

Research Article

Effects of α -lipoic acid on growth and antioxidant enzyme activities in common carp (*Cyprinus carpio*)

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Abstract

This study evaluated the effects of α -lipoic acid (LA) supplementation on antioxidants responses in intestine and liver of *Cyprinus carpio* juveniles. Four experimental groups were fed in duration of 60 days with a diet containing different levels (0 as control, 0.5, 1 and 1.5g kg⁻¹) of LA evaluating glutathione (GSH) activity, superoxide dismutase (SOD) and total antioxidant activity (TAC) in two organs. Parameters, including survival, weight gain and specific growth rates were also evaluated. The results showed that adding LA supplementation to carp diet led to increased activity of antioxidant enzymes in 1.5 g dose and the difference was significant in 0.5 and 1g groups ($p < 0.05$). There was no significant difference in survival among experimental and control groups ($p > 0.05$). Weight gain in 1 g group was significantly higher than other groups ($p < 0.05$). Specific growth rates exhibited the same response pattern as that of weight gain ($p < 0.05$). The obtained results regarding growth and antioxidant status indicated that LA could be supplemented in diets for *Cyprinus carpio* at doses between 1 and 1.5g LA kg⁻¹ dry food.

Keywords: GSH, SOD, TAC, Antioxidant activity, Growth, Common carp

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Introduction

Aquaculture is one of the most important industries that provide food for human societies. In aquaculture industry, in production of fish or crustaceans there are numerous biotic and abiotic factors in dense production systems that affect growth, survival and aquatic health, and any change in these parameters may result in oxidative stress (Oliva-Teles, 2012). All aerobic organisms, including aquatic species, are susceptible to harmful effects of oxidative activity; therefore, they need an adequate internal defense system (Ross *et al.*, 2001). The oxidative stress actually reflects the imbalance between prooxidants and antioxidants, which interferes with signaling control and inhibits cellular and molecular damage (Jones, 2006). Imbalance between prooxidation and antioxidant defense is the result of oxidation action, and will cause biomolecules like lipids, proteins, and nucleic acids to change (Djordjevic, 2004). The level of reactive oxygen species (ROS) is very important for health of living organisms, including aquatic organisms. Reactive oxygen species play an important role in natural body functions such as gene transcription, signal transduction, regulation of enzyme activity, and bacterial elimination (Lygren *et al.*, 1999; Zheng and Storz, 2000). Of course, high levels of reactive oxygen species, due to rapid chemical reactions, may result in irreversible damage to the body protein, cell membrane lipid, and mitochondrial DNA, as well as mitochondrial dysfunction, ultimately

causing cell death (McCord and Fridovich, 1969). To protect cells from oxidative damage during oxygen metabolism, an antiseptic defense system has probably evolved in aerobic organisms. The system includes a wide range of mechanisms, such as enzymatic systems and low molecular weight compounds, which can include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), etc. Oxidative stress seems to play an important role in the pathogenesis and progression of many diseases of liver and intestines (Papada *et al.*, 2014; Xiao *et al.*, 2014). Therefore, if the antioxidant capacity of liver and intestine can be increased, it will be beneficial to the liver and the intestine. Recently, dietary supplements have been used to improve the body's antioxidant capacity.

Alpha-lipoic acid is a disulfide compound, made up of octanoic acid, and found in microorganisms. Because alpha-lipoic acid is a naturally occurring antioxidant that is found in all living organisms, animals and plants such as spinach, broccoli and tomatoes, it is subject of many researches (Reed, 1974; Navari-Izzo *et al.*, 2002). Alpha-lipoic acid supplementation is considered as a powerful antioxidant, it exerts antioxidant effects via direct clearing of free radicals, metal ions, it also has effect on some other antioxidants and it increases intracellular glutathione (Longaray-Garcia *et al.* 2013). Antioxidant effects of alpha-lipoic acid include ROS cleansing, chelation of metal ions, repair of oxidative damage,

regulate gene expression, protecting of organisms from DNA oxidation, etc. (Packer *et al.*, 1995). The α -lipoic acid is easily absorbed into the cell through absorption of food, converting it into dihydrolipoic acid (DHLA), which also has antioxidant activity (Handelman *et al.*, 1994). Through *in vitro* and *in vivo* experiments, it has been shown that LA has potential positive effects on the prevention and treatment of oxygen-related diseases (Biewenga *et al.*, 1997; Cakatay, 2006). However, most studies in this field have been conducted in terrestrial vertebrates (Arivazhagan *et al.*, 2002a; 2002b; Chae *et al.*, 2008; Fujita *et al.*, 2008).

In fish, like other vertebrates, antioxidant defense is important in health and prevention of cell damage. PUFA auto-oxidation produces compounds such as fatty acid hydroperoxides, aldehydes and hydrocarbons, which can cause some disease conditions in fish (Kanazawa, 1993). Hence strengthening of immune system can lead to maintaining the health of fish and prevent lesions caused by oxidative stress. Nowadays, the use of compounds with antioxidant properties is considered. Alpha-lipoic acid supplement is one of these compounds. Also Kütter *et al.* (2012) examined the effects of different doses of LA on growth performance, body composition and antioxidant status. The results obtained regarding growth, antioxidant status, lipid and protein metabolism showed that LA can be used in the diet of *Trachinotus marginatus* at doses of 317 and 524 mg kg⁻¹ of diet.

Interestingly, these effects are dose-dependent, such that doses of 317 and 524 mg LA kg⁻¹ are better to reduce lipid peroxidation in fish muscle tissue. A study by Park *et al.* (2006) showed that use of lipoic acid in diets improved antioxidant status, detoxified and suppressed the symptoms of vitamin C deficiency. Also, Lobato *et al.* (2013) in shrimp, *Penaeus vannamei* showed that the use of LA reduced lipid peroxidation and increased antioxidant response in muscles. Given that common carp is the main species of warm water fish and one of the important species of teleosts in the Caspian Sea; this research aimed to evaluate the effects of alpha-lipoic acid supplementation on growth and its antioxidant enzyme activities.

Materials and methods

Fish maintenance and experimental design

Common carp juveniles (*Cyprinus carpio*) were obtained from Semeskandeh Hatchery (Sari, Mazandaran) and transferred to Raje Research Center (Babol, Mazandaran). The fish were conditioned for 1 week during which time they were reared on a commercial diet at a rate of 5% of their body weight given three times a day (08:00, 12:00 and 16:00 hours). After 1 week of acclimation in the stock tanks, the fish were transferred to experimental tanks. A total of 96 healthy fish were randomly distributed in 12 glass aquaria in 4 groups (200 Liters each) with a density of 8 fish per aquarium with an average weight of 40±0.05 g/fish on

triplicate groups per treatment. The aquaria were cleaned daily and excreta were siphoned. Light was about 12:12 h light: dark cycle throughout the day. Each tank was aerated through an air stone. The temperature, pH and dissolved oxygen were monitored daily using multi-parameter electrode (Haana, HI769828). Temperature was controlled using submersed heater thermostat ($26 \pm 1^\circ\text{C}$). The experiment was conducted for 60 days.

Experimental diets

The composition of formulated experimental diets used in this

experiment is outlined in Table 1. Four experimental diets were formulated to contain 0, 0.5, 1, and 1.5 g kg⁻¹ of LA (synthetic α 1-lipoic acid purity $\geq 99\%$ (Sigma Aldrich)) respectively. All the dry ingredients were mixed with a 200T Mixer Bench for 15 min, and then Soybean oil was added, and mixed for another 15 min. Water was added to 300 ml kg⁻¹ dry ingredients mixture and mixed for another 15 min. The wet mixture was pelleted into 2 mm size and the pellets were air-dried at room temperature for 24 h, and then stored at -20°C until used.

Table 1: Proximate composition and formulation of the experimental diets with different levels of α -lipoic acid (g/kg diet, wet basis)

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	125	125	125	125
α -lipoic acid	0	0.5	1	1.5
Soybean meal	200	200	200	200
Rapeseed meal	160	160	160	160
Wheat gluten	200	200	200	200
Rice bran	120	120	120	120
Soybean oil	15	15	15	15
Vitamin premix ^a	10	10	10	10
Mineral premix ^a	10	10	10	10
Calcium dihydrogen phosphate	30	30	30	30
Zeolite powder	10	10	10	10
Cellulose	40	39.5	39	38.5
Cotton seed meal	80	80	80	80
Proximate composition (% DM)				
Dry matter (%)	84.64	84.74	84.94	84.74
Ash (%)	10.98	10.99	10.99	11.00
Crude fat (%)	5.74	5.72	5.71	5.68
Crude protein (%)	30.84	30.94	29.93	29.93

^a Vitamin and mineral premix (IU or mg/kg dry diet) Vitamin A 900,000 IU, Vitamin D 250,000 IU, Vitamin E 400 mg, Vitamin K 40 mg, Vitamin B₁ 50 mg, Vitamin B₂ 200 mg, Vitamin B₃ 500 mg, Vitamin B₆ 50 mg, Vitamin B₇ 5 mg, Vitamin B₁₁ 15 mg, Vitamin B₁₂ 11 mg, Vitamin C 1,000 mg, inositol 2,000 mg, choline 5,000 mg, biotin 50 mg, Pantothenate 1,000 mg, folic acid 165 mg, niacin acid 2,500 mg, CuSO₄·5H₂O 2.5 g, FeSO₄·7H₂O 28 g, ZnSO₄·7H₂O 22 g, MnSO₄·4H₂O 9 g, Na₂SeO₃ 0.045 g, KI 0.026 g, CoCl₂·6H₂O 0.1 g.

Tissue sampling

At the end of the experiment, fish feeding was stopped for 24 h prior to the

sampling, so that the stomach would be empty. Then, from each replicate of the experimental treatments, 3 fish samples

were selected randomly, making it 9 fish for each treatment. Samples were anesthetized, immersed in benzocaine at a concentration of 100 mg/L, measured and weighed, then killed at 400 mg/L benzocaine. After killing the fish, the

liver and intestine were completely removed and stored in liquid nitrogen. The growth performance variables were calculated using the following formulas (Santos *et al.*, 2016):

Survival rate (%) = (number of fish at the end of evaluation period/initial number of fish) $\times 100$

Weight gain (Wg %) = $100 \times (W_f - W_i) / W_i$

Specific growth rate (SGR % day⁻¹) = $100 \times (\ln W_f - \ln W_i) / t$

Where W_f is final body weight (g); W_i is initial body weight (g) and t is time (days).

Tissue Homogenization

After different organs were separated and washed in tube homogenizer by phosphate-buffered saline containing 50 mmol, homogenized solution was prepared with a homogenizer. The homogenized samples were transferred to 16 cm falcon tube and were centrifuged by refrigerated centrifuge (10000 g at 4°C for 15 min). Supernatant obtained from any of the organs were transferred to a test tube and after writing specifications, they were stored at -70°C until proper time to determine the biochemical and antioxidant parameters (Da Rocha *et al.*, 2009).

The level of glutathione (GSH) as the first level of antioxidant defense against oxidative stress was evaluated based on Ellman's method (Ellman *et al.*, 1961). Superoxide dismutase (SOD) activity in supernatants of homogenate of animal tissue was measured based on the Kono's method (Kono, 1978). The total antioxidant activity (TAC) in supernatant obtained from the homogenate of animal tissue was determined by FRAP method (Habig

and Jakoby, 1981; White *et al.*, 2003; Monserrat *et al.*, 2013).

Statistical analysis

All collected data at each stage were recorded in Excel software and descriptive analyses were carried out in this program. The normality of the data distribution was evaluated using Kolmogorov-Smirnov test. Thereafter, obtained data were analyzed using SPSS software and ANOVA test and comparisons among treatment means were done by Duncan test at 95% confidence level (Zar, 1984). Tables and graphs were plotted using the Excel software.

Results

The mean water temperature, dissolved oxygen and pH over the entire experiment (60 days) were $26.33 \pm 0.8^\circ\text{C}$, 7.2 ± 0.45 ppm and 7.6 ± 0.55 , respectively. All water quality parameters were in the right range for the growth of carp; food intake was also confirmed in all groups with direct

observation throughout the duration of the experiment.

Comparison of average glutathione in the fish liver showed that there was a significant difference among treatments and control group ($p < 0.05$) and fish treated with 1.5 g kg^{-1} LA was better than other treatments, although this treatment had no significant difference with fish treated with 1 and 0.5 g kg^{-1} LA ($p > 0.05$). Also comparative evaluation of mean reduced glutathione in the fish intestine indicated that there was a

significant difference among treatments and control group ($p < 0.05$), concentration of 1.5 g kg^{-1} LA was better than other treatments, there was no significant difference between concentrations of 0.5 and 1 g kg^{-1} LA ($p > 0.05$). Figure 1 shows changes in activity of reduced glutathione (GSH) under different levels of LA dietary supplements in the intestine and liver tissues of common carp.

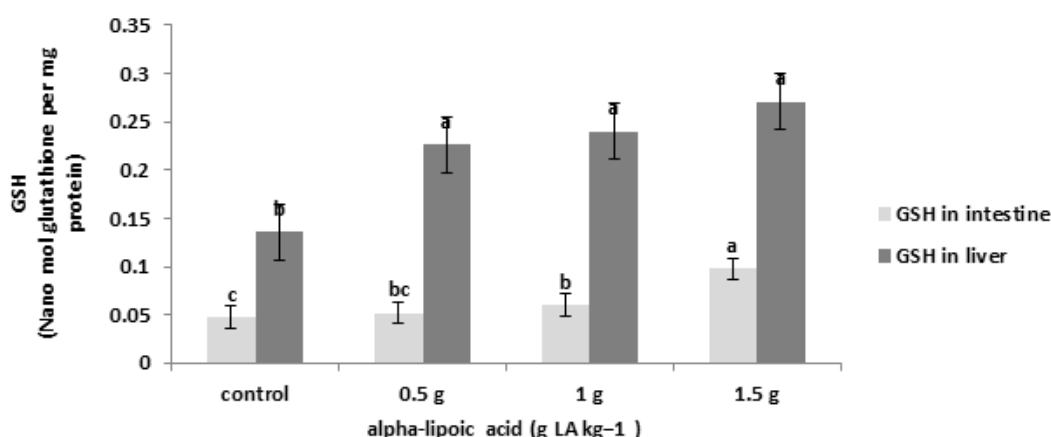


Figure 1: GSH level in the intestine and liver of fish in different treatments of LA. Different letters indicate significantly different values among groups ($p < 0.05$).

The results indicated that treatment with dose of 1.5 g kg^{-1} LA had the highest activity and liver tissue had the greatest influence from this supplement. Comparative evaluation of superoxide dismutase in the liver indicated that there was a significant difference among treatments with control group ($p < 0.05$) and dose of 1.5 g kg^{-1} LA was better than other treatments, although this treatment had no significant difference with concentrations of 0.5 and 1 g kg^{-1} LA ($p > 0.05$). Also, comparison of

superoxide dismutase in the fish intestine indicated that there was a significant difference among treatments and control group ($p < 0.05$) and dose of 1.5 g kg^{-1} LA was better than other treatments, although this treatment had no significant difference with dose of 1 g kg^{-1} LA ($p > 0.05$). Figure 2 shows the changes in activity of superoxide dismutase (SOD) in the intestine and liver of common carp under different levels of alpha-lipoic acid supplement.

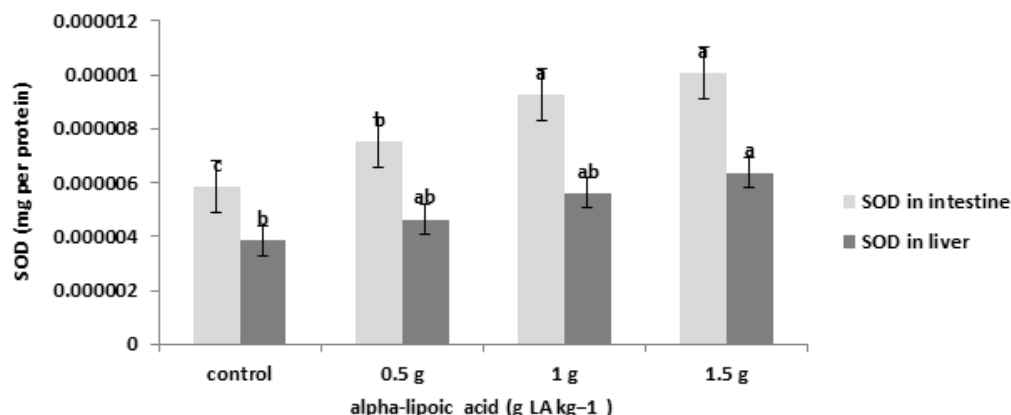


Figure 2: Superoxide dismutase (SOD) in the intestine and liver of fish in different treatments of LA. Different letters indicate significantly different values among groups ($p < 0.05$).

The results indicate that fish treated with 1.5 g kg^{-1} LA had the highest activity and the intestine tissue had the greatest influence from this supplement. Comparison of average total antioxidant in fish intestine showed that there was a significant difference among treatments with control group ($p < 0.05$) and the concentration of 1.5 g kg^{-1} LA was better than other treatments; however, this treatment had no significant difference with dose of 1 g kg^{-1} LA ($p > 0.05$) but showed a significant difference with concentration of 0.5 g kg^{-1} LA and control group. In addition, comparison of average total antioxidants in the fish liver indicated that there was a significant difference among treatments with control group ($p < 0.05$) and concentration of 1.5 g kg^{-1} LA was better than other treatments; treatment with a concentration of 1 g kg^{-1} LA had significant difference with control group ($p < 0.05$) but there was no significant difference between this treatment and concentration of 0.5 g kg^{-1} LA ($p > 0.05$).

Figure 3 shows the changes in the total antioxidant activity (TAC) under the influence of different levels of alpha-lipoic acid supplement in the intestine and liver tissues of common carp.

The results indicated that fish treated with concentration of 1.5 g kg^{-1} LA had the highest activity and liver tissue had the greatest influence from this supplement. The fish survival, weight gain, specific growth rates (SGR) data after 60 days of LA supplementation are shown in Table 2. No significant difference was observed in survival rates among the treatments ($p > 0.05$). The weight gain was significantly higher in the 1 g kg^{-1} LA group, this group had more weight gain than other groups, and showed a significant difference with other groups ($p < 0.05$). The specific growth rates were observed in 1 g kg^{-1} LA nutritional treatments with the highest levels and had a significant difference with other treatments ($p < 0.05$).

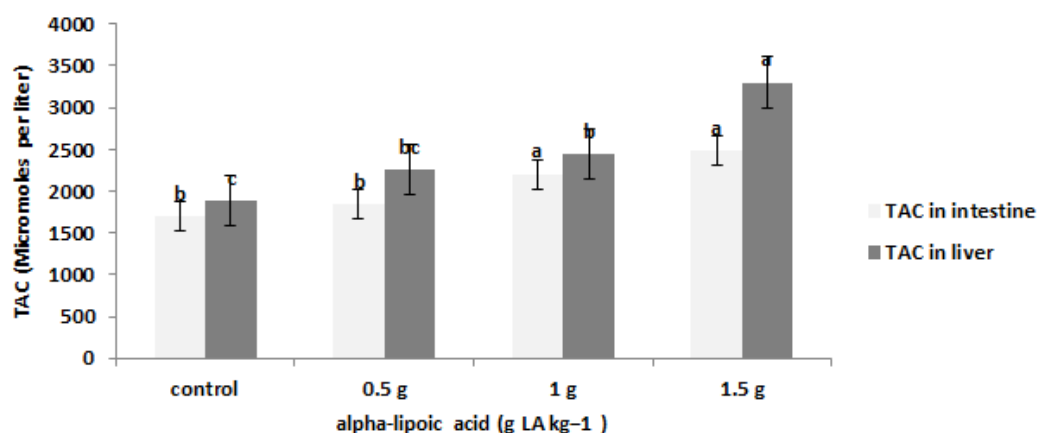


Figure 3: TAC level in the intestine and liver in different treatments of LA. Different letters indicate significantly different values among groups ($p < 0.05$).

Table 2: Survival, weight gain, specific growth rate of *Cyprinus carpio* after 60 days of being fed with diet containing different concentration of lipoic acid. Data are expressed as mean \pm standard deviation.

Growth parameters	Experimental group (g LA kg ⁻¹)			
	Diet 1(0 g Control)	Diet 2 (0.5 g)	Diet3(1 g)	Diet 4(1.5 g)
Survival (%)	98.83 \pm 4.16	97.67 \pm 8.33	100.00 \pm 0.00	98.83 \pm 4.17
Weight gain(g)	275.00 \pm 36.08 ^c	285.00 \pm 10.00 ^b	316.67 \pm 8.33 ^a	216.67 \pm 54.17 ^d
SGR(% day ⁻¹)	2.18 \pm .16.00 ^b	2.24 \pm .043 ^b	2.37 \pm 0.033 ^a	1.87 \pm 0.29 ^c

*Different letters in the same row indicate significantly ($p < 0.05$) different values among groups.

Discussion

In the present study, we provided direct evidence that LA supplementation affected the growth performance and anti-oxidant status of common carp. Therefore, detection of LA and its measurement in diets and biological samples plays an important role in recognizing the properties and its biochemical effects in the tissues/organs of fish. The results obtained in this study, can be attributed to the positive antioxidant effects of this supplement. In this study, it was shown that there was a significant difference between the doses used in the control treatment ($p < 0.05$). In addition, the dose of 1.5 g produced the best antioxidant activity in *Cyprinus carpio*. Also, the results indicate that the

antioxidant activity depends on the concentration level, so that at 1.5 g concentration, it was more effective than other concentrations.

In a study by Kütter *et al.* (2012), the effects of different doses of LA on growth performance, body composition and antioxidant status were investigated. The results showed that the concentration of 317 and 524 mg/kg of lipoic acid in the *Trachinotus marginatus* diet would improve growth, antioxidant status, lipid metabolism and also protein. Interestingly, these effects are dose dependent where the doses of 317 and 524 mg kg⁻¹ LA were better for the reduction of lipid peroxidation in fish muscle tissue. However, in other studies by Zhang *et al.* (2009) and Seo *et al.*

(2012) the adverse effects of high doses of LA in different organisms, which reduce the function of different organs of the body were demonstrated, but there are no harmful effects from the LA on the biological parameters (WG, FW and SGR) comparing the fish fed with LA or without it. Nevertheless, LA supplementation increases antioxidant function in a variety of tissues and organs through improved enzymatic status and non-enzymatic routes (Packer *et al.*, 1995; Shay *et al.*, 2009). Furthermore, the effect of this supplement had the greatest impact on the activity of reduced glutathione (GSH) as a non-enzymatic antioxidant and total antioxidant activity (TAC) in liver tissue, while the antioxidant activity in superoxide dismutase (SOD) as an enzymatic antioxidant has been more active in the intestinal tissue. Zhang *et al.* (2010) reported increased levels of GSH in the hepatopancreas of Abalone (*Haliotis*) after feeding by LA in concentration of 200 mg kg⁻¹. But Mårtensson *et al.* (1990) and Veskokoukis *et al.* (2012) found that two weeks feeding with LA supplementation increased the detoxification concentration of GSH and Glutathione-S-transferase (GST) enzymes in the intestine of juvenile carps. In other words, increased GST activity increases detoxification against physiological or dietary oxidative loads, in addition, GSH is an indispensable field for many enzymes, and GST also increases the intestinal function.

Recently, several studies have demonstrated the biological importance

of dietary supplements with antioxidant effects on the physical growth, intestinal functions and redox balance of the intestine. The results of this study and some other studies point out to the positive antioxidant effects of this supplement in fish. Monserrat *et al.* (2008) and Amado *et al.* (2011) showed that alpha-lipoic acid improves the detoxification and antioxidant capacity, thus protecting the organs in *Corydoras paleatus* and *Cyprinus carpio* against oxidative stress, as well as the microcystin toxicity. A study by Park *et al.* (2006) showed that the use of lipoic acid in the diets improved antioxidant status, detoxification and suppressing of the symptoms of vitamin C deficiency. Also, Zhang *et al.* (2010) reported that in abalone, high doses of LA in the diet (between 1600 and 3200 mg kg⁻¹ of diet) decreased the antioxidant capacity and growth. In another study, Trattner *et al.* (2007) showed that using LA supplementation in pacu (*Piaractus mesopotamicus*) reduces lipid peroxidation in the muscles and improves the antioxidant status of the brain.

Monserrat *et al.* (2008) found that LA is effective in reducing protein oxidation in muscle and liver, also in reducing oxidative stress parameters. Fish tissues compared to tissues of mammals and birds are rapidly corrupted due to the significant amount of polyunsaturated fatty acids and oxidation after death. Rapid oxidation of these fatty acids and omega-3 fatty acids reduces shelf life in fish. In addition, corruption in marine products is affected by internal and

external factors such as concentration of sensitive compounds to temperature, ionic strength and the presence of oxygen. Fish and their products, despite high nutritional value, are highly sensitive to oxidative corruption and their qualitative characteristics are reduced during storage due to bacterial and oxidative corruption (Mexis *et al.*, 2009). To postpone the oxidative corruption in fish and its products, several solutions have been proposed including control and reduction of its temperature, vacuum packaging, modified atmosphere packaging and also adding antioxidant (Lin and Lin, 2005). However, the use of a substance with antioxidant properties, such as α -lipoic acid, can improve antioxidant status in the organs and tissues of different fish species and protect against oxidative stress.

Lipoic acid (LA) is a natural disulfide compound with high biological activity (Bustamante *et al.*, 1998); therefore, it has been used effectively in experiments on living organisms and in laboratory conditions. In addition to evaluating antioxidant enzyme activities, the effects of this supplement on survival and growth factors were also investigated. The study found that there were no harmful effect on biological parameters such as survival, weight gain, and specific growth rates, whether in fish fed with or without LA. Several reports on the side effects of LA of different growth model animals have shown that, when the supplement is taken in high doses, it leads to reduced animal growth performance. In this study, the positive

performance of alpha-lipoic acid supplementation on growth of common carp was demonstrated.

In the study of Zhang *et al.* (2010), an increase in the growth of abalone when fed with these supplements at a dose of 800 mg was demonstrated, and doses higher than 1600 and 3200 mg led to a reduction of growth. According to the study of Kim *et al.* (2004), this decline is attributed to the reduction in activity of the hypothalamic AMP-activated protein Kinase. Cell signaling may also be responsible for energy consumption according to the study. It can be concluded that low dose of 1 g kg^{-1} LA had more positive effects on the growth factors compared to doses above 1 g kg^{-1} LA.

In this study, the optimum temperature and nutritional status were achieved in August and September. Based on selection procedure, due to the ideal conditions of temperature (between 20 and 30°C) we were confronted with problems in feeding the fish and the impact of temperature on the feed was also felt. The fish feed in the control group and the experimental groups were of the same amount. The water source in this research for all groups was from the same source and the nature of the chemical and physical conditions were the same for control group and experimental groups. So there was no difference in chemical factors, water temperature and the environment among groups. We did not have the desired temperature on feeding of the groups. In general, it is suggested that future studies should focus on the

measurement of dose response and use of more comprehensive techniques such as metabolomics to discover the metabolic pathways that may be associated with different antioxidant responses. In addition, development of analytical techniques to measure the effective dose of LA in different organs that have positive or negative effects would be very useful (Schock *et al.*, 2012; 2013).

In general, it can be stated that the supplements have many properties including antioxidant properties and disposal of free radicals that can boost the immune system in different organisms. It can be concluded that: (1) the beneficial effects of alpha-lipoic acid supplementation in common carp fish depend on its concentration. (2) LA supplement not only does not have a negative effect on the survival of carp, but also improves weight gain and specific growth rates. (3) The selected doses of the present study were safe at least for 8 weeks due to lack of side effects from the perspective of biochemical responses in this species. (4) It can also be concluded that doses of 1 g kg⁻¹ LA and below have better positive effects on the growth factors.

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