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Effect of megalevels of dietary vitamin C on the immune response of channel catfish *Ictalurus punctatus* in ponds

P. R. Liu, J. A. Plumb, M. Guerin, R. T. Lovell

Department of Fisheries and Allied Aquacultures and Alabama Agricultural Experiment Station Auburn University, Alabama 36849, USA

ABSTRACT: Channel catfish *Ictalurus punctatus* fingerlings in ponds were fed diets containing 0, 100, 500, 1000 or 4000 mg of vitamin C kg^{-1} of diet for 9 wk. They were then immunized with *Edwardsiella ictaluri*. One month after immunization, they were challenged with virulent *E. ictaluri*. Fish showed no clinical signs of vitamin C deficiency during the experimental period and final weights of the fish on the various diets were not significantly different ($p > 0.05$). Increasing the level of vitamin C in the diet did not affect complement hemolytic activity and antibody titers. However, fish receiving vitamin C at 1000 mg kg^{-1} showed increased resistance to *E. ictaluri*. These fish exhibited a 100-fold LD_{50} compared to fish receiving a diet without supplemented vitamin C.

INTRODUCTION

Vitamin C is an essential component of the diet for proper health and growth of cultured channel catfish *Ictalurus punctatus*. The amount of vitamin C required in the diet for growth is from 30 to 90 mg kg^{-1} of diet (Lovell 1973, Andrews & Murai 1975, Lovell & Lim 1978, Li & Lovell 1985). It has been shown that a diet deficient in vitamin C will result in injury to the spine of catfish causing 'broken back' (Lim & Lovell 1978). Increased levels of vitamin C (megadoses) in the diet have been shown to enhance disease resistance against the bacterial pathogens *Edwardsiella ictaluri* and *E. tarda* of catfish held in aquaria (Durve & Lovell 1982) and of salmonids (Blazer 1982, Navarre 1985). Specific antibodies against *E. ictaluri* and serum complement activity were suppressed in channel catfish fed a vitamin C-free diet while in aquaria, and antibody titers and complement activity were significantly higher when fish were fed doses of vitamin C that were much higher than the normal requirement (Li & Lovell 1985). The present study tested the effects of megalevels of dietary vitamin C on the immune response of channel catfish in ponds.

MATERIALS AND METHODS

Channel catfish *Ictalurus punctatus* averaging 21.8 g were stocked into six 0.04 ha earthen ponds at a density of 450 $pond^{-1}$. After 3 wk acclimation, fish were fed 6 experimental diets which contained 0, 100, 500, 1000, 2000 or 4000 mg of supplemental vitamin C kg^{-1} (Table 1).

The diets were prepared by mixing the ingredients with 6 % water and pelleting mixtures into 4 × 15 mm sinking dry pellets. To compensate for predicted losses during processing of the diets, 15 % more vitamin C than the desired level was added to the formula. The pellets were then dried for 3 h, sealed in plastic bags and immediately frozen at −20 °C. Each day's allowance was removed from storage 1 h before feeding and brought to ambient temperature. Fish were fed once daily, 7 d wk^{-1} for 9 wk at 16:00 h at a rate of 3 % of the body weight. Afternoon water temperature was 18 °C at the beginning of the vitamin C feeding period and reached 25 °C at the end of the 9 wk period.

Edwardsiella ictaluri bacterin was prepared by procedures described by Saeed & Plumb (1986). After 9 wk feeding, 120 fish treatment group were given a single

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Table 1. Composition of basal diets to which 6 levels of vitamin C were added. Proximate analysis: crude protein = 36.2 %; crude fat = 2.0 %; crude fiber = 4.5 %; ash = 8.2 %; moisture = 11.8 % (Guerin & Lovell in press). Feed was stored at −20 °C until used. Vitamin mix supplied the following activites per kg of diet: vitamin A = 5228; vitamin D3 = 2409; vitamin E = 50; menadione = 1.5; thiamin = 5.5; riboflavin = 8.8; pyridoxine = 5.5; pantothenic acid = 26.5; niacin = 110; folic acid = 4; vitamin B_{12} = 0.03; choline = 800; ethoxyquin = 55; crude protein = 35.2; crude fat = 2.9; crude fiber = 4.5; ash = 8.2; moisture = 11.8 (R. T Lovell, Auburn University, pers. comm.). Note, corn meal decreased as vitamin C increased

Ingredients	Percent
Fish meal (menhaden)	12.0
Soybean meal	56.0
Corn meal	27.5–27.9
Molasses	2.0
Bentonite	1.0
Vitamin mix (vitamin C-free)	0.5
Vitamin C	0.0–0.4

intraperitoneal injection of 0.1 ml $fish^{-1}$ (10^6 cells). Immunized fish were transferred to six 7 m^3 concrete tanks where each group continued to receive its respective experimental diet at the same rate, but now 3 × wk^{-1} for an additional 4 wk. The average water temperature was 27.5° ± 2.6 °C, and dissolved oxygen was 10.2 ± 3.0 mg l^{-1}.

Blood samples were taken from 25 immunized fish from each treatment 4 wk after vaccination. The blood from 5 fish was pooled to form a single sample, held at room temperature for 1 h, and then overnight at 4 °C. Serum was extracted and stored at −20 °C until antibody titration, which was performed within 1 wk of sampling. Antibody titers were determined by the staining microagglutination technique in 'U' bottom microtiter plates (Eurell et al. 1977) using serial 2-fold dilutions of sera from 1:2 to 1:2048. The concentration of antigen was 10^6 cells ml^{-1}. The antigen was stained with 1 % neotetrazolium chloride (Sigma Chemical Company).

The procedure for titration of complement activity in serum of fish was similar to that described by Garvey et al. (1979). Sheep erythrocytes were washed 3 times with modified barbital buffer and adjusted spectrophotometrically to 1 % suspension. Total complement hemolytic (CH) activity was reported in CH_{50} units ml^{-1}.

Vaccinated fish from each vitamin C treatment were divided into 8 groups of 10 fish each and transferred to 8 aerated 40 l aquaria (static) at 24 °C where fish were acclimated for 1 wk. Fish in 6 aquaria from each vitamin C-vaccinated treatment group were injected with virulent *Edwardsiella ictaluri* 0.1 ml $fish^{-1}$, at concentrations of 10^4, 10^6 or 10^8 cells 0.1 ml^{-1} (2 aquaria per concentration) and a lethal dose −50 % end point (LD_{50}) calculated. Fish in 2 aquaria of each treatment were injected with the same volume of sterile saline solution.

Weight, antibody titer, and complement activity were compared using Student's t- and Fisher-tests. Challenge mortalities and LD_{50} were analysed for significant differences among treatments by probit analysis.

RESULTS

After being fed experimental diets containing the various levels of vitamin C for 9 and 13 wk, there was no difference ($p > 0.05$) in growth (Table 2), and vitamin C-deficiency signs were not observed even among

Table 2. *Ictalurus punctatus*. Mean weights (± 95 % confidence limits) of channel catfish fed 6 levels of vitamin C after 9 and 13 wk (g $fish^{-1}$). Means not significantly different ($p > 0.05$) at either time period. Mean initial weight was 21.8 g kn group. Vit C: dietry vitamin C, mg kg^{-1} of diet; n: no of fish

Vit C	Pre-vaccination		Post-vaccination	
	n	Wk 9	n	Wk 13
0	28	60.5 ± 5.0	10	66.8 ± 6.8
100	28	50.3 ± 5.9	10	70.9 ± 13.5
500	28	58.7 ± 5.9	10	72.5 ± 5.0
1000	28	61.3 ± 6.6	10	67.0 ± 10.2
2000	28	57.8 ± 4.2	10	71.4 ± 12.0
4000	28	52.1 ± 3.9	10	64.3 ± 5.0

fish fed the vitamin C-free diet. However, when moved from ponds to tanks at 9 wk, fish fed the diet without supplemental vitamin C experience a 13 % mortality within a 4 wk period. No mortality was observed in any fish fed supplemental vitamin C. This suggested that the vitamin C deficient fish were more susceptible to handling stress than those receiving vitamin C. However, without replication this could not be confirmed statistically.

Agglutinating antibody titers against *Edwarsiella ictaluri* in sera of fish from the 6 vitamin C treatments 4 wk after vaccination were ca 1:30, and there were no differences ($p > 0.05$) among the treatment groups. Before immunization, all fish had 1:4 detectable agglutinating antibody titer against *E. ictaluri*. There were no differences in the complement hemolytic activities among the various treatment groups ($p > 0.05$) (Table 3). The mean was 20.89 CH_{50} ml^{-1} units, which was not different from the level in normal fish reported by Legler et al. (1967).

The 10 d *Edwardsiella ictaluri* LD_{50} increased with greater concentrations of vitamin C in the diet (Fig. 1). The most dramatic increase occurred at vitamin C

Table 3. *Ictalurus punctatus*. Agglutination antibody titers and complement hemolytic activity (CH_{50} ml^{-1}) (± 95 % confidence limits) of channel catfish 4 wk after immunization with formalin-killed *Edwarsiella ictaluri* and following 13 wk on a diet with one of 6 dietary levels of vitamin C. Means not significantly different ($p > 0.05$). Vit C: dietary vitamin C, mg kg^{-1} of diet; n: no. of serum samples, 5 pools of sera with each pool containing sera of 5 fish

Vit C	n	Titer	
		Agglutination	Complement
0	5	30.4 ± 3.6	23.4 ± 0.8
100	5	32.0 ± 9.3	20.1 ± 0.5
500	5	32.0 ± 13.2	20.4 ± 1.2
1000	5	28.0 ± 14.6	20.7 ± 3.3
2000	5	32.0 ± 16.2	20.6 ± 2.5
4000	5	32.0 ± 13.2	22.7 ± 1.3

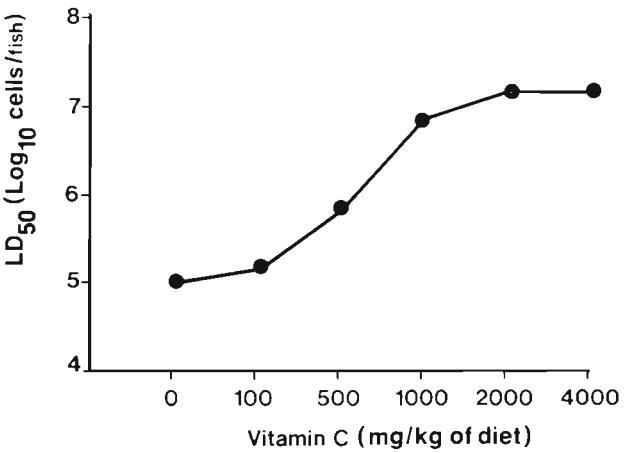

Fig. 1 *Ictalurus punctatus*. Relationship between LD_{50} and dietary supplemental vitamin C levels in channel catfish challenged with *Edwardsiella ictaluri*. LD_{50} values for diets containing 0 to 100 or 1000 to 4000 mg vitamin C kg^{-1} are not significantly different, $p = > 0.05$; LD_{50} values for diets containing 100 to 500 mg vitamin C kg^{-1} are significantly different, $p = < 0.05$

levels between 100 and 1000 mg kg^{-1} of diet. Concentrations above 1000 mg kg^{-1} diet did not increase the LD_{50} appreciably. None of the saline injected fish died.

DISCUSSION

Fish fed the diet not supplemented with vitamin C grew normally and did not show any clinical signs of vitamin C-deficiency during the 9 wk growing period in the ponds. This might be attributed to acquisition of vitamin C from natural sources in the pond. Guerin (1986) measured the vitamin C in serum, liver, and head kidney of the fish used in this experiment when fish were removed from the pond and vaccinated. The serum of fish receiving no supplemental vitamin C contained 4 mg vitamin C g^{-1}, whereas the liver and head kidney had 43 and 122 mg g^{-1}, respectively. According to Guerin (1986), fish that were fed 500 mg of vitamin C kg^{-1} of feed, did have significantly elevated vitamin C in the liver, head kidney, and serum, but tissue levels did not increase with higher doses of vitamin C. For example, the concentration of vitamin C in the kidney relative to the amount fed was: 190 µg g^{-1} tissue with 100 mg of vitamin C kg^{-1} of diet and 353 µg g^{-1} tissue with 500 mg of vitamin C kg^{-1} diet. Fish receiving 1000, 2000, and 4000 mg of vitamin C kg^{-1} of diet yielded 313, 366, and 317 µg of vitamin C per g of kidney, respectively.

It was reported by Navarre (1985) that rainbow trout *Oncorhyncus mykiss* increased their disease resistance with increasing levels of supplemental vitamin C in their diet. The LD_{50} of *Edwardsiella ictaluri* in channel catfish increased nearly 100-fold from 10^5 cells 0.1 ml^{-1} at 0 mg vitamin C to ca 10^7 cells 0.1 ml^{-1} in fish receiving 1000 mg of vitamin C kg^{-1} of diet (Fig. 1). Higher supplemental application of vitamin C (2000 and 4000 mg kg^{-1} of diet) did not further increase the LD_{50}.

Impaired antibody response and reduced complement activity caused by vitamin C deficiency have been reported in guinea pigs (Delafuente & Panush 1980). Megadose feeding of vitamin C (50 to 100 times the normal requirement for growth and prevention of signs of vitamin C deficiency) to small channel catfish in aquaria significantly increased antibody production and complement titers (Li & Lovell 1985). In the present experiment, there were no correlations between the level of the humoral immune responses or complement titers and megadose levels of vitamin C. Prinz et al. (1980) reported that peak antibody titers associated with elevated vitamin C were both higher and occurred earlier in the human primary immune response, but not in the secondary response. In our experiment, a 1:4 pre-exposure antibody titer against *Edwardsiella ictaluri* was found in all fish. This indicates that fish had probably been exposed to the pathogen prior to immunization. Thus, our attempts at immunizing catfish may have triggered only the secondary immune response – a response that, in catfish, may not be sensitive to vitamin C either

The lack of a positive effect of megadoses of vitamin C on complement activity does not agree with the findings of Li & Lovell (1985) and Navarre (1985). This discrepancy may have been caused by 2 factors: (a) natural food in ponds may have provided sufficient vitamin C for fish; or, (b) the effect of vitamin C on complement might be regulated by a 'critical tissue level', Guerin (1986) demonstrated that non-vitamin C supplemented fish did have vitamin C in their tissues.

Regardless of the levels of vitamin C provided for fish, the same level of complement titer might be produced if the 'critical tissue level value' is satisfied.

The results of this study indicate that vitamin C does affect the defense mechanisms in channel catfish held in ponds. However, antibody and complement activities were not influenced. The defense mechanisms affected might, therefore, have been cellular in nature. The findings further suggest that channel catfish in open ponds need only be fed vitamin C at intermediate levels.

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