in the free form, at neutral pH has already been demonstrated by Amado et al. [11], which has a beneficial effect in reducing the toxicity of microcystin. The nanoencapsulated formulation is prepared at a much more acid pH (4.0) according to Külkamp et al. [16]. These authors mentioned several advantages of the nanoencapsulated formulation of LA in terms of stability, although studies on their biological effects in terms of antioxidant responses have been done up to date.

In our study it was used nanocapsules with a mean size around 200 nm, where the composition of the polymeric nanocapsule includes, between other compounds, capric and caprylic acid (octanoic acid). Although the biosynthesis of LA in organisms is not well understood, it is known that octanoic acid is substrate for LA synthesis [29, 30]. The composition of the polymeric membrane, thus, can be a factor influencing the endogenous production of LA. For example, in our study we observed that after 96 h of the first injection, the liver of fish from groups CN and LAN showed a similar decrease in TBARS levels (Fig. 5b). Also, the CN group presented a reduction in TBARS concentration similar to the induced by the treatment LAN, both in brain and muscle after one week of the first injection (Fig. 5a and 5c). However the effects elicited by the nanocapsule itself seem to be strongly organ-dependent, since in livers from CN group it was observed a pro-oxidant response after 48 h and one week of the first injection (Fig. 5b). The interaction of compound from the polymeric membrane with endogenous molecules of different organs seems to be an issue to be considers in future studies.

588   The GCL activity in 48 h increased in liver in the two treatments that were used

589   the antioxidant (LA and LAN) compared with C group (Fig. 2b). However, no

590   difference in the concentration of GSH was observed in this time (Fig. 3). In the

591   intermediate time (96 h) it was observed again a possible antioxidant effect generate by

592   the polymeric membrane, because in the brain (Figure 2a), a similar increase in activity

593   of GCL in the CN and LAN groups was observed when compared with LA group. In

594   the liver (Figure 2b) it was verified an increase in GCL activity in LAN group relative

595   to LA, resulting in a higher concentration of GSH in LAN group compared to C and LA

596   groups in this organ (Fig. 3b), showing a positive effect of LA when nanoencapsulated.

597   Note that this result is expected since, as mentioned, GCL is the rate-limiting enzyme

598   for GSH synthesis [24].

599   However, it seems that the benefits induced by nanoencapsulated LA follows a

600   different time frame according to the organ. For example, after one week of the first

601   injection, only in the muscle it was verified an increase in GSH levels in LAN group

602   when compared with the others treatments (Fig.3c). Similar to our results, Suh et al.

603   [10] also found an increase in GCL activity, followed by an increase in the

604   concentration of GSH in the liver of aged rats exposed to free LA (40 mg LA/kg) via

605   i.p. for 24 h.

606   After 24 h of exposure to free LA Cao et al. [9] observed an increase in GST

607   activity in rat cardiac H9C2 cells when exposed to a concentration of 100 μM and this

608   effect remained after 48 and 72 h. With a lower concentration (50 μM), GST activity

609   was augmented only after 48 and 72 h of exposure. At the lowest concentration (25

610   μM), the effect was significant only after 72 h. In our *in vivo* study, the results were

611   somewhat different, since after 48 h of the first injection, no significant differences was

612   observed in any treatment of the three organs tested (Fig. 4). Already in 96 h of the first

613   injection there was a decrease in GST activity (Figure 4a) in brains from CN group

614   when compared with C group, pointing again to the important biochemical effects that

615   are elicited by the compounds present in the polymeric membrane, since the observed

616   response was opposite to that expected after LA exposure. However in muscle, GST

617   activity (Figure 4c) was reduced after 96 h in the LAN group compared with the others

618   treatments. It is important to remark that some doses of LA can in fact lower the

619   antioxidant responses. For example, in the mollusk *Haliotis discus hannai* fed with LA

620   enriched diet, it was observed a reduction in the activity of the antioxidant enzyme

621   glutathione peroxidase and total antioxidant capacity at the highest doses (1,600 and

622   3,200 mg LA/kg) [31]. Although in present study we assayed only one dose, selected

623   according to the study of Amado et al. [11], the responses were evaluated at different

624   times and employing free and nanoencapsulated LA. These different conditions can

625   results in distinct dynamics of distribution and absorption by the different organs that in

626   some cases could result in high internal doses, affecting the detoxifying responses. The

627   dynamics of the different organs after different times was evident after one week, where

628   it was observed an increase of GST activity in brains from LAN group compared with

629   CN group (Fig. 4a), whereas in muscle this increase appeared in the free form of LA

630   also compared to its respective control (C) (Fig. 4c).

631   A previous study has shown that the LA increased the expression of different

632   forms of GST (alpha, mu and pi) in the liver of fish exposed via i.p. (two injections,

633   with an interval of 24 h between each) for 48 h [11]. In our study the expression

634   analysis was performed after 96 h of the first injection and it was observed a decrease,

635   in the brain, for the expression of GST pi in groups treated with nanocapsules (CN and

636   LAN) (Fig. 6), again pointing to the importance of polymeric nanocapsule composition

637   as a factor that influences the effects elicited by nanocapsules. Similarly to what found

by Amado et al. [11], in liver there was an increased expression of GST alpha in all the treatments (including that with free LA) when compared to C group (Fig. 6c). Again, note that free LA induced the expression of this GST form, but the free nanocapsule, without LA was also able to up-regulate the expression of this form. For the expression of the GST pi, only the free form of LA up-regulated their expression, as previously reported by Amado et al. [11]. Already when the time was increased to a week, only GST alpha showed significant difference. In the brain (Fig. 6a) it was registered a decrease in its expression in LAN group compared to C and LA groups, while the liver (Fig. 6b) followed the same pattern seen for Amado et al. [11], with an increase of expression in the free form (LA) compared with the others treatments. So, a similar conclusion to the biochemical responses can be stated concerning the expression results: important differences are seems to be influenced by the composition of the polymeric membrane as well as for the kind of organs, leading to distinct responses at different time frames.

In addition to these analyzes, we also analyzed after 1 week of the first injection, the concentration of protein carbonyl groups in brain, liver, and muscle, and it were not observed any significant differences. Unlike these results, Monserrat et al. [12], reported a decrease in the concentration of carbonyl groups in the liver of fish feeding with a diet enriched with LA (70 mgLA/kg) for four weeks. This difference may be due to differences in the administration route and time frames of these two studies. However, the constitutive levels of carbonyl groups concentration in the control group, was higher in brain, suggesting that any molecule as LA that improve the antioxidant capacity in this organs should be beneficial at long term. It is known that the brain is one of the organs most susceptible to suffer oxidative stress, since it has high oxygen

662 consumption, high levels of polyunsaturated fatty acids and transition metal ions and

663 present relatively low levels of antioxidants [32].

664   Studies have shown that characteristics such as release, rate of diffusion,

665 permeability of the drug in nanocapsules can vary depending on the concentration of the

666 polymer that is used to produce the nanocapsule. For example, there are studies showing

667 that the relative permeability of the drug decrease with an increase in the concentration

668 of the polymer [33]. Cauchetier et al. [34] tested *in vitro* three different polymers for the

669 production of nanocapsules containing atavaquone, a drug used in the treatment of

670 leishmania and the results showed that encapsulation efficiency and stability of the drug

671 varies with the type of polymer used. In the production of nanocapsules are added

672 surfactants, often in large quantities to stabilize the suspension, and some of these

673 surfactants may trigger toxic effects. Studies made with six different surfactants, tested

674 the toxicity in HaCaT cells after incubation for 8 h. The results showed differences in

675 the toxicity of different surfactants [35]. Here is important to emphasize that one of the

676 surfactants tested in this work (polysorbate 80) was the same that used in the production

677 of nanocapsules suspension employed in present study, and Maupas et al. [35] ranked as

678 the fourth most toxic, on a scale of toxicity, compared with the others surfactants tested.

679 Polysorbate 80 at clinically relevant concentrations may increase oxidative stress, *in*

680 *vitro*, in cells of rat thymocytes, enhancing the cytotoxicity of hydrogen peroxide,

681 causing a decrease in cellular glutathione content. Thus, the polysorbate 80 may reduce

682 the synthesis of glutathione or its recycling [36]. Under this context, it can be postulated

683 that some of the pro-oxidant effects observed in CN group (higher TBARS content after

684 48 h and 1 week in the liver) can be considered consequence of the employed surfactant

685 to prepare the suspension.

686   Based on these results, we may conclude that the organs respond differently,

687 depending on exposure time. At short time, the liver was the principal organ to provide

688 antioxidants responses, whereas at longer times, the brain and muscle were more

689 responsive. Differences between the free and nanoencapsulated LA were observed in

690 the same organ at the same time, where the antioxidant effect of nanoncapsulated LA

691 appears to be more effective when used at longer exposure times (i.e.: higher GSH

692 levels in muscle after 1 week). This result is expected, since nanocapsules allows a

693 slowly release of antioxidants. Most importantly, our results indicate that the

694 composition of nanocapsule must be taken into account, since there was a significant

695 antioxidant effect in the treatments containing only the nanocapsules, without LA. It is

696 suggested that this effect occurs due to endogenous production of LA, favored by

697 nanocapsule composition, which has octanoic acid, a substrate for the synthesis of LA.

698 Also the observed pro-oxidant effects in some treatments suggest that some components

699 of nanocapsule, for example, the surfactant which is used to stabilize the formulation

700 may increase the susceptibility of the organ to oxidative stress.

701

717

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819

Figure captions

821

822 **Figure 1.** Size distribution by intensity of lipoic acid nanocapsules measured through

823 dynamic light scattering. Mean average of the nanocapsules are indicated in the figure.

824

825 **Figure 2.** Glutamate-cysteine ligase (GCL) activity (in nM GSH/h/mg of proteins) in

826 brain **(a)**, liver **(b)**, and muscle **(c)** from common carp (*Cyprinus carpio*) after 48 h, 96 h

827 and one week of the first injection via i.p. C: control group (exposed only to dilution

828 solution without LA); LA: lipoic acid group (exposed to free LA); CN: exposed only to

829 the nanocapsules without the presence of LA; LAN: exposed to LA in nanocapsules.

830 Different letters indicate significant differences (p<0.05) between experimental groups

831 at each time after the first injection using Newman-Keuls test. Asterisk (*) indicate

832 significant differences (p<0.05) after applying orthogonal test.

833

834 **Figure 3.** Glutathione reduced (GSH) concentration (in nM GSH/mg of proteins) in

835 brain **(a)**, liver **(b)**, and muscle **(c)** from common carp (*Cyprinus carpio*) after 48 h, 96 h

836 and one week of the first injection via i.p. C: control group (exposed only to dilution

837 solution without LA); LA: lipoic acid group (exposed to free LA); CN: exposed only to

838 the nanocapsules without the presence of LA; LAN: exposed to LA in nanocapsules.

839 Different letters indicate significant differences (p<0.05) between experimental groups

840 at each time after the first injection using Newman-Keuls test.

841

842 **Figure 4.** Glutathione-S-transferase (GST) activity (in nmol CDNB-GSH

843 conjugate/min/mg of proteins) in brain **(a)**, liver **(b)**, and muscle **(c)** from common carp

844 (*Cyprinus carpio*) after 48 h, 96 h and one week of the first injection via i.p. C: control

845   group (exposed only to dilution solution without LA); LA: lipoic acid group (exposed to

846   free LA); CN: exposed only to the nanocapsules without the presence of LA; LAN:

847   exposed to LA in nanocapsules. Different letters indicate significant differences

848   ($p < 0.05$) between experimental groups at each time after the first injection using

849   Newman-Keuls test. Asterisk (*) indicate significant differences ($p < 0.05$) after applying

850   orthogonal test.

851

852   **Figure 5.** Concentration of thiobarbituric reactive substances (TBARS) (nmol of

853   TMP/mg of tissue) in brain **(a)**, liver **(b)**, and muscle **(c)** from common carp (*Cyprinus*

854   *carpio*) after 48 h, 96 h and one week of the first injection via i.p. C: control group

855   (exposed only to dilution solution without LA); LA: lipoic acid group (exposed to free

856   LA); CN: exposed only to the nanocapsules without the presence of LA; LAN: exposed

857   to LA in nanocapsules. Different letters indicate significant differences ($p < 0.05$)

858   between experimental groups at each time after the first injection using Newman-Keuls

859   test.

860

861

862   **Figure 6.** Gene expression (relative expression) of glutathione-S-transferase (GST)

863   alpha and pi in brain and liver of common carp (*Cyprinus carpio*) after 48 h, 96 h and

864   one week of the first injection via i.p. **(a)** GST alpha in brain, **(b)** GST pi in brain, **(c)**

865   GST alpha in liver and **(d)** GST pi in liver. C: control group (exposed only to dilution

866   solution without LA); LA: lipoic acid group (exposed to free LA); CN: exposed only to

867   the nanocapsules without the presence of LA; LAN: exposed to LA in nanocapsules.

868   Different letters indicate significant differences ($p < 0.05$) between experimental groups

869   at each time after the first injection using Newman-Keuls test.

870

871 **Table 1.** Polymerase chain reaction (PCR) conditions and primers sequences employed

872 to analyze the expression of alfa and pi forms of glutathione-S-transferase (GST).

873

Enzymes	Primers sequences (5`→3`)	Annealing temperature (°C)	PCR product (bp)	GenBank accession number (mRNA)
GST-alpha	F - GGTGGAAATAGACGGGATGCAGCTCG	60	394	DQ411310
	R – GGCCTTCATCTTCTCTTGAAACGCCTG			
GST-pi	F - CTATGTTAAGGCATTTGGGTCGCAAAC	60	338	DQ411313
	R – ATCCACATAGCTCTTGAGAGTTGGGAAGG			

874

875

876

877

878

879   **Figure 1**
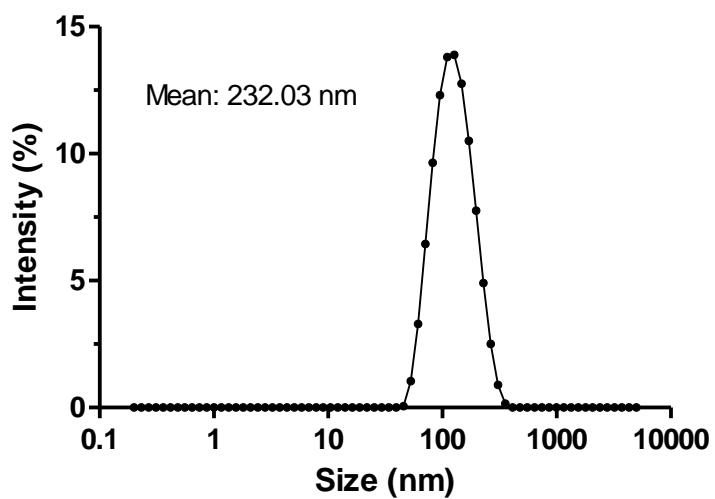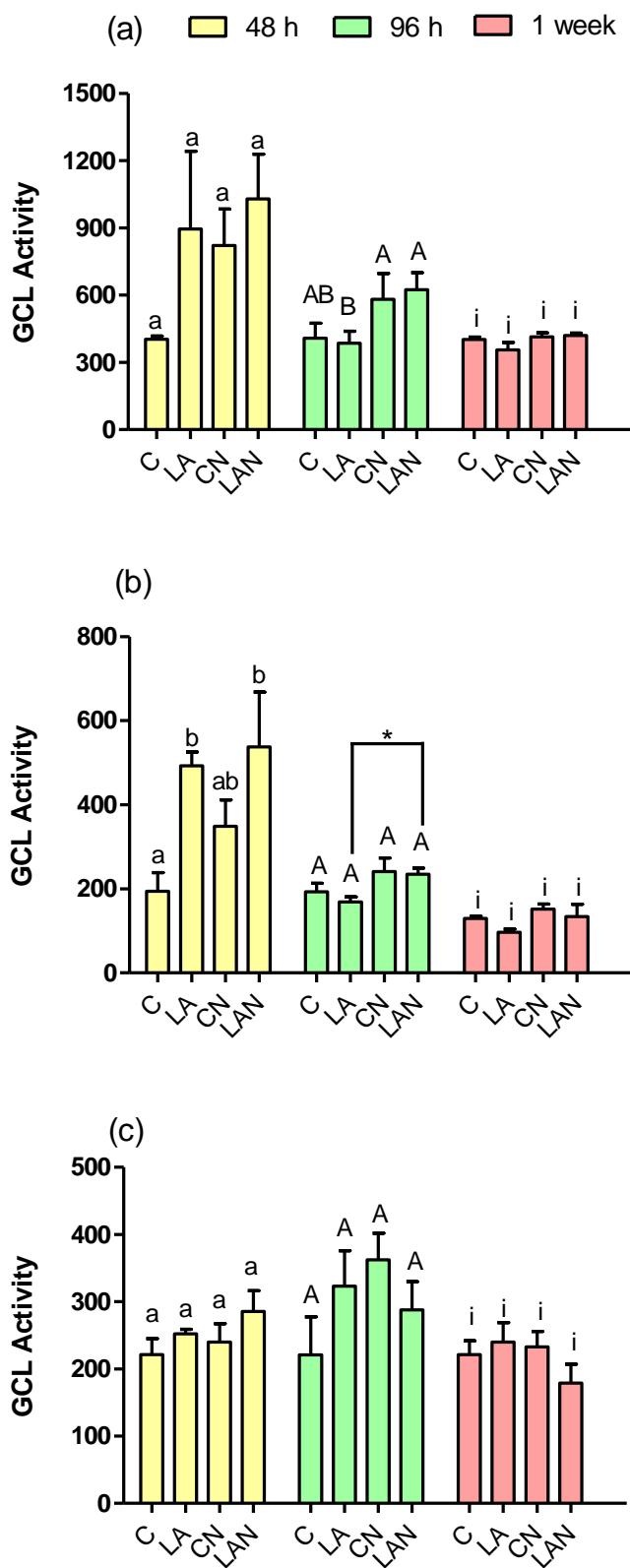
880
881
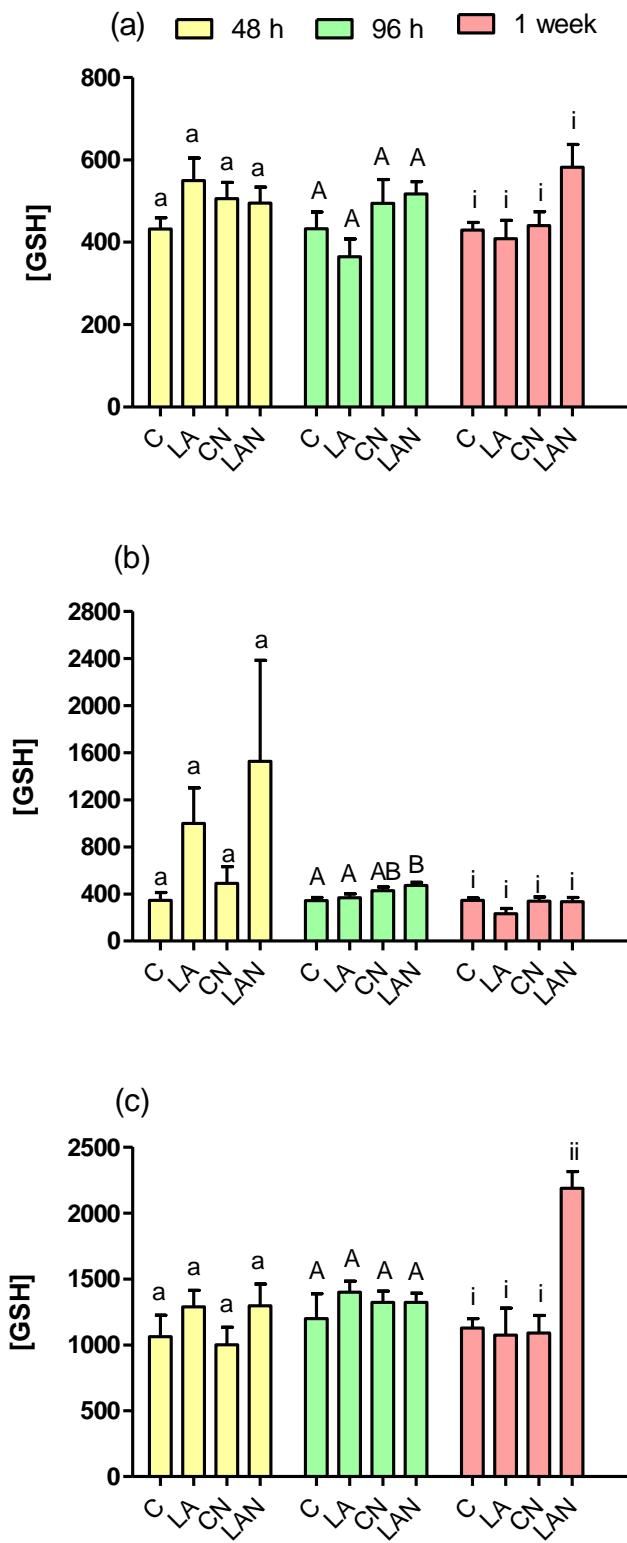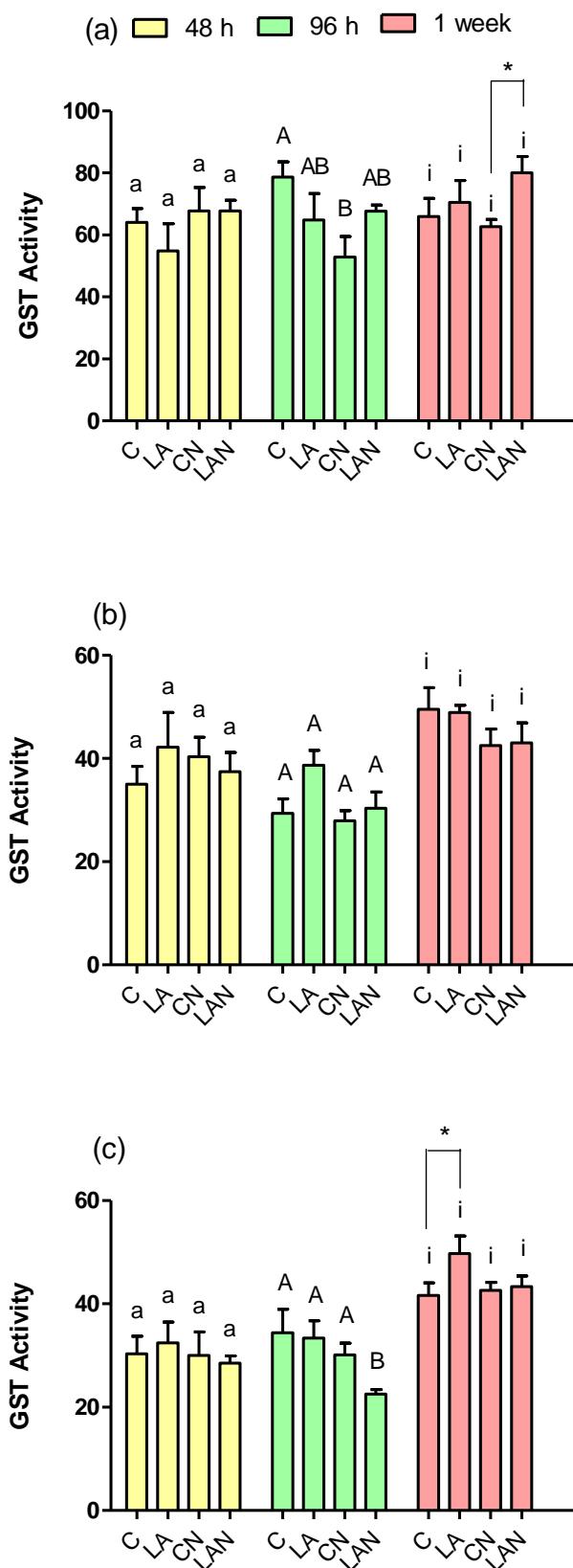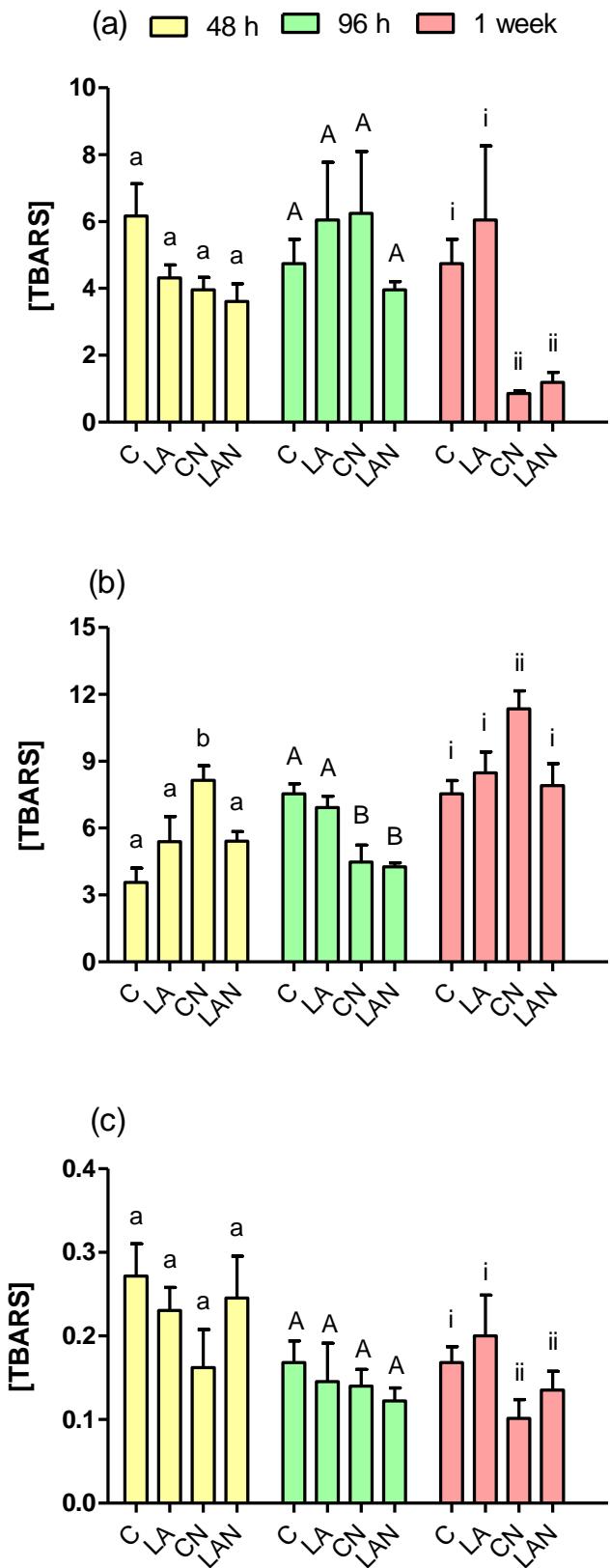
Figure 3

Figure 4

Figure 5

Figure 6
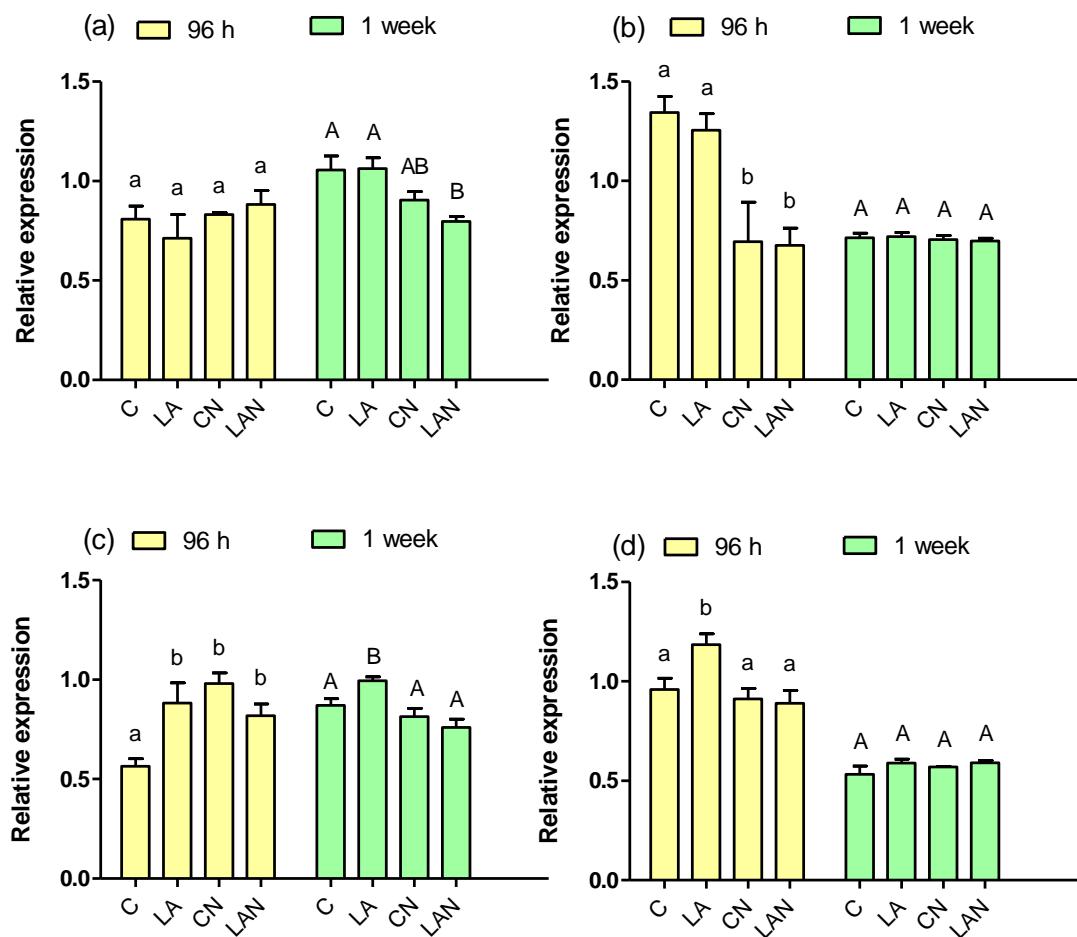
909 **6. Conclusões Gerais**

910 Com base nos resultados obtidos neste trabalho, podemos concluir que:

911

912 1- Os órgãos analisados (cérebro, fígado e músculo) responderam de forma diferente,

913 sendo observado que, a curto prazo, o fígado foi o principal órgão a apresentar respostas

914 antioxidantes, como por exemplo, o aumento da atividade da GCL nos tratamentos com

915 AL em 48 h, se mantendo no tratamento com nanocápsulas em 96 h, além da

916 diminuição dos níveis de TBARS neste mesmo tempo. A longo prazo, o cérebro e o

917 músculo se mostraram mais responsivos, em termos antioxidantes, do que o fígado, já

918 que foi verificado um aumento da concentração de glutationa reduzida no músculo, um

919 aumento da atividade da GST no cérebro e no músculo e uma diminuição dos níveis de

920 TBARS nestes dois órgãos.

921

922 2- A resposta antioxidante de cada órgão também foi variável de acordo com a forma de

923 ácido lipóico utilizada (livre ou em nanocápsulas), toda vez que foi verificado que um

924 mesmo órgão em um mesmo tempo pode responder de forma diferente, sendo que a

925 forma livre induzia um efeito diferente do que forma nanoencapsulada. Isto foi

926 observado na atividade da GST no músculo em 96 h, onde a forma nanoencapsulada

927 induziu um aumento da atividade desta enzima, enquanto que a forma livre não teve

928 efeito. Já a atividade da GST no músculo uma semana após a primeira injeção,

929 apresentou maior atividade com a forma livre, não havendo diferença com as

930 nanocápsulas.

931

932 3- O efeito antioxidante parece ser mais efetivo quando utilizado nanocápsulas a longo

933 prazo, sugerindo-se que esta formulação libera mais lentamente o ácido lipóico, o que é

934 esperado de uma formulação com nanocápsulas.

935

936 4- A composição da nanocápsula deve ser levada em consideração, uma vez que neste

937 trabalho foi observado em várias situações respostas distintas dos tratamentos em que

938 foram utilizadas somente as nanocápsulas sem ácido lipóico (CN), sendo elas:

939 (a) Efeito antioxidante bem evidente no cérebro em 96 h quanto à atividade da

940 GCL, onde houve um aumento na atividade da enzima no tratamento apenas

941 com a nanocápsula (CN) em proporções semelhantes do tratamento que continha

942 nanocápsula e AL (LAN).

943 Foi também observado no conteúdo de TBARS com uma semana de exposição

944 no cérebro e no músculo, havendo uma diminuição da peroxidação lipídica nos

945 dois tratamentos que continham nanocápsulas (CN e LAN) comparados com os

946 organismos que receberam o AL livre. Este efeito pode ser devido à produção

947 endógena de LA, favorecida pela composição da nanocápsula que possui ácido

948 octanóico, que por sua vez é substrato para a síntese de AL. No entanto, também

949 se observou um efeito pró-oxidante em alguns casos nesses mesmos tratamentos

950 expostos apenas à nanocápsulas (CN). Isso pode ser observado quando analisado

951 o conteúdo de TBARS no fígado em 48 h e uma semana após a primeira injeção,

952 onde houve um aumento da peroxidação lipídica quando comparado com os

953 demais tratamentos. Este resultado pode estar relacionado aos componentes

954 utilizados para a fabricação da nanocápsula, como por exemplo, o surfactante

955 (polisorbato 80) que é utilizado para estabilizar a suspensão e que já foi

956 demonstrado que pode aumentar a suscetibilidade do órgão ao estresse

957 oxidativo.

958

7. Perspectivas

960 Com base nesses resultados, em que a composição da cápsula se mostrou muito

961 importante, quando observada a resposta antioxidante dada pela substância em estudo,

962 temos como perspectiva a produção de nanocápsulas com diferentes composições

963 (variando por exemplo o polímero e o surfactante utilizado) e mantendo o mesmo

964 composto de interesse, como por exemplo o ácido lipóico.

965 Além disso, é necessário desenvolver metodologias analíticas para a

966 dosagem de ácido lipóico em cada órgão o que daria informação importante a respeito

967 da absorção e distribuição deste antioxidante.

968

969

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