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N-3 essential fatty acids in Nile tilapia, *Oreochromis niloticus*: Effects of linolenic acid on non-specific immunity and anti-inflammatory responses in juvenile fish

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ABSTRACT

This study was conducted to investigate the effects of dietary linolenic acid (LNA) on non-specific immunity and anti-inflammatory responses in juvenile Nile tilapia, *Oreochromis niloticus*. Purified ethyl linolenate was added to the basal diet to formulate seven isoproteic and isolipidic diets containing 0.00, 0.10, 0.32, 0.63, 0.98, 1.56 and 2.04% LNA of dry weight, respectively. All diets contained similar amounts of 18:2n-6 (0.61% of dry weight) from purified ethyl linoleate. Basal diet without LNA was considered as a control. Fish were fed experimental diets for 10 weeks. The results showed that amounts of LNA as well as eicosapentaenoic acid, docosahexaenoic acid and even arachidonic acid in head kidney macrophages showed strong positive curvilinear relationships with dietary LNA levels. No significant differences were observed in serum lysozyme and superoxide dismutase activities among dietary treatments ($P < 0.05$). The phagocytic activity of head kidney macrophages increased with the increase of dietary LNA from 0.00 to 0.63% and then decreased as the dietary LNA increased to 2.04% ($P < 0.05$). Macrophages in fish fed the diet with 0.32% LNA showed the highest respiratory burst activity ($P < 0.05$). The expression of pro-inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin (IL) 1β in lipopolysaccharide (LPS)-stimulated macrophages of fish fed diets with 0.32–0.63% LNA significantly reduced compared to those of the control group ($P < 0.05$). In addition, a significant dose-dependent reduction in LPS-induced nitric oxide (NO) production of macrophages was demonstrated by dietary LNA levels. The results suggested that moderate levels of dietary LNA (0.32–0.63% of dry weight) could enhance non-specific immunity and anti-inflammatory responses of juvenile Nile tilapia.

Statement of relevance: To our knowledge, this is the first study to provide specific evidence for the anti-inflammatory effect of linolenic acid in juvenile tilapia. Moreover, this study helps to further confirm the dietary requirement of linolenic acid for juvenile tilapia.

1. Introduction

It has been evident for over 30 years that fatty acids play an important role in the immune and inflammatory processes (Calder, 2008; Meade and Mertin, 1978). Polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 series, especially arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are considered as the precursors for the synthesis of eicosanoids, regulators of cell signaling and gene expression and the most powerful modulators of cell membrane fluidity (Calder, 2006; Yaqoob and Calder, 2007). In mammals, alteration in PUFAs contents of the immune cells was demonstrated to be associated with the alteration of phagocytic capacity (Kew et al., 2003), nitric oxide (NO) production (Aldridge et al., 2008; Ambrozova et al., 2010; Ren et al., 2007), inflammatory cytokine production (Yan et al., 2013) as well as antigen presentation capability (Calder, 2008). These immune-modulatory effects of dietary fatty acids also occurred in farmed fish (Li et al., 2013a; Montero et al., 2010; Xu et al., 2010; Zuo et al., 2012). It has been shown that the composition of dietary fatty acid influenced non-specific immunity (e.g. phagocytosis, respiratory burst and serum lysozyme) (Kiron et al., 2011; Sun et al., 2011; Xu et al., 2010), specific immunity (e.g. antibody production and resistance to pathogens) (Li et al., 2013b; Montero et al., 2010; Thompson et al., 1996; Zuo et al., 2012), eicosanoid production (Ganga et al., 2005; Gjøen et al., 2004; Mourente et al., 2005, 2007) and immune related gene expression

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(Montero et al., 2008, 2010; Seierstad et al., 2009; Zuo et al., 2012) in fish. Decreased production of the classical pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6) were also reported in human and rodent models consuming oils rich in n-3 PUFAs (for references, see Calder, 2006), which indicated that n-3 PUFAs directly (i.e., competitive inhibition of ARA-derived eicosanoid) and/or indirectly (i.e., the activation of transcription factors) suppress the production of inflammatory eicosanoids, cytokines and reactive oxygen species (Calder, 2015). Dietary inclusion of n-3 PUFAs does not always lead to a reduction in expression of pro-inflammatory cytokines, since the impact of n-3 PUFAs on inflammatory processes appears to depend on some other factors including species, age, and the experimental protocols (Yaqoob and Calder, 1995). The expression of TNF-α and IL-1β in gilthead sea bream (Montero et al., 2010) was affected by the alteration of dietary n-3 PUFAs composition, while it did not occur in Atlantic salmon (Seierstad et al., 2009).

Tilapia is the second most widely and successfully farmed species worldwide (Ng and Romano, 2013). Yildirim-Aksoy et al. (2007) reported that serum protein, lysozyme and natural hemolytic complement activity were significantly reduced in Nile tilapia fed beef tallow diet deficient in both n-3 and n-6 fatty acids. But no significant differences in serum immune parameters (total protein, lysozyme and complement) were found in hybrid tilapia fed diets containing various levels of linoleic acid (18:2n-6, LA) and linolenic acid (18:3n-3, LNA) (Li et al., 2013b). In spite of the increase of researches on the role of dietary fatty acids in fish immunity, little is known in tilapia. Therefore, this study aimed to investigate the effects of linolenic acid on non-specific immunity and the production of pro-inflammatory cytokines, an index of anti-inflammatory responses, after exposure to lipopolysaccharide in juvenile Nile tilapia. It will provide more information on knowledge of immune function and application of linolenic acid in tilapia.

2. Materials and methods

2.1. Animals, diets and feeding trial

Nile tilapia (*Oreochromis niloticus*) were obtained from a local hatchery and kept in the feeding facility of South China Agricultural University. After two weeks of acclimation with a basal lipid-free diet, fish with 2.10 ± 0.01 g initial body weight were randomly distributed into 28 tanks (300 l) and each tank was stocked with 30 fish. Fish in quadruplicate tank were randomly assigned to each of the semi-purified experimental diets (approximately 37.5% crude protein and 6.2% total lipid) containing graded levels of LNA (0.00, 0.10, 0.32, 0.63, 0.98, 1.56 and 2.04% of dry weight). All diets contained similar levels of 0.62% LA which was essential for normal growth of Nile tilapia (Takeuchi et al., 1983). The proximate composition and fatty acid composition of the experimental diets were shown in Table 1. The ingredients of the experimental diets were provided in the previous study (Chen et al., 2013), in which a detailed description of the feeding trial was also included. The feeding trial was conducted for 10 weeks.

2.2. Sample preparation

At the end of the feeding trial, fish were fasted for 24 h and anesthetized with MS-222. Blood samples were collected from the caudal vein of six fish per tank with heparinized syringes. The serum was collected as previously described (Xu et al., 2010) and stored at −20 °C for later analysis of lysozyme and superoxide dismutase (SOD) activities.

Head kidney macrophages were prepared as described by Xu et al. (2010) with some modifications. Briefly, the head kidney was aseptically excised and transferred to L-15 medium (Gibco) supplemented with 10 IU ml^{-1} heparin (Sigma), 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma). Cell suspensions were obtained by forcing the tissue with a syringe plunger through a sterile nylon mesh (100 μm), pooled and washed twice, and then carefully layered on 34/51% Percoll (Pharmacia). After centrifugation at 500 ×*g* for 30 min at 4 °C, cells at the 34–51% interface were collected, washed and re-suspended to 1×10^6 cells ml^{-1} with L-15 medium. Cell viability was determined using the trypan-blue dye exclusion method, and was greater than 95% for all assays.

Table 1
Proximate composition and fatty acid composition of the experimental diets.

	Dietary LNA (% of dry weight)						
	0.00	0.10	0.32	0.63	0.98	1.56	2.04
Proximate composition (% dry weight)							
Moisture	9.07	10.18	8.44	9.00	8.82	8.63	8.62
Crude protein	37.95	37.40	37.61	37.74	37.16	37.43	37.34
Total lipid	6.31	6.25	6.35	6.09	5.89	6.28	6.14
Ash	3.27	3.24	3.26	3.22	3.22	3.24	3.10
Fatty acid composition (% total fatty acid)							
10:0	0.37	0.45	0.43	0.42	0.42	0.42	0.38
12:0	68.92	71.75	67.06	61.52	56.03	48.45	41.57
14:0	1.54	1.43	1.51	1.52	1.45	1.36	1.26
16:0	6.95	6.56	6.65	6.61	6.46	6.11	5.67
18:0	3.31	2.92	3.13	3.12	2.94	2.84	2.54
18:1n-9	6.46	5.80	6.18	6.24	6.21	6.11	5.73
18:2n-6	10.21	9.46	10.02	10.21	9.84	9.75	9.63
18:3n-3	nd	1.63	5.03	10.37	16.63	24.89	33.23
(n-3)/(n-6)	0.00	0.17	0.50	1.02	1.69	2.55	3.45

nd: not detected (values < 0.01).

2.3. Analysis of fatty acid profiles in head kidney macrophages

Total fatty acid composition of experimental diets and cell samples from two fish per tank was analyzed by gas chromatography–mass spectrometry (GC–MS) after chloroform/methanol extraction, saponification and methylation with boron trifluoride according to the method of Chen et al. (2013). Fatty acids were identified by retention indices and by comparing of their mass spectra with the NIST 05 spectral database. The relative percentages of individual fatty acids were calculated and expressed as weight percent of total fatty acids.

2.4. Measurement of serum lysozyme and SOD activities

The lysozyme activity in serum was measured using a kit (Nanjing Jiancheng Bioengineering Institute, China), according to the method of Ellis (1990). The assay was based on the lysis of the lysozyme-sensitive Gram-positive bacterium. Hen egg white lysozyme was used as an external standard. The absorbance was measured after 5 s and 125 s using a spectrophotometer (Biophotometer Eppendorf, Eppendorf AG, Hamburg, Germany) at 530 nm. The rate of reduction in absorbance of samples was converted to lysozyme concentration (μg ml^{-1}) using a standard curve.

Serum SOD activity was measured using a kit (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer' instruction. The result of SOD activity was expressed as units per ml serum (U ml^{-1}) and one unit of SOD activity was defined as the amount of superoxide dismutase required to inhibit the reduction of nitroblue tetrazolium by 50%.

2.5. Phagocytosis analysis

Phagocytosis was measured according to the method of Li et al. (2012). Following isolation of head kidney macrophages from four fish per tank as described above, the cells were pooled and seeded into a 24-well flat-bottomed plate at 100 μl/well, four wells each tank. After incubation at 28 °C for 2 h, the cells were washed twice to remove any unattached cells and then the heat-killed opsonized yeasts were added. The ratio of macrophage to yeast in each well was approximately 1:10. Following incubation for 1 h, extracellular yeasts were discarded, and the wells were gently washed three times and then fixed with