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Effect of dietary vitamin C on the disease susceptibility and inflammatory response of mrigal, *Cirrhinus mrigala* (Hamilton) to experimental infection of *Aeromonas hydrophila*

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Abstract

Two groups of 3-day-old hatchlings of *Cirrhinus mrigala* were fed with vitamin C supplemented (at 1000 mg vitamin C/kg diet) and non-supplemented practical diet for a period of 4 months. At the end of the feeding period, fishes were examined for their disease susceptibility and inflammatory response to a virulent strain of *Aeromonas hydrophila*. Mortality curves were clearly distinct and the vitamin C non-supplemented (VNS) group showed significantly higher mortality rates compared to the vitamin C supplemented (VS) group. While studying the inflammatory response to *A. hydrophila*, it was found that in the VS group, the infiltration of phagocytic cells was quicker with very limited lesion development at the injection site and there was complete resolution by day 9 post-injection. In the VNS group, the bacterium was able to produce necrotic lesions clinically and histologically typical of a disease condition.

Keywords: Vitamin C; *Aeromonas hydrophila*; Disease susceptibility; *Cirrhinus mrigala*; Inflammatory response

1. Introduction

In India, with the emergence of large-scale commercial carp culture, diseases of varied aetiology are being increasingly recognised as a major hurdle to successful and sustainable

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farming (Rao et al., 1992). The use of chemotherapeutants for controlling diseases has been widely criticized for their negative impacts like accumulation of tissue residues, development of drug resistance and immunosuppression (Rijkers et al., 1980; Van Muiswinkel et al., 1985; Ellis, 1988). Hence, there is an urgent need to look for ecofriendly disease preventative measures to promote sustainable culture of Indian major carps. In order to reduce the risk of disease, the level of resistance to infection in the cultured organisms should be increased by the use of better feeds, vaccines, immunostimulants or by selective breeding for higher disease resistance (Raa et al., 1992).

Vitamin C has been demonstrated to play an important role in the functioning of the immune system when supplied at dietary levels higher than standard doses in several fish groups (Blazer, 1992). Specific effects of vitamin C on a variety of non-specific resistance mechanisms and the specific immune response have been reported in fish (Hardie et al., 1991; Verlhac and Gabaudan, 1994; Ortuno et al., 1999).

The present study was undertaken to evaluate the modulatory role of vitamin C on the disease susceptibility and inflammatory response in the Indian major carp, *Cirrhinus mrigala*. *Aeromonas hydrophila*, one of the important bacterial pathogens of cultured carp, was used as a model pathogen since it is usually associated with the development of bacterial hemorrhagic septicaemia in cultured carps (Roberts, 1989).

2. Materials and methods

2.1. Fish and experimental systems

Three-day-old hatchlings of mrigal, *C. mrigala* were used for the study. Circular ferrocement tanks having capacity for holding 1000-l water were used for conducting the experiments. Prior to stocking of the hatchlings, the tanks were disinfected with potassium permanganate at a concentration of 5 ppm, and then washed thoroughly and dried. Freshwater was then filled up to a height of 90 cm and these levels were maintained throughout the rearing period. Municipal supply tap water was used, after dechlorination in holding tanks. Aeration was provided throughout the experimental period. The water was static and 50% of the water in the tanks was replaced daily throughout the experimental period after siphoning out leftover feed and fecal matter. The water temperature ranged between 26 and 31 °C during the experimental period.

2.2. Vitamin C

A stable and bioavailable form of a commercial vitamin C derivative viz., Rovimix® Stay C® was used as the source of L-ascorbic acid. The product had 25% ascorbic acid activity and the active ingredient was L-ascorbyl-2-polyphosphate.

2.3. Experimental diet

The standard pelleted feed for carps developed by Varghese et al. (1976) was used as the basal diet. Vitamin C was incorporated at the rate of 1000 mg/kg diet. The basal diet

without incorporation of vitamin C was used as a control. Post-pelleting, the control diet contained 92.72 mg vitamin C/kg dry diet and the vitamin C supplemented diet contained 1014.63 mg vitamin C/kg diet as determined by vitamin C analysis (Bessey et al., 1947).

2.4. Preparation of *A. hydrophila*

A virulent strain of *A. hydrophila* (Sah 93) isolated from EUS affected *Sillago sihama* was used. One-day-old cultures of *A. hydrophila* on nutrient agar slants were harvested and resuspended in sterile phosphate-buffered saline (PBS), and the cell density was adjusted to the desired level by measuring absorbance (OD) in a spectrophotometer. For the disease susceptibility studies, a cell density of 10^6 cells/ml was used, and for studies on inflammatory response, 10^5 cells/ml.

2.5. Experimental design

Mrigal hatchlings were stocked in four ferrocement tanks at a uniform density of 100/tank. Fish received a practical diet with (1000 mg vitamin C/kg diet) or without supplementation of vitamin C, each in duplicate tanks. The pelleted feed was crushed into fine pieces and fed to the hatchlings at the rate of twice the body weight per day for the first 15 days, followed by 10% body weight/day for the next month. After 1 month, the surviving fish were collected and distributed at a uniform density of 50 fish/tank. The feeding rate was reduced to 5% body weight/day. Fish were sampled at monthly intervals to assess the growth in terms of length and weight and to adjust the quantity of feed given. After a total of 4 months of experimental feeding, the fishes were analysed for growth in terms of length and weight, survival, tissue vitamin C levels in kidney and liver, disease susceptibility to a virulent strain of *A. hydrophila* and inflammatory response to *A. hydrophila*.

2.6. Tissue vitamin C analysis

On termination of experimental feeding, the ascorbic acid concentration of the kidney and liver was analysed following the DNPH method described by Bessey et al. (1947). The difference in ascorbic acid levels in different tissues and different treatments was tested using ANOVA and Duncan's multiple range test (Duncan, 1955).

2.7. Disease susceptibility to *A. hydrophila*

At the end of the experimental feeding, 10 fish each from the VS and VNS group were challenged by injection (i.m.) of 0.1 ml of the bacterial suspension having a cell density of 10^6 cells/ml (i.e. 10^5 cells/fish). The dose was fixed based on the results of previous infectivity trials carried out in our laboratory (Azad et al., 1999). Control fish received 0.1 ml PBS. The challenged fish were kept in 500-l-capacity fiberglass tubs. Aeration was maintained with replenishment of 50% of water daily. The mortality pattern was recorded daily up to 10 days post-challenge. Only specific mortalities confirmed through

reisolation of the pathogen from kidney on Rimler Shott's medium containing novobiocin (Shotts and Rimler, 1973) were considered.

The difference in cumulative percentage mortalities between treatments was tested using the Kolmogorov–Smirnov test (Smith, 1989).

2.8. Inflammatory response to A. hydrophila

At the end of the experimental feeding period, 20 fish (average weight 21.93 g ± 0.96 and 23.37 g ± 1.39 in the VNS and VS group, respectively) from each treatment were used for evaluating the inflammatory response to a sublethal dose of a virulent strain of *A. hydrophila*. The fish were anaesthetised prior to handling in a solution of Benzocaine (10 ppm). Each fish was injected (i.m.) with 0.1 ml of the bacterial suspension having a cell density of 10^5 cells/ml (i.e. 10^4 cells/fish) and were released back to the tanks. Two fish each were sampled at time intervals of 0 h, 1 h, 3 h, 12 h, 24 h, 3 days, 6 days and 9 days following injection. The skeletal muscle at the site of injection of the sampled fish were collected and fixed immediately in 10% neutral buffered formalin for further histological investigations. Standard histological techniques (Bullock, 1989) were followed.

3. Results

3.1. Growth and survival

There was no significant difference ($P < 0.05$) in the growth performance in terms of length and weight between the VNS and VS groups of mrigal, at the end of 4 months of experimental feeding. The average length and weight attained were 13.91 cm ± 0.32 and 21.93 g ± 0.96 in the VNS group and 14.20 cm ± 0.39 and 23.37 g ± 1.39 in the VS group. The survival rate of mrigal hatchlings at the end of 1 month of rearing was 54% in the VNS group and 59.5% in the VS group. The survival rate of 1-month-old fry reared for the subsequent 3-month period was 77% in the vitamin C non-supplemented group and 81% in the vitamin C supplemented group.

3.2. Tissue vitamin C level

Both kidney and liver ascorbic acid levels determined at 4 months were significantly higher ($P < 0.05$) for the vitamin C supplemented group compared to the non-supplemented group (Fig. 1).

3.3. Disease susceptibility to A. hydrophila

The cumulative percentage mortality observed after injection challenge with *A. hydrophila* showed that the vitamin C non-supplemented group had a significantly higher ($P < 0.05$) mortality rate compared to the supplemented group (Fig. 2). The vitamin C non-supplemented groups developed typical clinical and pathological con-

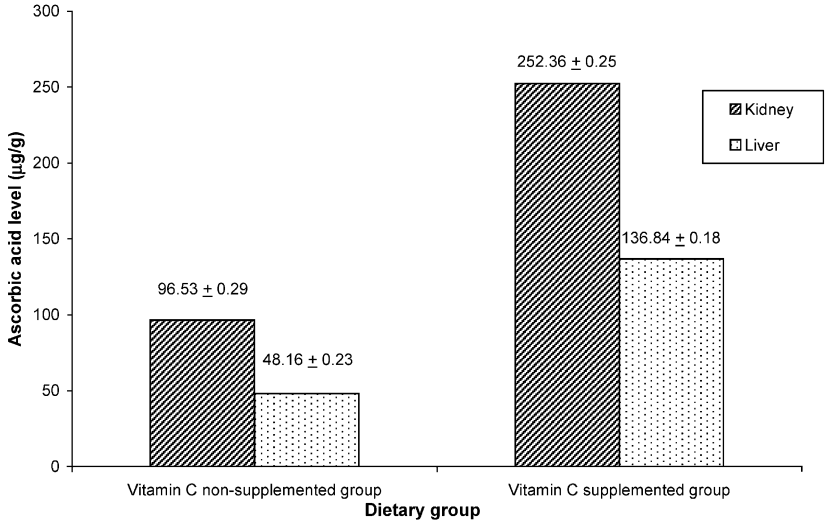

Fig. 1. Tissue ascorbic acid level in the kidney and liver of the two dietary groups of mrigal at the end of 4 months of experimental feeding. Data represent means of 5 fish/group ± S.E.

ditions such as necrotic lesion development at the site of injection. Even the fish that survived in the VNS group showed external ulceration at the site of injection after 8 days post-challenge (dpc). Histological examination of the injection site at 8 dpc showed

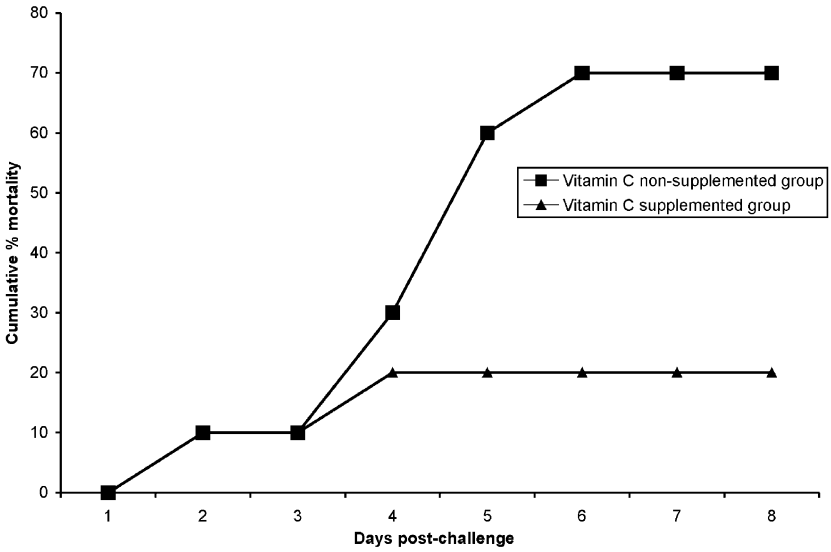

Fig. 2. Cumulative percentage mortality in the two dietary groups of mrigal upon challenge with *A. hydrophila* (10^5 cells/fish) at the end of 4 months of experimental feeding.

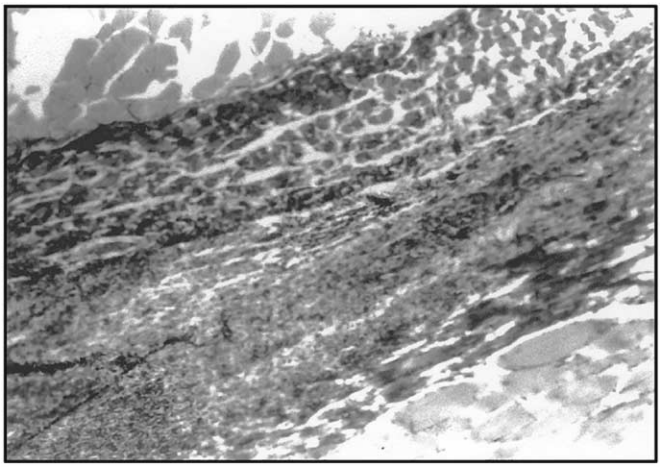

Fig. 3. The skeletal muscle tissue at the injection site of the vitamin C non-supplemented group of mrigal from the disease susceptibility experiment, at 8 days post-challenge with *A. hydrophila* (10^5 cells/fish). A necrotic lesion with large scale infiltration of inflammatory cells is very conspicuous. H and E; $\times 100$.

lesion development and large-scale infiltration of inflammatory cells (Fig. 3). In the VS group, no external manifestation of ulcer development was noticed and the histological examination of the injection site at 8 dpc showed signs of resolution (Fig. 4).

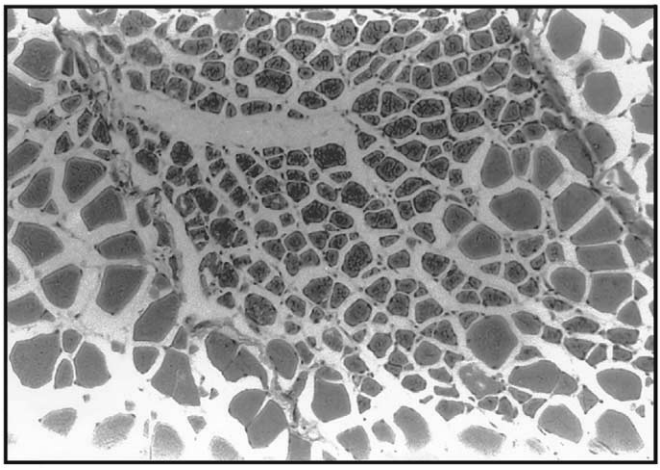

Fig. 4. Skeletal muscle tissue at the site of injection of the vitamin C supplemented group of mrigal from the disease susceptibility experiment, at 8 days post-challenge with *A. hydrophila* (10^5 cells/fish). Note signs of resolution and healing with very few inflammatory cells and the absence of any necrotic lesion. H and E; $\times 200$.

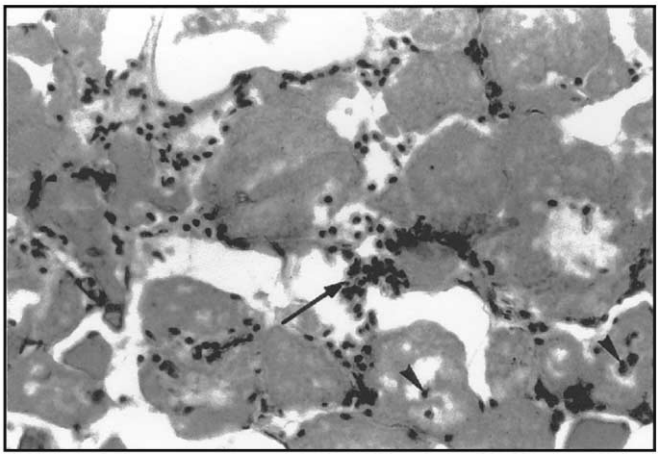

Fig. 5. Sequential inflammatory response at the injection site in mrigal fed the VS diet, following challenge with *A. hydrophila* (10^4 cells/fish). Large-scale muscle necrosis, extensive vascular activity (arrow) and myophagia (arrowhead) at 24 hpi. H and E; $\times 400$.

3.4. Inflammatory response to A. hydrophila

The sequential inflammatory response following intramuscular injection with *A. hydrophila* over 9 days in the vitamin C supplemented (VS) and non-supplemented (VNS) diet groups of *C. mrigala* is summarised below.

One hour after the injection of the bacterial suspension, the histological section of the skeletal muscle at the site of injection showed initial stages of necrosis and hemorrhages

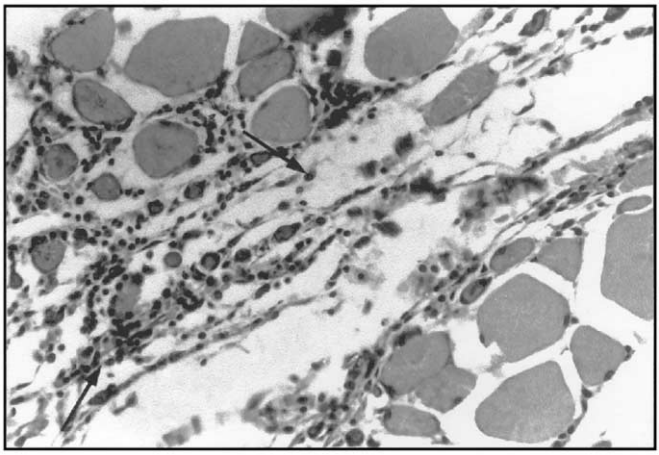

Fig. 6. Sequential inflammatory response at the injection site in mrigal fed the VS diet, following challenge with *A. hydrophila* (10^4 cells/fish). Cellular response confined to the area of lesion development (arrow) at 3 dpi. H and E; $\times 400$.

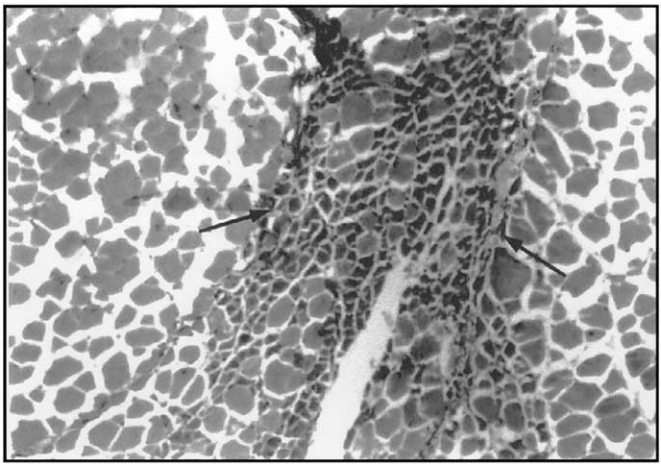

Fig. 7. Sequential inflammatory response at the injection site in mrigal fed the VS diet, following challenge with *A. hydrophila* (10^4 cells/fish). Injection site at 6 dpi showing signs of resolution with regenerating muscle cells and the presence of very few necrotic cells. Note the confinement of the lesion area (arrows). H and E; $\times 100$.

due to the trauma of injection. At 3 h post-injection (hpi), there was initiation of vascular activities and extravascular neutrophils were present in the area. Bacterial masses were also seen within the necrotised muscle cells. At 12 hpi, there was increased infiltration of phagocytes dominated by neutrophils in the area. The initial responses up to 12 hpi were almost similar in both VS and VNS groups. In both groups, widespread generalised necrosis and a large number of phagocytes dominated by neutrophils were present.

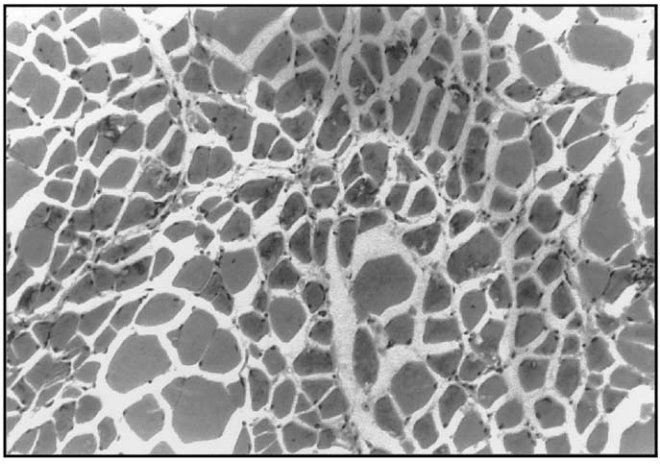

Fig. 8. Sequential inflammatory response at the injection site in mrigal fed the VS diet, following challenge with *A. hydrophila* (10^4 cells/fish). Injection site at 9 dpi completely healed with new myocytes appearing healthy. H and E; $\times 200$.

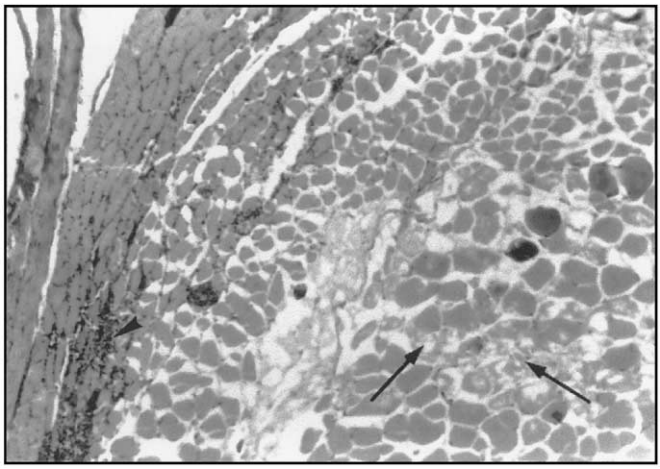

Fig. 9. Sequential inflammatory response at the injection site in mrigal fed the VNS diet following challenge with *A. hydrophila* (10^4 cells/fish). The lesion at 3 dpi showing extensive muscle necrosis (arrow) and a large number of inflammatory cells (arrowhead). H and E; × 100.

At 24 hpi, the VS group showed an increased macrophage response and myophagia with debris of dead muscle fibres being cleared by phagocytes (Fig. 5). At 3 days post-injection (dpi) in the VS group, the lesion was confined with the cellular response limited to the area of lesion development. The myocytes appeared dark and condensed with pyknotic nuclei (Fig. 6). At 6 dpi, the lesion site showed signs of resolution with very few inflammatory cells and emergence of regenerating muscle fibers (Fig. 7). At 9 dpi in the

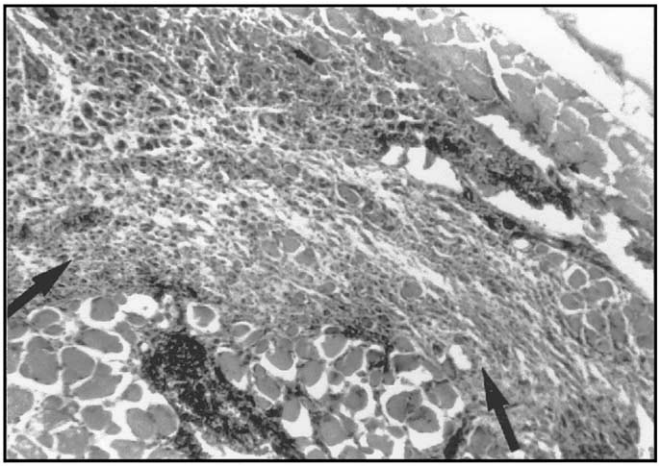

Fig. 10. Sequential inflammatory response at the injection site in mrigal fed the VNS diet following challenge with *A. hydrophila* (10^4 cells/fish). Development of necrotic lesion typical of a disease condition (arrow) at 6 dpi. H and E; × 100.

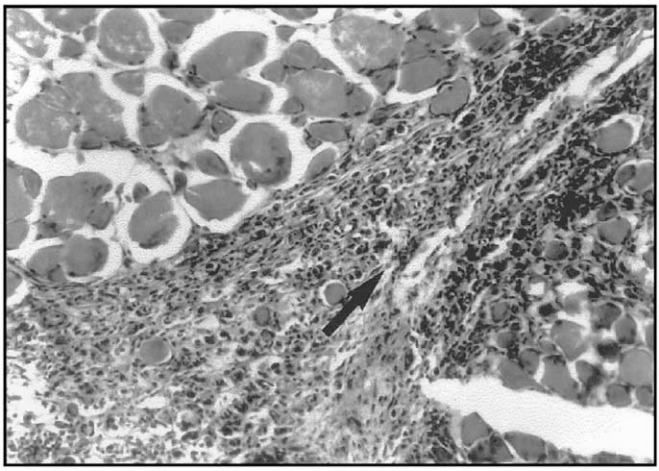

Fig. 11. Sequential inflammatory response at the injection site in mrigal fed the VNS diet following challenge with *A. hydrophila* (10^4 cells/fish). Necrotic lesion development confined by the perimysium (arrow) at 6 dpi. H and E; × 200.

VS group, the injected site showed a healing lesion area with regenerated muscle fibers (Fig. 8).

In the VNS group, at 24 hpi, though there was increased infiltration of phagocytic cells, the area was not invaded by macrophages. By 3 dpi, the VNS group had the cellular response spreading laterally and towards the centre leading to a large lesion area with a

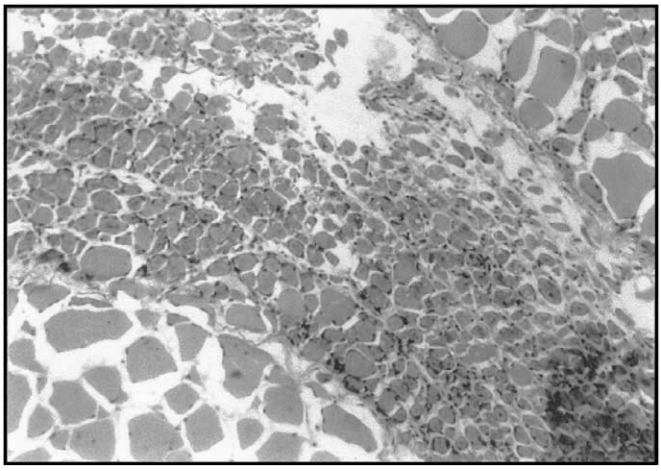

Fig. 12. Sequential inflammatory response at the injection site in mrigal fed the VNS diet following challenge with *A. hydrophila* (10^4 cells/fish). The lesion site showing signs of organisation with reduced pathology at 9 dpi. H and E; × 200.

large number of inflammatory cells, hemorrhages and large-scale muscle necrosis (Fig. 9). At 6 dpi, necrotic lesion development typical of a disease condition was seen in the VNS group with a large number of inflammatory cells and complete degeneration of muscle fibres in the area (Fig. 10). The development of pathology was confined by the perimysium (Fig. 11). At 9 dpi, the VNS group also showed initial signs of reorganisation of the lesion with reduced pathology (Fig. 12).

4. Discussion

Mrigal fed vitamin C supplemented diets exhibited decreased mortality rates when challenged with *A. hydrophila* compared to fish fed a vitamin C non-supplemented diet. The disease susceptibility of an organism depends on its innate and acquired resistance mechanisms and also on the ability of the pathogen to establish and proliferate (Raa et al., 1992). While studying the inflammatory response, the initial stages of vascular events following administration of the bacterial suspension were found to be similar in both the VS and VNS groups. After 12 h post-injection (hpi), both groups started showing clear differences in their response. On the third day post-injection (dpi) in the VS group, the response was confined to a limited lesion having pyknotic and phagocytic cells. By 6 dpi, regenerating muscle fibers were seen, and by 9 dpi, the lesion area had completely healed with no sign of inflammatory cells. In the VNS group, the bacteria were able to produce a necrotic lesion and exudate formation typical of a disease condition by 6 dpi. The histological section of the lesion resembled that of ulcer development in the vitamin C non-supplemented fishes when challenged with a lethal dose of *A. hydrophila* in the disease susceptibility studies. By 9 dpi, the lesion area showed initial signs of reorganisation with reduced pathology. However, there was no external manifestation of the lesion. This may be due to the fact that the pathogen was administered once and the dosage was low.

In the VS group, the phagocytic cells would have efficiently cleared up the pathogen by 6 dpi. The role of ascorbic acid in enhancing the phagocytic response has been reported by several authors (Thomas and Holt, 1978). Phagocytic cells generate reactive metabolites such as superoxide anion, hydrogen peroxide and hypochlorous acid in response to membrane stimulation. These antimicrobial substances, however, may compromise host responses by causing oxidative damage. A study with human neutrophils indicated that ascorbic acid acted to protect neutrophils from oxidative damage (Anderson et al., 1990). The phagocytic index of peritoneal macrophages has been shown to be significantly lower in rainbow trout fed a vitamin C deficient diet (Blazer, 1982). Ascorbic acid deficiency also significantly reduced phagocytosis of *Edwardsiella ictaluri* by channel catfish neutrophils (Li and Lovell, 1985). It is possible that ascorbic acid acts as an antioxidant in preventing oxidative damage to neutrophils and other phagocytes during respiratory burst activity, and thereby helps to increase the mobility and phagocytic activity of these cells.

Vitamin C nutrition has also been reported to enhance complement activity by a number of investigators. Li and Lovell (1985) observed significantly lower levels of serum haemolytic activity in fish fed a vitamin C non-supplemented diet compared to those fed vitamin C supplemented diets. Similar results were obtained with Atlantic salmon

(Hardie et al., 1991), where complement activity differed significantly in a dose-dependent manner. Complement is a non-specific component of the immune system, which can attract and activate phagocytes (chemotaxis), function as opsonin and thereby increase phagocytosis of complement coated particles causing target cell lysis. Thus, enhanced complement activity in the VS group may have contributed to an enhanced inflammatory response and this could be one of the mechanisms responsible for the increased disease resistance observed in the VS group.

The importance of vitamin C in disease resistance has been highlighted in other species of farmed fish. Several studies have demonstrated that dietary supplementation of vitamin C, at levels much higher than the minimal requirement for promoting normal growth, can enhance disease resistance (Durve and Lovell, 1982; Li and Lovell, 1985; Liu et al., 1989; Navarre and Halver, 1989; Hardie et al., 1991). In channel catfish, Durve and Lovell (1982) observed that diets with 150 mg/kg diet reduced mortalities to *E. tarda* challenge compared to fish fed a vitamin C deficient diet. According to Li and Lovell (1985), a ration of 3000 mg vitamin C/kg diet afforded protection to catfish experimentally infected with *E. ictaluri*. Rainbow trout fed high levels of vitamin C exhibited increased resistance to the ciliate parasite, *Ichthyophthirius multifiliis*, compared to fish receiving a vitamin C deficient diet (Wahli et al., 1986). Resistance to *Vibrio anguillarum* has been shown to be directly related to vitamin C intake in rainbow trout (Navarre and Halver, 1989). Several workers have reported the beneficial role of vitamin C in enhancing disease resistance of other species of fish (Ortuno et al., 1999).

In the present study, the difference in the disease resistance observed between the VS and VNS groups may be due to the difference in the magnitude of the inflammatory response and other components of the innate/acquired resistance mechanisms of the host. Even the antibody response and cell mediated immunity are regulated by the basic inflammatory response. Therefore, the greater magnitude of the inflammatory response could be one of the reasons for increased disease resistance observed in the vitamin C supplemented groups.

5. Conclusion

The results from this study in mrigal have clearly shown that vitamin C can play an important role in increasing the disease resistance of fish. The role of vitamin C in enhancing the inflammatory response in mrigal is also apparent. These findings may be used as baseline information for designing further studies in Indian major carps to investigate the degree and duration of the resistance offered, and also for assessing disease resistance to other microbial and parasitic pathogens.

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