INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 4, p. 113-120, 2017

RESEARCH PAPER OPEN ACCESS

Influence of dietary lipid and Vitamin E levels on lipid peroxidation of *Labeo rohita* fingerlings

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Key words: α-tocopherol acetate, SOD, Catalase, Peroxidase, TBARS

http://dx.doi.org/10.12692/ijb/10.4.113-120 Article published on April 28, 2017

Abstract

Increased dietary lipids provide more energy and highly unsaturated fatty acids to fish. However, the fish having high lipid concentrations is at more risk of lipid peroxidation, which is counteracted vitamin E as an antioxidant. A 2×3 factorial experiment under completely randomized design was executed to study the influence of increased lipid and vitamin E levels on peroxidation status of *L. rohita* fingerlings. The status of lipid peroxidation was determined by studying the level of thiobarbituric acid reactive substances (TBARS) and antioxidant enzyme activities. Six experimental diets were made containing two levels of lipids (8 and 16%) and three levels of vitamin E (0, 100, 1000 mg/kg). Upon onset of feeding trial, twenty-five fish were stocked randomly in triplicates against each dietary treatment and fed twice a day. Upon termination of 60 days feeding trial, fish were sacrificed and analyzed for TBARS and antioxidant enzyme activities. Increased supplementation of dietary lipids, increase the TBARS contents in fish body, which were significantly decreased by supplementation of vitamin E. Similarly, increased dietary lipids also increased the activities of superoxide dismutase (SOD), catalase and peroxidase in the whole-body of fish. Supplementation of dietary vitamin E significantly reduced the activities of these enzymes. Moreover, lower values of TBARS and antioxidant enzyme activities were recorded with adequate levels (100 mg/kg) of vitamin E supplementation at 8% lipid level while high dose (1000 mg/kg) of vitamin E showed reduced rate of lipid peroxidation at 16% lipid level. Conclusively, this study suggested an increased requirement of dietary vitamin E to cope the oxidative stress caused by an increased dietary lipids level.

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Introduction

Dietary lipids contain fat soluble vitamins, essential fatty acids, phospholipids and sterols, that provide energy for structural components of cell membranes and maintaining other physiological processes of the body (Watanabe, 1982; Sargent *et al.*, 1999). Among essential fatty acids, n-3 highly unsaturated fatty acids (HUFA) are important component of dietary lipids, used for skeletal formation, maximum growth as well as for survival (Sargent *et al.,* 1999; Tocher and Ghioni, 1999). The oxidative deterioration of n-3 HUFA is termed as lipid peroxidation. Lipid peroxide causes damage to the cell by adversely affecting the n-3 HUFA of cell membranes resulting in oxidative stress (Mourente *et al.,* 2002). For the improvement of aquaculture and fish growth, this type of stress is needed to be reduced.

Vitamin E, α-tocopherol (the most biologically active form of vitamin E), is an important nutritional factordue to its important role in the termination of lipid peroxidation. This fat-soluble vitamin helps in chain breaking and prevents proteins, lipids and cell membranes from oxidative damage by removing free radicals. This vitamin also functions to maintain the tissue integrity in fish including turbot (Tocher *et al.,* 2002), black sea bream (Peng *et al.,* 2009), Atlantic halibut (Lewis-McCrea and Lall, 2007) and gilthead sea bream (Tocher *et al.,* 2002). Vitamin E supplementation (as an antioxidant) in diet is necessary to prevent lipid peroxidation in fish fillets (Bai and Gatlin, 1993).

Proteins are the most promising source of energy in fish compared to carbohydrates and lipids. The protein utilization is nutritionally and economically important for tissue synthesis instead of utilization for energy purposes. The dietary digestible energy is increased by the addition of lipids in fish feed causes protein sparing effect resulting in decreased nitrogen excretion in aquatic environment (Cho and Kaushik, 1990). Hence, feed efficiency and growth of fish is improved by increasing lipid levels in fish diet (Hardy, 1999). The lipid concentration is increased in tissues by the supplementation of lipids in the diet, ultimately enhancing the degradation of lipids. Vitamin E is utilized at higher rate when the degradation of lipids increases in high-lipid diets. Schwarz *et al.* (1988) described an increased vitamin E requirement with the increase in level of dietary poly unsaturated fatty acids (PUFA). As the dietary lipids level increases from 5% to 12%, dietary vitamin E requirement also increases from 40-44 to 60-66 mg/kg (Shiau and Shiau, 2001).

Keeping in view the merits and demerits of use of dietary lipids and role of vitamin E supplementation in reducing lipid peroxidation the present experiment was executed to study the influence of dietary concentrations of lipids and vitamin E and their interaction on lipid peroxidation in fish.

Materials and methods

This feeding experiment was carried out in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan.

Experimental design

Six isonitrogenous (34%) experimental diets were formulated by supplementing the basal diet with two levels of cod liver oil (8, and 16% diet) and three levels of vitamin E (0, 100, 1000 mg/kg diet) at each level of fish oil (2×3 factorial experiment under CRD). Vitamin E was supplemented in the form of DL-α-tocopherol acetate and was obtained from Sigma-Aldrich. All of the added vitamin E, α-tocopherol acetate was mixed with the cod liver oil prior to addition to experimental diets. Each experimental diet was fed in triplicates for 60 days.

Experimental diets

The ingredient composition of experimental diets is given in Table 1. Dietary feed ingredients (fishmeal, soybean meal, wheat flour and rice polish) were purchased from local market and analyzed chemically by following AOAC (1995) before the preparation of experimental diets. Dry ingredients were ground and sieved (0.05 mm) in cereal grinding machine (FFC-45, JIMO, China).

Feed ingredients, vitamin E free vitamin premix, mineral mixture and choline chloride (Unichem Laboratories) were thoroughly mixed in an electric mixer before the oil was added. After the addition of required level of Cod liver oil (Poultry-vet Co, Nazimabad, Karachi, Pakistan), approximately 150 ml of distilled water/kg of diet was added. The moist mixture was extruded through hand pelletizer with a 3-mm diameter die. The resulting moist pellets were dried at room temperature using an electric fan to a moisture content of approximately 10%. Pellets were ground into small pieces, sieved to obtain appropriate sizes, and stored frozen in self-sealing plastic bags at −18 °C before and throughout the feeding trial. The proximate composition of experimental diets was determined following AOAC (1995). All of the experimental diets were isonitrogenous, isolipidic and isocaloric.The chemical composition of each experimental diet is given in Table 2.

Experimental fish and rearing conditions

Physically healthy *L. rohita* fingerlings were obtained from Government Fish Seed Hatchery, Faisalabad, Pakistan, and transported alive to the Fish Nutrition Laboratory. Prior to the initiation of feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks in cemented tanks (1000 L) while feeding a basal diet. At the onset of feeding trial twenty-five fish were stocked randomly into triplicate tanks (70-L) for each dietary treatment with near uniform biomass (initial body weight 3.33 ± 0.03 g). Fish were fed the test diets to satiation, 6 days a week. The daily ration was divided into two, and fed to the fish at 08:00 and 16:00 h. The fish were weighed every 2 weeks and their ration adjusted accordingly. After the feeding session of two hours the tanks were washed and water was exchanged with filtered fresh water. Round the clock aeration was provided through capillary system to all the experimental tanks. During experimental period the dissolved oxygen and pH were monitored constant throughout the experimental duration with the help of HANNA DO meter (model HI 9147) and AMPROBE pH meter (model WT-80), which were regulated between 5.8-7.3 mg/L and 7.4-8.6 respectively. The water temperature ranged from 24.9-28.7°C throughout the trial.

Sample collection and chemical analyses

At the termination of feeding trail, fish were starved for 24 h, anesthetized using MS-222 (Sigma-Aldrich) and sacrificed. Five fish from each tank were randomlycollected, minced, pooled, homogenized and utilized for analysis of TBARS and antioxidant enzyme activities. The measurement of body TBARS was carried out using a method adopted by Gatta *et al.* (2000). Enzyme extract for antioxidant enzyme assays was prepared by homogenizing whole body sample in phosphate buffer of pH 6.5 (1:4 w/v) and homogenates were centrifuged at 10,000 rpm and 4°C. After centrifugation process, clear supernatants were processed for enzyme assays. The activity of SOD was determined by measuring its ability to inhibit the photo reduction of nitroblue tetrazole (NBT) following the method of Giannopolitis and Ries (1977). Catalase activity was determined by its ability to decrease the H_2O_2 concentration at 240 nm (Chance and Mehaly, 1955). The activity of peroxidase was determined by measuring its ability to reduce the concentration of H_2O_2 at A_{470} nm (Civello *et al.*, 1995).

Statistical analysis

Statistical analyses were performed using CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA). Data are presented as means and pooled standard error (PSE). The data were analyzed for variance using two-way analysis of variance. Multiple comparisons among mean values were made with Student-Newman-Keul's test. Differences among treatments were considered significant at a probability level of $p<0.05$.

Results

Thiobarbituric acid reactive substances (TBARS)

Main and interaction effects of lipids and vitamin E on TBARS contents in whole body of *L. rohita* are given in Table 3. Fish fed on 8% lipid diets showed lower levels of TBARS compared to fish fed 16% lipids. Significant differences were also apparent between lower and higher levels of vitamin E supplementation. In 8% lipid supplemented groups, supplementation of vitamin E at the level of 100 mg/kg showed lower values of TBARS compared to

high dose (1000 mg/kg) of vitamin E,while reverse was observed in 16% lipid supplemented diets. Interaction effect showed a significantly (p<0.05) enhanced vitamin E requirement with increased lipid level.

Table 1. Composition of experimental diets.

Experimental Diets	LE1	LE2	LE3	LE4	LE5	LE6
Fishmeal	40	40	40	40	40	40
Soybean meal	25	25	25	25	25	25
Wheat flour	13	13	13	13	13	13
Rice polish	11.7	11.7	11.7	3.7	3.7	3.7
Cod liver oil[1]	8	8	8	16	16	16
Mineral mixture[2]	1	1	1	1	1	1
Vitamin premix (E free)[3]	1	1	1	1	1	1
Choline Chloride	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin E (mg/kg)[4]	0	100	1000	0	100	1000

[1]Cod liver oil was purchased from Poultry-vet Co, Nazimabad, Karachi, Pakistan.

[2] Each kg of mineral mixture contains: $CaCO_3$, 316; KH_2PO_4, 479; $MgSO_4.7H_2O$, 153; NaCl, 51; $CoCl.6H_2O$, 0.0816; Ammonium molybdate, 0.061; $AlCl_3.6H_2O$, 0.255; $ZnSO_4.7H_2O$, 121.33; $CuSO_4.5H_2O$, 210.67; $MnSO_4.5H_2O$, 116.67; $FeSO_4.H_2O$, 100.67.

[3]Each kg of Vitamin premix contains; Vitamin A (Retinoic acid) 5.0 g, Vitamin B1 (Thiamine) 0.5 g, Vitamin B2 (Riboflavin) 3.0 g, Vitamin B3 (Niacin) 5.0 g, Vitamin B6 (Pyridoxine) 1.0 g, Vitamin B7 (Biotin) 0.05 g, Vitamin B9 (Folic acid) 0.18 g, Vitamin B12 (Cobalamin) 0.002 g, Vitamin C (Ascorbic acid) 5.0 g, Vitamin D3 (Cholecalciferol) 0.002 g, Choline 100 g, Cellulose 815.26 g.

[4]Vitmain E was supplemented in the form of DL-α-toopherol acetat (Sigma-Aldrich).

Antioxidant enzyme activities

Effect of dietary treatments on antioxidant enzyme activities on *L. rohita* fingerlings is given in Table 4. Fat supplementation increased the antioxidant enzyme activities in whole body of fish. Similar to TBARS level, vitamin E supplementation reduced the activities of these enzymes in whole body at both levels of fat supplementation. Lower values of these enzymes were recorded with adequate levels of vitamin E supplementation at 8% lipid level while high dose of vitamin E showed reduced levels of these enzymes at 16% lipid level. Both dietary supplements showed a clear, significantly positive interaction between them.

Table 2. Proximate composition of experimental diets.

Lipids level (%)	Vitamin E level (mg/kg)	Experimental Diets	Dry matter (%)	Crude protein (%)	Crude fat (%)	Gross energy (kcal/kg)	α-tocopherol (mg/kg)
8%	0	LE1	90.95	34.08	10.84	4307.06	18.53
	100	LE2	90.85	33.9	10.91	4103.33	109.03
	150	LE3	90.83	34.26	11.05	4101	956.5
16%	0	LE4	90.94	34.15	18.01	4514	18.7
	100	LE5	91.03	33.99	18.01	4507.33	102.5
	150	LE6	90.9	33.87	17.93	4522.33	927.43

Discussion

Thiobarbituric acid reactive substances (TBARS)

Vitamin E as an antioxidant possess the ability to protect the unsaturated fatty acids containing biological membranes and diets against free radicals (Huang and Huang, 2004). The lipid peroxidation of unsaturated fatty acids, present in the diet or in tissues, ultimately degrade them into melondialdehyde (MDA) and other small aldehyde groups. Measurement of MDA serve as an strong index for the determination of extent of Lipid peroxidation. The most common method used to measure MDA is thiobarbituric acid-reactive substances (TBARS) assay.

Table 3. Effect of dietary lipids and vitamin E levels on TBARS of *L. rohita* fingerlings.

Lipids level (%)	Vitamin E level (mg/kg)	Experimental diets	TBARS
8%	0	LE1	2.65^{b}
	100	LE2	2.42^{d}
	150	LE3	2.55^{c}
16%	0	LE4	2.83^{a}
	100	LE5	2.63^{b}
	150	LE6	2.40^{d}
PSE			0.019
ANOVA			
Lipids			$p<0.05$
Vitamin E			$p<0.05$
Lipids × Vitamin E			$p<0.05$

The data are mean of three replicates

Mean values sharing different superscript letters within a row are significantly different ($p<0.05$)

PSE= Pooled standard error = √MSE/n (where MSE= mean-squared error)

TBARS= Thiobarbituric acid-reactive substances (mg/g protein).

In the present study, higher TBARS level were observed in whole body of fish fed 16% lipid compared to 8% lipid diet. Higher dietary lipid level might have caused the higher deposition of fat in the tissues which provide more chances of peroxidation. Similar increase in TBARS level by increasing dietary fat level have also been reported in *Onchorhynchus mykiss* fed 15 to 30% fat (Chaiyapechara *et al*., 2003) and *Epinephelus malabaricus* fed 4 to 9% fat (Lin and Shiau, 2005). In the present study, an inverse relationship between TBARS values and dietary vitamin E levels was observed, which agrees with the findings of studies on Atlantic halibut (Ruff *et al*., 2002), sea bass (Gatta *et al*., 2000), grouper (Lin and Shiau, 2005), rohu (Sau *et al*., 2004), hybrid tilapia (Huang and Huang, 2004), red drum (Li and Gatlin, 2009), juvenile cobia (Zhou *et al*., 2013) and black sea bream (Peng *et al*., 2009). Reduced TBARS level can be attributed to antioxidant behavior of vitamin E.

In the current study, the interactions between dietary lipid and vitamin E levels were found significant. Lipid supplementation at the level of 8% showed decreased TBARS contents with 100 mg/kg vitamin E, whereas, 16% lipid supplementation required 1000 mg/kg vitamin E. Higher oxidative stress at higher lipid level may require higher dietary vitamin E supplementation.

Antioxidant enzyme activities

Dietary lipid levelsignificantly affects the enzyme activities of the antioxidant defense system of fish. Higher dietary lipid supplementation increases the probability of lipid deposition leading to increased peroxidation in fish tissues. The increased TBARS activities, in the present study, are also an evidence of increased lipid peroxidation. Antioxidant defense system enhanced its enzymes (SOD, catalase and peroxidase) activities to cope with the stress.

In both dietary lipid levels, fish fed vitamin E deficient diet resulted in higher antioxidant enzymes activities. In 8% lipids containing diet, vitamin E supplementation in diet at the level of 100 mg/kg showed reduced activities of antioxidant enzymes. However, at the same lipid level, high vitamin E supplementation (1000 mg/kg) caused higher activities of antioxidant enzymes. The increased levels of enzyme activities indicate that vitamin E may act as pro-oxidant at the level of 1000 mg/kg rather than acting as an antioxidant. While, fish fed 16% lipid diet with 100 mg/kg vitamin E showed significantly increased enzyme activities compared to fish group feeding on 1000 mg/kg with same lipid level.

Table 4. Effect of dietary lipids and vitamin E levels on antioxidant enzyme activities of *L. rohita* fingerlings.

Lipids level (%)	Vitamin E level (mg/kg)	Experimental diets	SOD	Catalase	Peroxidase
8%	0	LE1	3.79^{b}	69.45^{b}	85.85^{a}
	100	LE2	2.50^{e}	60.46^{d}	73.99^{c}
	150	LE3	2.94^{d}	65.61^{c}	79.95^{b}
16%	0	LE4	4.62^{a}	73.68^{a}	85.56^{a}
	100	LE5	3.55^{c}	69.30^{b}	80.61^{b}
	150	LE6	2.41^{e}	59.93^{d}	81.10^{b}
PSE			0.062	0.459	0.543
ANOVA					
Lipids			$p<0.05$	$p<0.05$	$p<0.05$
Vitamin E			$p<0.05$	$p<0.05$	$p<0.05$
Lipids × Vitamin E			$p<0.05$	$p<0.05$	$p<0.05$

The data are mean of three replicates.

Mean values sharing different superscript letters within a row are significantly different ($p<0.05$).

PSE= Pooled standard error = √MSE/n (where MSE= mean-squared error).

SOD= Superoxide dismutase (Units/min/mg protein); Catalase= (Units/min/mg protein); Peroxidase= (mUnits/min/mg protein).

This shows that moderate vitamin E level (100 mg/kg) was insufficient to cope with the oxidative stress in fish at higher lipid level. Similar observations have also been reported in rainbow trout (Puangkaew *et al*., 2004), sea cucumber, *Apostichopus japonicus* (Wang *et al*., 2015), grass carp (Wang *et al*., 2015), halibut and sea bream (Tocher *et al*., 2002).

Conclusively, this study suggests that increasing the lipid supplementation level increases the need of vitamin E supplementation to achieve adequate protection against oxidative stress.

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