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# Immunostimulatory effect of vitamin e on infected common carp, *Cyprinus carpio* (L.) against *Aeromonas hydrophila*

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**ABSTRACT:**

The effect of various concentration of dietary Vitamin E on the survival and mortality rate, phagocytic activity, leucocyte count, protein and relative level protection of *Cyprinus carpio* against *Aeromonas hydrophila. Aeromonas hydrophila* treated group showed 60 % mortality and 40 % survival rate on 20th days than negative and Vitamin E treated. Experimental groups and treated with different quantity of Vitamin E (200 mg, 400 mg and 600 mg) and intraperitoneally (IP) injected with 0.1 ml of 105 CFU/ml of *A. hydrophila* showed no mortality. The control group showed more phagocytic activity and the Vitamin E administered groups showed more phagocytic activity on 25th day. Vitamin E experimental groups of different quantity showed more number of leucocytes from 15th day onwards. Vitamin E administered groups showed amount of muscle protein decreased from 5th, 10th and 15th day respectively. The control group showed 12.5 % protection and the Vitamin E treated 200 mg showed 77.77 %, 400 mg showed 57.40 % and 600 mg showed 42.85

**Keywords:**

*Cyprinus carpio*, Common carp, Bacterial disease, *Aeromonas hydrophila,* Vitamin E, Immunostimulant.

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## INTRODUCTION

Aquaculture is the fastest growing food producing sector in the globe (Saurabh and Sahoo, 2007). In many countries intensive fish farming is become a key industry in recent decades (Choi and Oh, 2007). In India, Tamil Nadu has 5th position in inland fish production (Senthilkumar and Sujith, 2008). The common carp is useful to eradicating weeds (Shammi and Bhatnagar, 2000). *Cyprinus carpio* is native of temperate regions of Asia but it is now conveniently cultivated in different parts of the world. Indian major corps is contributing to more than 80 percent of the production (Saurabh and Sahoo, 2007). Over 30 aquatic species are currently being cultured to produce protein as human food source (Meyer, 1991). The health status of aquatic organism is uniquely related to their environments, which contain high concentrations of microorganisms. Saprophytic and pathogenic are capable of infecting fish when conditions become favourable for multiplication (Ellis, 2001). Fish is high risk of infectious diseases caused by pathogenic (parasites, fungi, bacteria and virus) organisms present in the environment (Kaper *et al.,* 1981; Maqsood *et al.,* 2011). *Aeromonas hydrophila* is the etiological agent, causes the most economic loss in wild fish (Austin and Austin, 1993).

*Aeromonas hydrophila* is an important gram negative, free living and motile. Also, *A. septicaemia* pathogen causing disease not only to fishes but also to other animals including human. (Anbarasu *et al.,* 1998; Yesmin *et al.,*. 2004; Newman, 1993). The two bacterial pathogenic organisms were causing the red sore disease in fishes (Reed and Floyd, 2003). Antimicrobial agents have been widely used in aquaculture to treat infections by a variety of bacterial pathogen of fish, including *A. hydrophila, A. solmonicida, E. tarda, P. piscicida, V. anguillarum* and *Y. ruckeri.* Drugs are administered by mixing them with feed that is dispersed in the water they directly dose the environment, resulting in selective pressures in the exposed ecosystem (Angulo, 2000). Direct treatments were available through the food chain (Leger *et al*., 1986). Healthy diet is given for disease prevention (Akhtaar and Viji, 2008). Vitamins are organic compounds which are required for normal growth, physiological function, improve the reproductive performance, health, increasing fertilization, survival of fry and maintenance of fish metabolism (Halver 1982; Richard 1991; Takeuchi *et al.,* 1981). Vitamin E is fat-soluble that consists of a group of tocopherols and tocotrienols (Wang and Quinn, 2000). Vitamin C and E has improved immune response and disease resistance of variety of fish species (Bendich 1990; Frischknecht *et al.,* 1994).

Vitamin E depleted diet has significantly increased mortality rate of Atlantic salmon (Hardie *et al.,* 1990). Immunostimulant is important in fish culture for heightening the activity of non specific defense mechanism and conferring protection against disease (Jeney and Anderson, 1993; Villegas *et al.,* 2006) dietary supplementation of various tested substances revealed that they had induced some enhancement in immune parameters (Grondel *et al.,* 1987; Pal *et al*., 2007). Wide ranges of chemicals are known to modulate the immune response of fish. Immunostimulant includes a wide range of chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokines (Sakai, 1999), and vaccination are used in treatment (Ispir and Dorucu, 2005). Aly *et al.* (2008) reported that garlic improve the immune response, monocytes and enhance phagocytic activity of *O. niloticus*.

Phagocytic activity was increased by activities of immunostimulants in *C. carpio* (Yano *et al*., 1991). Baehner *et al.,* (1977) reported that vitamin E supplementation was enhance the phagocytic rate and to decrease bactericidal activity. Sen *et al.* (1992) found significant increase in white blood corpuscles count in *C. punctatus* exposed to sublethal doses of zinc. Enhance the phagocytic activity, neutrophils, number of leucocyte and serum lysozymes levels by injection of

levamisole (Siwicki, 1987). Survival, mortality rate, phagocytic activity, total WBC count, total protein and relative level of protection were undertaken to find out immunostimulatory effect of "Evion" on diseased common carp, *C. carpio* L. There is a paucity of potential of vitamins is still dearth of information on engender about *A. hydrophila*. Therefore, the present study focused to explore the use of various concentration of vitamin E against infected *C. carpio* with *A. hydrophila* and their survival and mortality rate, phagocytic activity, leucocyte count, protein and relative level protection.

## MATERIALS AND METHODS

### Collection and maintenance of experimental animals

The experimental animal *Cyprinus carpio* L. common carp is selected for the present study. They were acclimated to laboratory conditions for 15 days in non-chlorinated water in glass tanks 52 cm length X 42 cm width and the water holding capacity of 50 liter. The experiments were carried out in glass tanks 60 cm length X 28 cm height X 42 cm width. The water was changed on alternate days proper aeration was maintained and fed *ad-libitum*.

### Fish feed

The fish feed consisting of groundnut oil cake (50 g), dried soybean (30 g) wheat bran (20 g) and vitamin E tablet. All ingredients excepting tablet were mixed with distilled water and made into paste. The food was sterilized, cooled and mixed separately with the ingredients of vitamin E 200 mg, 400 mg and 600 mg per 100 g of food. Vitamin E is light yellow oil and it is resistant to heat (up to 200°C) and acids but acted upon by alkalies. The tocopherols are excellent antioxidants. The three pasted food were passed through sieve and dried. It was made into pellets. The pellet feed was offered to the experimental fish.

### Preparation of live bacteria

The pure culture of *A. hydrophila* was prepared for the stock solution. The control fishes were given normal feed without vitamin E ingredient; while disease induced experimental fishes were fed with vitamin E. After the stock culture of *A. hydrophila* was diluted to 105 dilutions and 0.1 mL of *A. hydrophila* was injected intraperitoneally on fifth day.

### Survival and mortality rate

It was estimated by observing the number of fish died throughout the experiment. The mortality rate of *C. carpio* was estimated as follow

$$\text{Mortality rate (\%)} = \frac{\text{Number of fish died}}{\text{Total number of fish}} \times 100$$

### Phagocytic activity

The phagocytic and unphagocytic leucocytes were counted under microscope. The phagocytic activity ratio was calculated by the formula

**Table 1. The cumulative percentage mortality of *C. carpio* treated with different quantity of vitamin E and IP injected with 0.1 ml of 105 CFU/ml of *A. hydrophila***

| Treatment | Quantity of Vitamin E(mg) | Days after treatment | | | | | | Total (%)* |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 15 | 20 | 25 | |
| Control | 0 | 0 | 20 | 50 | 50 | 60 | 60 | 60 |
| Negative control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Experiment | 200 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 600 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Fish were infected with intraperitoneally
Percent (%) of survival rates in control and experimental groups

**Total leucocyte count**

The total leucocyte count was made with Neubauer's counting chamber. Calculation was made taking into consideration the dilution factor and the total leucocyte (TLC) and was expressed as Total leucocyte x $10^3$/ $mm^3$.

$$\text{Phagocytic activity (\%)} = \frac{\text{Phagocytic leucocyte number}}{\text{Observed total leucocyte number}}$$

**Estimation of protein**

The concentration of the protein present in the sample was estimated by using (Lowry *et al*., 1951) method

**Relative level of protection**

Fresh *A. hydrophila* culture (0.1 ml) was injected in all the experimental fishes and the number of mortality in all experimental groups was observed. The potency of bacteria was determined by calculation of the relative level of protection (RLP) by the following formula

$$\text{Average number of leucocytes present in one square is} = \frac{\text{Total no. of leucocyte present in all counting square}}{\text{Number of all cubic squares counted}}$$

**RESULTS AND DISCUSSION**

Development of immunity by vitamin E on disease induced common carp, *C. carpio* (L.) has been studied. The feed mixed with different quantity of vitamin E, like 200 mg, 400 mg and 600 mg were given to experimental fishes. The mortality, phagocytic activity, total leucocyte count, total muscle protein and relative level of protection are the parameters considered in the present study. Vitamin E was shown to enhance phagocytic rate which was decrease the bactericidal activity (Baehner *et al.,* 1977).

**Survival and Mortality rate**

The cumulative survival and mortality rate of the common carp, *C. carpio* treated with different quantity of Vitamin E during the experimental period was given in Table 1. The control fish, *A. hydrophila* treated group caused 60 % mortality and 40 % survival rate on 20th days and later. On the contrary, all the experimental groups and treated with different quantity of vitamin E (200 mg, 400 mg and 600 mg) and IP injected with 0.1 ml of 105 CFU/ml of *A. hydrophila* showed no mortality and 100 % survival was observed. The present study aimed to development of immunity by Vitamin E on disease induced *C. carpio.*

The similar result was observed by Narmadha *et al.* (2007) that control fish showed 60 % mortality on

**Table 2. Phagocytic activity of *C. carpio* treated with different quantity of vitamin E and IP injected with 0.1 mL of 105 CFU/ml of *A. hydrophila***

| Treatment | Vitamin E | Days after treatment* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 Day | 5th day | 10th day | 15th day | 20th day | 25th day |
| Control | 0 | 16.0±3.0$^{a}$ | 22.0±2.6$^{ab}$ | 27.6±2.4$^{bd}$ | 37.2±2.4$^{c}$ | 32.6±3.3$^{cd}$ | 25.6±3.0$^{bd}$ |
| Negative Control | 0 | 16.5±4.6$^{a}$ | 20.4±3.2$^{ab}$ | 23.1±3.3$^{ab}$ | 23.2±2.8$^{ab}$ | 27.7±3.6$^{b}$ | 25.5±2.1$^{ab}$ |
| | 200 | 15.0±2.7$^{a}$ | 24.7±1.9$^{b}$ | 34.5±2.6$^{cd}$ | 41.1±2.7$^{d}$ | 54.5±4.1$^{e}$ | 62.0±2.9$^{e}$ |
| Experiment | 400 | 16.3±3.5$^{a}$ | 22.6±2.3$^{ab}$ | 29.1±3.8$^{bc}$ | 37.0±4.6$^{cd}$ | 45.0±4.5$^{d}$ | 56.0±3.8$^{e}$ |
| | 600 | 16.6±2.4$^{a}$ | 22.6±4.0$^{ab}$ | 30.6±2.5$^{bc}$ | 35.7±3.6$^{c}$ | 44.5±2.8$^{d}$ | 53.1±3.3$^{d}$ |

*Mean data in the same row with different letter statistically significantly difference (P=0.05)among groups according to Turkey's Test, Values are mean±SD.

**Table 3. Total leucocyte count of *C. carpio* treated with different quantity of vitamin E and IP injected with 0.1 mL of 105 CFU/ml of *A. hydrophila***

| Treatment | Vitamin E | Days after treatment* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 Day | 5th day | 10th day | 15th day | 20th day | 25th day |
| Control | 0 | 2.6±0.1$^{a}$ | 2.9±0.1$^{ab}$ | 3.5±0.1$^{bc}$ | 3.8±0.2$^{c}$ | 3.1±0.5$^{abc}$ | 2.7±0.2$^{bc}$ |
| Negative Control | 0 | 2.4±0.2$^{a}$ | 2.7±0.7$^{ab}$ | 2.4±0.3$^{a}$ | 3.3±0.2$^{ab}$ | 2.9±0.1$^{ab}$ | 3.5±0.3$^{b}$ |
| | 200 | 2.9±0.1$^{a}$ | 3.3±0.2$^{ab}$ | 3.7±0.2$^{bc}$ | 4.1±0.1$^{cd}$ | 4.6±0.3$^{de}$ | 4.8±0.2$^{e}$ |
| Experiment | 400 | 2.8±0.0$^{a}$ | 3.2±0.2$^{ab}$ | 3.6±0.0$^{bc}$ | 4.0±0.2$^{cd}$ | 4.3±0.3$^{d}$ | 4.5±0.1$^{de}$ |
| | 600 | 2.8±0.1$^{a}$ | 3.1±0.3$^{ab}$ | 3.5±0.1$^{bc}$ | 3.7±0.2$^{bc}$ | 3.9±0.3$^{c}$ | 4.1±0.2$^{c}$ |

Mean data in the same row with different letter statistically significantly differences (P=0.05) among groups according to Turkey's Test, Values are mean±SD.

the 20th day. The Vitamin E treated groups showed no mortality. Vitamin E deficiency disrupts normal immune responsiveness, resulting in increased mortality. Both humoral and cell mediated immune functions are affected by vitamin E deficiency (Bendich, 1990). Vitamin E supplementation was shown to enhance phagocytic rates and decrease bactericidal activity (Baehner *et al.,* 1977). Eighty percent mortality of *Japanese eel* and common carp caused by an ulcer type of disease in Japan and Greece (Rickards, 1978; Fotis *et al.,* 1994). Low dose of Vitamin E showed more immune response than the high dose (Rickards, 1978). Frischknecht *et al.,* (1994) reported that Vitamin C and E protected rainbow trout from anemia and mortality. WDA (2%) fed fish were significantly protected against *V. vulnificus* infection (Wang *et al*., 2012)

**Phagocytic activity**

The control group showed more phagocytic activity on 20th day, vitamin E administered experimental groups of different quantity showed more phagocytic activity on 25th day (Table 2). The control group fishes showed maximum phagocytic activity on 15th day as 37.2±2.4. The Vitamin E 200 mg, 400 mg and 600 mg treated group showed maximum phagocytic activity on 25th day as 62.0±2.9, 56.0±3.8 and 53.1±3.3 respectively. The maximum phagocytic activity was observed in all experimental groups than the control. The phagocytic activity was increased with increase in days after treatment in the experimental fish groups.

The comparison of both control and the experimental groups showed more phagocytic activity in the quantity of 200 mg vitamin E treated fishes. Therefore phagocytic activity was found increased in the vitamin E treated groups than the control. The low quantity of vitamin E favoured greater phagocytic activity. The same results were reported by Liu (1991) that polysaccharides of spirulina were IP injected into mice, increased percentage of phagocytosis and macrophages. Phagocytes and lysozymes activity was significantly higher in 2% Astragalus fed groups (Wang *et al*., 2012). Balcazar *et al.* (2007) demonstrated that humoral immune response enhance the phagocytic activity, early activity of the inflammatory response before antibody production has also been reported in fish *Oreochromis niloticus* with *Lactobacillus rhamnosus* (Pirarat *et al.,* 2006). Enhanced phagocytic activity was observed in the fish treated with immunostimulants (Kajita *et al.,* 1990). The oral administration of glucan enhanced phagocytic activity of both 1 and 14 days. Anderson and Jeney (1992) reported that rainbow front fed beta -1, 3/1 -6 – glucan, phagocytic activity was higher than in control fish within a week (Siwicki *et al.,* 1987). Sakai

(1999) reported that bacterial infection activate the phagocytes and enhance bovine lacroferrin. In our observation the control and all experimental groups showed the significant difference.

**Total leucocyte count**

In the study, total leucocyte count was observed on 15th day in control and experimental group. Vitamin E administered experimental groups of different quantity showed more number of leucocytes from 15th day onwards. The control group fishes showed maximum leucocytes count on 15th day as 3.8±0.2 and the Vitamin E 200 mg, 400 mg and 600 mg administered group showed more leucocytes count on the 25th day as 4.8±0.2, 4.5±0.1 and 4.1±0.2 respectively. So more total leucocytes count was observed in all experimental groups than the control (Table 3). In the present study, the total leucocyte count has been found to be high in the Vitamin E administered group than the control. Aly *et al.,* (2008) reported that total leucocytic count were significantly higher the experimental group than control group. The use Vitamin C results in proliferation of rainbow trout lymphocytes. Vitamin E had an increased lymphocytic and phagocytosis (Sakai, 1999). Anderson and Jeney, (1992) investigated the effects of immunostimulants, including levamisole against *A. salmonicida* and found that leucocyte levels were increased with immunostimulant treatment. Siwicki (1987) High concentration of levamisole had a negative effect but optimal concentration had a positive effect on leucocyte level in carp. The intraperitoneal administration of 1 mg per dosage of alginic acid to rainbow trout (100-500 g) stimulated immune system (Peddie *et al.,* 2002). Duncan and Lovell (1994) reported that haematological abnormalities in channel catfish fed diets without Vitamin C. The total WBCs count was lowest in catfish fed on diet containing no Vitamin C.

**Total muscle protein**

The control group showed the amount of protein decreased from 5th day onwards and increased on 25th day. In the same way, the Vitamin E administered experimental groups of different quantity showed the amount of muscle protein decreased from 5th, 10th and 15th day respectively then increase the muscle protein from 20th day onwards. The control group fishes showed minimum amount of protein on 20th day as 205±6.5 mg/g. The Vitamin E 200 mg, 400 mg and 600 mg administered group showed minimum amount of muscle protein on the 15$^{th}$ day as 223±2.5 mg/g, 216±2.1 mg/g and 227±2.6 mg/g respectively. Therefore, the experimental groups showed that the amount of muscle protein was increased than the control group (Figure 1). The comparison of both control and experi-

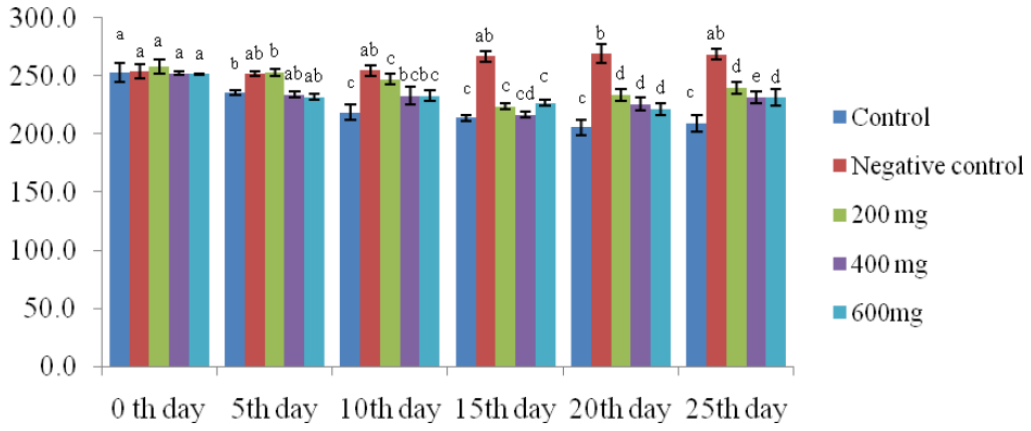


**Figure 1. Cumulative protein present in *C. carpio* fed a control diet and Vitamin E against 0.1 mL of 105 CFU/ mL of *A. hydrophila*. The values are mean with SD. Mean data in the same row with different letter statistically significantly difference (P=0.05) among groups according to Turkey's Test**

mental groups showed the amount of muscle protein loss was very low in the 200 mg Vitamin E treated fishes. Therefore, the amount of protein was varied in Vitamin E treated groups than the control. Similar result was reported by Nanjundappa and Varghese (1988), the growth promoting ability of diethylstilbestrol (DES) in the common carp, *C. carpio* and they found that significantly enhances growth in common carp. Sandnes *et al.* (1992) reported that 10-20 ppm Vitamin C was required for normal growth. The Vitamin C is not only inducing the immunostimulation but also induces the growth promoting tissue (Narmadha *et al.,* 2007). Das *et al.* (1994) developed a nutritionally balanced pelleted feed which gave much higher growth in common carp *Cyprinus carpio* (L). Gopal *et al.* (1996) observed muscle protein and glycogen increased when the biogas plant effluent and rice bran was used. Ahmadifar *et al.* (2009) studied that dietary administration of alginic acid significantly improved growth parameters in the fish feed supplemented diet.

**Relative level of protection**

The fishes were exposed to the three different concentrations of Vitamin E and treated with the fresh culture of *A. hydrophila* (Figure 2). The control group showed 12.5 % protection and the Vitamin E 200 mg treated group showed 77.77 % protection. The Vitamin E 400 mg treated group showed 57.4 % protection and 600 mg treated group showed 42.85 % protection. The results clearly showed that 200 mg Vitamin E treated group showed more protection than other two higher concentrations. It is noted that 400 mg and 600 mg were less protective than the 200 mg Vitamin E. Lipton (2000) reported immunostimulants capable of preventing attachment of bacteria in gut. The plant extracts treated groups showed 100 % protection. Mastuo and Miyazano (1993) reported that rainbow trout showed increased protection against *V. anguillarum*. Anderson (1992) proposed that immunostimulant showed increased resistance. Chiense herb has enhanced cellular immune response and disease resistance in spotted maigre against *V. vulnificus* (Wang *et al*., 2012). Sodium alginate enhanced immunity and resistance against *Streptococcus* sp. and an Iridovirus (Chiu *et al*., 2008) and *L. vannamei* and increase its resistance to *V. alginolyticus* infection (Cheng *et al*., 2005).

## CONCLUSION

The development of immunity by Vitamin E as supplement on disease induced *C. carpio* was investigated. The administration of Vitamin E induces/regulate the physiological enzymes and biochemical compositions in disease induced fish and develop the immune system against the pathogens. Based on the overall observation, Vitamin E is the powerful tool as potential immunostimulant for controlling the pathogenic microbes challenged in Aquaculture.

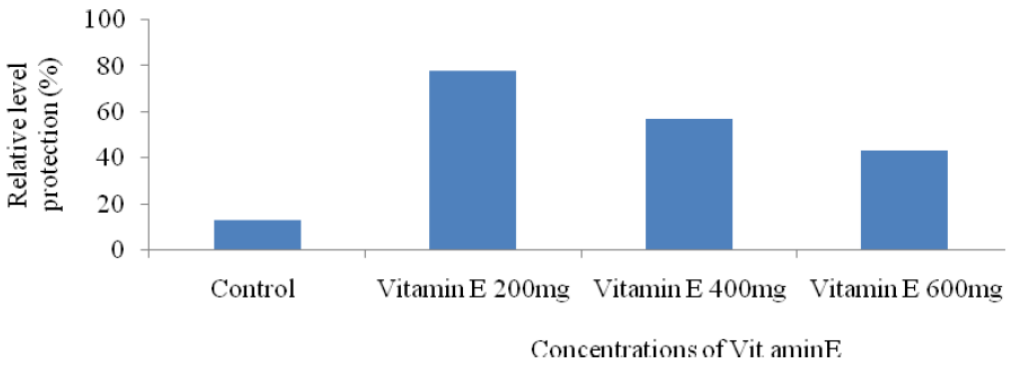


**Figure 2. Relative level of protection of Vitamin E treated *C. carpio***

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