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Effects of dietary ascorbic acid deficiency on *Cryptobia salmositica* infection and on vaccination against cryptobiosis in *Oncorhynchus mykiss*

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ABSTRACT: Eight groups of *Oncorhynchus mykiss* were fed diets either lacking or supplemented with ascorbic acid (AA, 500 mg kg^{-1} diet) for 10 wk prior to vaccination against cryptobiosis. The concentrations of AA in livers and kidneys of the fish correlated with those of the exogenous dietary AA. Fish were vaccinated intraperitoneally with 100 000 attenuated *Cryptobia salmositica* per fish. They were challenged with 100 000 virulent *C. salmositica* per fish 4 wk post-vaccination. Both vaccinated AA-deficient and AA-supplemented fish were protected while unvaccinated controls had high parasitaemias and cryptobiosis (e.g. anaemia, abdominal distension with ascites) after being challenged with the pathogen. AA-deficiency did not significantly affect titres of complement fixing antibodies (CFAb) in vaccinated and vaccinated/challenged fish. However, detectable CFAb was delayed 1 wk in vaccinated and vaccinated/challenged fish fed the AA-deficient diet. Also the parasitaemias in infected and vaccinated/challenged AA-deficient fish were consistently lower than those in AA-supplemented fish. This indicates that AA may have directly and indirectly promoted more rapid multiplication of the virulent parasite.

KEY WORDS: *Cryptobia salmositica* · Ascorbic acid · Vaccination · Cryptobiosis · Protection

INTRODUCTION

Cryptobia salmositica is a pathogenic haemoflagellate found in *Oncorhynchus* spp. in freshwater streams in western North America (Woo 1987, 1994). The parasite causes cryptobiosis and mortality in naturally and experimentally infected rainbow trout *Oncorhynchus mykiss* (Wales & Wolf 1955, Woo 1979). Some of the clinical signs are exophthalmia, abdominal distension with ascites, general oedema, splenomegaly, anaemia (Woo 1979), immunodepression (Jones et al. 1986) and anorexia (Li & Woo 1991b, Thomas & Woo 1992). The virulent *C. salmositica* was attenuated by *in vitro* culture and was used as a live vaccine against experimental cryptobiosis in rainbow trout (Woo & Li 1990, Li & Woo 1995).

The effects of dietary ascorbic acid (AA) on the immune response in fish are controversial. Dietary AA-deficiency depressed cellular (Anderson et al. 1980) and humoral responses in channel catfish *Ictalurus punctatus* and rainbow trout to *Vibrio anguillarum* infection (Durve & Lovell 1982, Navarre & Halver 1989). A megadose of dietary AA in rainbow trout and channel catfish significantly enhanced humoral (Navarre & Halver 1989) and cellular immune responses as well as complement haemolytic activity (Li & Lovell 1985). However, Lall et al. (1989), Hardie et al. (1991) and Li et al. (1993) were unable to confirm the immunodepressive effects of dietary AA-deficiency either on Atlantic salmon *Salmo salar* infected with *V. anguillarum* and *Aeromonas salmonicida* or on channel catfish infected with *Edwardsiella ictaluri*.

The aims of the present study were to examine the effects of dietary AA on: (1) *Cryptobia salmositica* infection in *Oncorhynchus mykiss*; (2) protective immunity in rainbow trout vaccinated against cryptobiosis and the production of complement fixing antibodies.

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MATERIALS AND METHODS

A total of 84 laboratory-raised rainbow trout (229 ± 57.2 g) maintained in continuously aerated well water (12 ± 3°C) were divided into 2 groups, each with 42 fish. Average weights were not significantly different. One group was fed once daily to satiety with a diet deficient in AA (Table 1) and the second group was fed with AA-supplemented diet (500 mg AA per kg diet). After fish had been on their respective diets for 10 wk, AA concentrations in livers and kidneys of 2 fish (each with 4 replications) from each group were measured using High Performance Liquid Chromatography (Wang et al. 1988). Each group was then further divided into 4 subgroups of 10 fish, i.e. vaccinated, vaccinated/challenged, infected and uninfected naive subgroups. Each fish in vaccinated and vaccinated/challenged subgroups on both diets was vaccinated intraperitoneally (ip) with 100 000 attenuated *Cryptobia salmositica* while fish in other subgroups (infected and uninfected) were inoculated with Ringer's saline (RS). After 4 wk post-vaccination (wpv), AA concentrations in livers and kidneys of 2 fish (each with 4 replications) from each of uninfected naive subgroups on both diets were measured. Each fish in vaccinated/challenged and infected subgroups was challenged ip with 100 000 virulent parasites while fish in vaccinated and uninfected naive subgroups were inoculated with RS. Fish blood (0.5 ml) was withdrawn weekly from the caudal vein following anaesthetization with tricaine methanesulfonate (MS-222), 1:10 000; heparin (0.1 g per 10 ml of RS) was used as anti-coagulant.

Table 1. Composition of ascorbic acid deficient diet. The diet contained 17 MJ digestible energy per kg and 22 g digestible protein per MJ digestible energy. CP: crude protein

Ingredients	%
Fish meal, 68% CP	30.0
Corn gluten meal, 60% CP	17.0
Soybean meal, 48% CP	13.0
Wheat middings, 17% CP	16.5
Whey, dried, 12% CP	10.0
Vitamin premix (free of ascorbic acid)[a]	1.0
Mineral premix[b]	0.2
Sodium chloride	0.3
Fish oil[c]	12.0

[a]Supplied, mg per 100 g diet except as noted: retinyl acetate, 250 International units (IU); cholecalciferol, 240 IU; dl-α-tocopheryl acetate, 9 IU; menadione sodium bisulfite, 2; biotin, 0.02; cyanocobalamin, 0.002; choline chloride, 100; folic acid, 1; niacin, 20; calcium D-pantothenate, 7; pyridoxine HCl, 1.5; riboflavin, 1; thiamin HCl, 2

[b]Supplied, mg per 100 g diet: $CuSO_4$ $5H_2O$, 2.5; $FeSO_4$ $7H_2O$, 6.3; $MnSO_4$ H_2O, 8.6; KI, 0.8; $ZnSO_4$ H_2O, 14.4.

[c]Deaerated with nitrogen and stabilized with 0.05% ethoxyquin

The haematocrit centrifuge technique (Woo 1969, Woo & Wehnert 1983) was used to detect the infection in fish during the first and second week after infection. Packed cell volumes (PCV; Woo 1979) and parasitaemias (Archer 1965) were measured and complement fixing antibodies (CFAb) were determined using the *in vitro* lysis test (Li & Woo 1995). Briefly, fish antiserum was heat-inactivated at 45°C for 30 min (Sakai 1981) and 25 µl of the heat-inactivated antiserum was dispensed in one well in a microtitre plate which was kept on ice. The antiserum was diluted by 2-fold serial dilution with Dulbecco's Phosphate Buffered Saline (PBS, GIBCO) and incubated with 25 µl of PBS containing approximately 200 parasites and 25 µl of fresh rainbow trout complement for 3 h at 10°C. The titre of CFAb was the maximum antiserum dilution at which all the parasites were lysed. All fish were cared for in accordance with the provisions of the University of Guelph Animal Care Committee.

Differences in mean values of AA concentration in tissues, PCV, parasitaemias and titres of CFAb were analyzed using Statistics Analysis System procedures. The data were analysed using the *t*-test and *F*-test at $p \leq 0.05$.

RESULTS

AA concentrations and parasitaemias

The concentrations of AA in the livers and kidneys of uninfected naive fish fed diet supplemented with AA were significantly higher than those of fish fed diet deficient in AA at 10 and 14 wk after the start of the study (Table 2). Using the haematocrit centrifuge technique, parasites were detected in the blood of infected controls given both diets at 2 wk post-infection (wpi). The parasitaemias increased rapidly and were significantly higher than those in vaccinated and vaccinated/challenged fish. Mean parasitaemias in the infected controls were consistently lower in AA-deficient fish than in AA-supplemented fish. The difference was significant ($p \leq 0.05$) at 4 wpi (Table 3). Similarly, after challenge with virulent parasites, parasitaemias in vaccinated/challenged fish fed with AA-deficient diet were consistently lower than those with AA-supplemented diet and the different parasitaemias between diets were significant at 5 wk post-challenge (wpc). Mean parasitaemias between vaccinated and vaccinated/challenged fish were not significantly different, but they were significantly lower than those in infected controls on both diets. No parasite was found in the uninfected naive controls.

Table 2. Ascorbic acid (mean ± SE) (n = 4) stored in livers and kidneys of uninfected naive rainbow trout *Oncorhynchus mykiss* fed ascorbic acid deficient or ascorbic acid supplemented diets

Tissue samples	Time on diets (wk)	Ascorbic acid (μg g^{-1} tissue)	
		Ascorbic acid deficient diet	Ascorbic acid supplemented diet
Liver	10	17.3 ± 13.8	87.3 ± 26.3*
	14	9.4 ± 0.9	119.3 ± 23.8*
Kidney	10	17.1 ± 11.8	79.8 ± 36.0*
	14	27.7 ± 2.5	165.6 ± 14.2*

*Significant difference ($p \leq 0.05$) between diets

Table 3. Parasitaemias (mean ± SE) (n = 10) in vaccinated, vaccinated/challenged and infected rainbow trout *Oncorhynchus mykiss* fed ascorbic acid deficient (AA–) and supplemented (AA+) diets

Time post-challenge (wk)	Parasitaemias × 1000 (mean ± SE) ml^{-1} fish blood					
	Vaccinated[a]		Vaccinated/challenged[b]		Infected[c]	
	AA–	AA+	AA–	AA+	AA–	AA+
3	0	0	75 ± 28	68 ± 17	244 ± 96	222 ± 87
4	58 ± 31	58 ± 30	45 ± 28	68 ± 17	1633 ± 96	3585 ± 87*
5	143 ± 45	100 ± 44	75 ± 39	238 ± 63*	3877 ± 1000	4650 ± 677
6	123 ± 60	45 ± 32	118 ± 43	120 ± 43	1878 ± 838	3005 ± 1453
7	88 ± 56	170 ± 74	88 ± 33	200 ± 94	1864 ± 534	2250 ± 896
8	70 ± 47	110 ± 60	56 ± 24	100 ± 52	1958 ± 485	2075 ± 949

[a]Vaccinated with 100 000 attenuated *Cryptobia salmositica* per fish
[b]Challenged with 100 000 virulent *C. salmositica* per fish
[c]Infected with 100 000 virulent *C. salmositica* per fish
*Significant difference ($p \leq 0.05$) between diets

Packed cell volumes

Mean PCV of fish in the 4 subgroups fed AA-supplemented diet were not significantly different before challenge with the pathogen. PCV in infected controls decreased rapidly after infection and were significantly lower (indication of anaemia) than those in other subgroups from 5 wpi. As the disease progressed in infected controls, the anaemia became more severe. However, mean PCV were not significantly different between vaccinated, vaccinated/challenged or uninfected control subgroups (Table 4).

Similar results were also found in fish fed the AA-deficient diet (Table 4). The PCV between vaccinated and vaccinated/challenged subgroups were not significantly different before and after the challenge. Also, PCV in these 2 subgroups were not significantly different from those in uninfected naive controls (using a linear regression test of mean PCV against wk). However, the mean PCV in infected controls decreased rapidly and were significantly lower after infection than those in other 3 subgroups (vaccinated, vaccinated/challenged and uninfected naive controls).

Mean PCV in AA-deficient vaccinated, vaccinated/challenged and uninfected naive fish were significantly lower than those in AA-supplemented fish (Table 4). However, the mean PCV between infected controls were not significantly different between the diets.

Complement fixing antibodies

Detectable CFAb was found in the blood at 5 wpv in 6 of 10 vaccinated fish and 1 wpc in 5 of 10 vaccinated/challenged fish fed AA-supplemented diet. In subsequent weeks, CFAb was detected in all vaccinated and vaccinated/challenged fish. Mean CFAb titres increased rapidly in both subgroups; however, the titres in vaccinated/challenged fish were consistently higher than those in vaccinated fish. The detectable CFAb was delayed 1 wk in fish given the AA-deficient diet. It was first found at 6 wpv in vaccinated fish and at 2 wpc in vaccinated/challenged fish. Mean titres of CFAb in these fish also increased rapidly; there were no significant differences either between vaccinated and vaccinated/challenged fish or

Table 4. Packed cell volumes (mean ± SE) in vaccinated, vaccinated/challenged, infected and uninfected rainbow trout *Oncorhynchus mykiss* fed ascorbic acid deficient (AA–) and ascorbic acid supplemented (AA+) diets

Time post-challenge (wk)	Packed cell volume (%) (mean ± SE)							
	Vaccinated (n = 10)		Vaccinated/challenged (n = 10)		Infected (n = 10)		Uninfected (n = 8)	
	AA–	AA+	AA–	AA+	AA–	AA+	AA–	AA+
0	21.3 ± 1.3	29.2 ± 2.1	23.7 ± 2.3	28.5 ± 1.5	32.0 ± 1.1	33.9 ± 1.7	29.3 ± 1.6	32.4 ± 1.6
3	24.8 ± 1.3	30.9 ± 0.8	22.5 ± 1.3	30.2 ± 1.6	27.0 ± 1.9	29.9 ± 1.6	32.8 ± 0.8	31.8 ± 1.7
4	23.2 ± 1.3	29.0 ± 1.3[a]	23.4 ± 1.6	29.4 ± 1.2[a]	21.0 ± 1.5[b]	24.7 ± 2.0[b]	28.4 ± 0.8	32.9 ± 1.5
5	21.8 ± 1.2	29.9 ± 1.3[a]	23.6 ± 1.9	28.8 ± 1.1[a]	18.2 ± 1.0[b]	17.1 ± 1.3[b]	27.6 ± 1.5	32.1 ± 1.4
6	21.2 ± 1.4	32.1 ± 1.4[a]	21.0 ± 1.9	29.7 ± 1.6[a]	17.4 ± 1.4[b]	18.0 ± 2.3[b]	28.6 ± 2.3	35.0 ± 1.6[b]
7	21.8 ± 1.1	28.9 ± 0.7[a]	23.3 ± 2.0	30.4 ± 1.0[a]	16.8 ± 1.0[b]	17.7 ± 2.6[b]	29.3 ± 1.8	32.2 ± 1.2
8	21.4 ± 1.0	28.0 ± 1.4[a]	22.6 ± 2.1	29.4 ± 1.1[a]	15.2 ± 1.6[b]	18.6 ± 3.0[b]	26.0 ± 1.2	32.9 ± 1.3[b]

[a]Significant difference ($p \leq 0.05$) between ascorbic acid deficient and supplemented diets
[b]Significant difference ($p \leq 0.05$) between infected and uninfected controls on these diets

Table 5. Titres (mean ± SE) (n = 10) of complement fixing antibodies in vaccinated, vaccinated/challenged, and infected controls fed ascorbic acid supplemented (AA+) or deficient (AA–) diets

Time post-challenge (wk)	Titres (log_2) of complement fixing antibodies (mean ± SE)					
	Vaccinated[a]		Vaccinated/challenged[b]		Infected controls[c]	
	AA+	AA–	AA+	AA–	AA+	AA–
1	0.7 ± 0.3	0	0.7 ± 0.3	0	0	0
2	2.2 ± 0.3	1.9 ± 0.2	2.8 ± 0.3	2.7 ± 0.2	0	0
3	2.9 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	3.3 ± 0.3	0	0
4	3.8 ± 0.4	3.6 ± 0.3	4.4 ± 0.3	4.2 ± 0.3	0	0
5	3.0 ± 0.2	3.5 ± 0.2	3.6 ± 0.3	3.1 ± 0.3	0	0
6	3.5 ± 0.5	4.1 ± 0.4	3.8 ± 0.5	3.9 ± 0.4	0	0
7	3.0 ± 0.6	3.7 ± 0.3	4.0 ± 0.3	4.2 ± 0.3	0.9 ± 0.1	0.6 ± 0.2
8	2.9 ± 0.4	2.9 ± 0.3	3.4 ± 0.3	3.4 ± 0.4	2.1 ± 0.2	2.0 ± 0.3

[a]Vaccinated with 100 000 attenuated *Cryptobia salmositica* per fish
[b]Vaccinated fish were challenged with 100 000 virulent *C. salmositica* per fish
[c]Infected with 100 000 virulent *C. salmositica* per fish

between diets from 2 wpc (or 6 wpv) (Table 5). The CFAb in infected controls was detected in 5 of 10 AA-deficient fish and 6 of 10 AA-supplemented fish from 7 wpi. CFAb was found in all infected fish in subsequent weeks. No detectable CFAb was found in uninfected naive controls.

DISCUSSION

Ascorbic acid is an essential dietary component for normal physiological functions and growth of fish (Tucker & Halver 1986). Rainbow trout are particularly reliant upon exogenous dietary AA because they lack the L-gulonolactone oxidase needed for AA synthesis from glucose (Dabrowski 1990). Therefore, the dietary AA intake influences AA contents in the liver and kidney (Navarre & Halver 1989, Hardie et al. 1991, Al-Amoudi et al. 1992, present study). The high standard error in some AA measurements was due to the relatively small sample size.

Cryptobia salmositica requires protein and glucose for *in vivo* and *in vitro* multiplication (Li & Woo 1991a, b). In the present study, the parasitaemias in infected or vaccinated/challenged fish with virulent parasites were consistently and significantly higher (at 4 wpi or 5 wpc) in AA-supplemented fish than in AA-deficient fish. This may indicate that the higher ascorbic acid content in tissues may have directly or indirectly promoted more rapid multiplication of the virulent parasite.

The present study confirmed that the live *Cryptobia salmositica* vaccine protected trout against cryptobiosis (Woo & Li 1990, Li & Woo 1995). In an earlier study, Wahli et al. (1986) showed that ascorbic acid increased immune response in rainbow trout infected

with *Ichthyophthirius multifiliis* and this resulted in reduced mortality. Results of the present study indicated that the dietary AA did not affect protection by the live vaccine in rainbow trout. Similar results were reported in Atlantic salmon against *Vibrio anguillarum* (Lall et al. 1989). The production of protective CFAb (present study) and agglutinating antibodies (Lall et al. 1989, Hardie et al. 1991) were independent of dietary AA. However, Li & Lovell (1985) and Navarre & Halver (1989) found that production of agglutinating antibodies was depressed under conditions of AA deficiency. Since agglutinating antibodies in fish may not be protective (Salati 1988, Landolt 1989), it is important to measure the effects of AA on the production of protective antibodies, such as CFAb which causes lysis of parasites under *in vitro* conditions (Li & Woo 1995, present study). The present study demonstrated that the production of protective CFAb was not significantly affected by the dietary AA.

Vaccination with the live vaccine establishes an immunological memory, and vaccinated fish respond rapidly against the pathogen (Li & Woo 1995). The dietary AA deficiency did not significantly affect the establishment of an immunological memory in vaccinated trout. Production of protective CFAb in vaccinated fish was more rapid, and the titres were significantly higher, than in those of unvaccinated infected controls for both diets (present study). Although the detectable CFAb was delayed for 1 wk in fish given the AA-deficient diet, the mean CFAb production in fish given either diet was not significantly different from <2 wpc (or 6 wpv) onward. All AA-supplemented and AA-deficient fish were protected from cryptobiosis by the live vaccine. In addition, there was no cryptobiosis in fish inoculated with the vaccine compared with unvaccinated infected fish, which had high parasitaemias, were anaemic and had abdominal distension with ascites.

Anaemia, one of the clinical signs of AA-deficiency, was also found in uninfected AA-deficient fish in the present study. Internal haemorrhage (Halver et al. 1969) and haemolysis (Chazan & Mistilis 1963, Goldberg 1963) might have contributed to anaemia in AA-deficient fish. However, anaemia in infected controls was caused by both the parasite (Thomas & Woo 1989a, b) and AA-deficiency, and it was more severe than in the uninfected AA-deficient fish.

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